Human adenovirus E4orf4 protein induces premature mitotic arrest by a PP2A-dependent mechanism leading to cell death in *Saccharomyces cerevisiae*

by

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Abstract

The E4orf4 protein of human adenovirus kills transformed cancer cell lines by a p53-independent mechanism. Accumulating evidence suggests that the mechanism of E4orf4-induced death is distinct from apoptosis and occurs via some novel pathway. Previous studies have shown that E4orf4 can interact with the mammalian Bα subunit of protein phosphatase 2A (PP2A) and that this interaction is important for E4orf4-induced cell death. As PP2A is highly conserved across eukaryotic species we chose to study the effects of E4orf4 in a genetically tractable organism, *Saccharomyces cerevisiae,* in an effort to elucidate the mechanism of E4orf4-mediated cell death. E4orf4 expression is lethal in yeast cells and this toxicity is dependent on E4orf4 having a functional interaction with Cdc55, the yeast homolog of Bα. Through its interaction with the B regulatory subunit, which determines substrate specificity, E4orf4 may inhibit or promote the dephosphorylation of selected PP2A substrates.

E4orf4 expression can induce high levels Clb2-Cdc28 activity and mitotic arrest in a Cdc55-dependent manner. Since E4orf4 targets only the Cdc55-containing pool of PP2A, the E4orf4-induced mitotic arrest suggests that PP2A-Cdc55 plays a direct role in regulating exit from mitosis. Two anaphase-promoting complexes (APCs) control mitotic exit, APC^{Cdc20} and APC^{Hct1}. We find that E4orf4 induces premature APC^{Cdc20} activity resulting in the premature degradation of Pds1 and Scc1 in a Cdc55-dependent manner. In contrast, E4orf4 did not induce APC^{Hct1} as evidenced be the stability of its substrates, Cdc20, Clb2 and Cdc5 as well as the hyperphosphorylation of Hct1. E4orf4 prevents Cdc55-containing PP2A complexes from localizing normally, another mechanism by which E4orf4 may modulate PP2A activity towards substrates. We propose that E4orf4 promotes mitotic arrest by acting as a Cdc55-specific inhibitor of PP2A and that PP2A plays a role in controlling the timing of anaphase by regulating APC^{Cdc20} activity.

Résumé

La protéine E4orf4 provenant d'adénovirus humain tue les lignées de cellules cancéreuses par un mécanisme indépendant du gène p53. Les données accumulées suggèrent que le mécanisme de la mort cellulaire induit par E4orf4 est distinct de l'apoptose et utilise une nouvelle voie moléculaire. Des études précédentes ont démontré que E4orf4 peut interagir avec la sous-unité Bα de la phosphatase PP2A (PP2A) mammifère et que cette interaction est importante pour la mort cellulaire causée par E4orf4. Comme PP2A est largement conservé à travers tous les eucaryotes, nous avons décidé d'étudier E4orf4 dans un organisme pour lequel plusieurs outils génétique sont disponible, c'est à dire *Saccharomyces cerevisiae*, afin d'élucider le mécanisme de la mort cellulaire induit par E4orf4. L'expression de E4orf4 est létale dans la levure et sa toxicité dépend de son interaction physique avec Cdc55, l'homologue de Bα chez la levure. À travers cette interaction avec la sous-unité régulatrice B, qui détermine la spécificité de la phosphatase, E4orf4 peut empêcher ou promouvoir la déphosphorylation de certaines protéines ciblées par PP2A.

L'expression de E4orf4 peut induire une suractivité de Clb2-Cdc28 et un arrêt de la mitose par le biais de Cdc55. Parce que E4orf4 cible seulement PP2A associé à Cdc55, l'arrêt mitotique induit par E4orf4 suggère que PP2A-Cdc55 joue un rôle direct dans la régulation de la sortie de mitose. Deux complexes promoteur l'anaphase (APC) contrôlent la sortie de mitose, APC^{Cdc20} et APC^{Hct1}. Nous avons trouvé que E4orf4 induit une activation prématurée de APC^{Cdc20} qui résulte dans la dégradation prématurée de Pds1et Scc1 selon un mécanisme dépendant de Cdc55. En contrepartie, E4orf4 n'a pas activé le complexe APC^{Hct1} compte tenu de la stabilité de ses substrats, Cdc20, Clb2 et Cdc5 ainsi que de la présence de la forme hyperphosphorylisé de Hct1. Eforf4 empêche également la localisation normale des complexes PP2A-Cdc55, ce qui pourrait constituer un autre mécanisme de modulation de l'activité de PP2A envers ses substrats. Nous proposons que E4orf4 déclenche un arrêt de la mitose en agissant comme un inhibiteur de PP2A spécifique à Cdc55 et que PP2A joue un rôle dans le contrôle de l'anaphase en régulant l'activité du complexe APC^{Cdc20}.

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Preface

In compliance with the "Guidelines Concerning Thesis Preparation" of the Faculty of Graduate Studies and Research of McGill University, chapter 2 of this thesis represents the text of a paper published in Oncogene (2001) 20; 5279-5290, with the exception of figure 8c which will be submitted for publication with the data from chapter 3. At the time of submission of this thesis Chapter 3 was being prepared to be submitted for publication.

Contribution of Co-authors

All figures were produced from data generated by myself except for the fluorescent microscopy data in Figure 3-1 which was produced by Matthew Gentry. Denis Paquette generated the following plasmids: p424*GAL1*-FLAG-*CDC55*, p424 *GAL1*-FLAG-*PPH21* and p424*GAL1*-FLAG-*PPH22*. Serge Shahinian made the SEY6210 Δ*cdc55* deletion strain.

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List of Abbreviations

4E-BP – 4E-binding protein
243R – 243 residue E1A protein product of 12s mRNA
289R – 289 residue E1A protein product of 13s mRNA
aa – amino acids
Ad – adenovirus
AIF – apoptosis-inducing factor
APC/C – anaphase promoting complex/cyclosome
C- terminal - carboxy terminal
CAK – CDK-activating kinase
cAMP – cyclic adenosine monophosphate
CAR – coxsackievirus and adenovirus receptor
CBP- CREB-binding protein
CDK – cyclin-dependent kinase
CHO cells – Chinese hamster ovary cells
CKI – cyclin-dependent kinase inhibitor
CREB – cAMP responsive element
DNA – deoxyribonucleic acid
E1A – early region 1A
E1B – early region 1B
E2 – early region 2
E3 – early region 3
E4 – early region 4
ECL – enhanced chemiluminescence
ECM – extracellular matrix
eIF-2α – eukaryotic initiation factor 2α
eIF4E – eukaryotic initiation factor 4E
FACS – fluorescence-activated cell sorting
FADD – Fas-associated death domain
FAK – focal adhesion kinase
FEAR – Cdc14 early release
G1 – gap phase before S phase
G2 – gap phase before M phase
GFP – green fluorescent protein
GST – glutathione S transferase
HA – hemagglutinin
HEAT motif – Huntington-elongation factor-A subunit-TOR motif
kD – kilodalton
LMCT I - leucine carboxyl methyltransferase-I
LMCT II - leucine carboxyl methyltransferase-II
M phase – mitotic phase
MAPK – mitogen-activated protein kinase
MEN – mitotic exit network
MPF – mitosis promoting factor
mRNA – messenger RNA

mTOR – mammalian target of rapamycin
NLS – nuclear localization sequence
N-terminal – amino terminal
OA – okadaic acid
orf – open reading frame
p70-S6K – The 70-kDa ribosomal protein S6 kinase
PARP – poly ADP ribose polymerase
PCR – polymerase chain reaction
PDK1 – pyruvate dehydrogenase kinase 1
PDK2 - pyruvate dehydrogenase kinase 2
PI3K – phosphatidylinositol-3-kinase
PKB – protein kinase B
PME1 – protein methylesterase 1
PP1 – protein phosphatase 1
PP2A – protein phosphatase 2A
PPM – phosphoprotein phosphatase M family
PPP – phosphoprotein phosphatase family
PtdIns(4,5)P2 - phosphatidylinositol -4,5 bisphosphate
PtdIns(3,4,5)P3 - phosphatidylinositol -3,4,5 triphosphate
PTP – phosphotyrosine phosphatase family
Rb – retinoblastoma tumour suppressor protein
RNA – ribonucleic acid
S phase – synthesis phase
SCF – Skp/Cullin/F-box
SDS-PAGE – sodium dodecyl sulfate polyacrylamide gel electrophoresis
SH – Src homology
SR protein – serine arginine protein
SV40 – simian virus 40
TNF – tumour necrosis factor
Ub – ubiquitin
UBC –ubiquitin conjugating enzyme
WD40 – tryptophan-aspartic acid 40 repeat

Original Contributions to Knowledge

E4orf4 interacts specifically with the Cdc55 (B55) regulatory subunit of PP2A, but not Rts1 (B56)

E4orf4 requires the presence of the Cdc55 regulatory subunit to interact with the PP2A holoenzyme and has no preference for Pph21 or Pph22 containing PP2A trimers

E4orf4-induced cell death can be almost completely rescued in Δ*cdc55* yeast cells, but not in Δ*rts1* cells

E4orf4 can induce Cdc55-independent toxicity, suggesting that E4orf4 has a Cdc55-independent mechanism

E4orf4 induces high levels of Clb2-Cdc28 (Cdk1) activity and can do so inappropriately in cells arrested in S phase

E4orf4 can induce premature APC^{Cdc20} activity as demonstrated by the instability of its substrate Pds1

E4orf4-induced Pds1 degradation is dependent on a functional APC^{Cdc20} complex

E4orf4 can induce premature degradation of Scc1 accompanied by an increase in the separation of sister chromatids

E4orf4-induced degradation of Pds1 and Scc1 is dependent on a functional E4orf4-Cdc55 interaction

E4orf4 does not induce APC^{Hct1} activity as demonstrated by the stability of its substrates Cdc20, Clb2, Cdc5 and Hct1 hyperphosphorylation.

Chapter 1: General Introduction

1.1 Human Adenovirus

There are 51 serotypes of human adenovirus (Ad) classified into six subgroups (A-F) (Table 1-1). They replicate in terminally differentiated cells and have been used to study mechanisms of cell growth, oncogenesis and apoptosis and have been engineered for use as gene therapy vectors (Wang et al.,1996; Ben-Israel et al., 2002;Cao et al., 2004). There is no evidence that adenoviruses are associated with human cancers, however subgroup A and B viruses can promote tumors in rodents and all subgroups can transform rodent cells in culture (Endter and Dobner, 2004). This work deals with proteins of human Ad2 and Ad5, non-tumourigenic serotypes, which share 95% identity.

			Oncogenic	Potential
Group	Serotypes	Sites of infection	Tumours in animals	Transformation in tissue culture
A	12, 18, 31	Intestine	High	+
B	3, 7, 11, 14, 15, 16, 21, 34, 35, 50	lung, urinary tract*	Moderate	+
C	1, 2, 5, 6	upper respiratory, liver*	Low or none	+
D	8, 9, 10, 13, 15, 17-20, 22-30, 32, 33, 36-39, 42-49, 51	eye, intestine*	Low or none, Mammary tumours	+
E	4	Respiratory	Low or none	+
F	40, 41	Intestine	Unknown	+

Table 1-1 Classification of Human Adenoviruses

(Adapted from Shenk, 2001 and Horwitz, 2004)

Asterisks indicate the sites of infection, which are characteristic for immunodeficient patients, and are not commonly the sites of disease in immunocompetent hosts. The exception is the urinary tract for group B Ads, which can also infect patients, especially males with normal immune systems. Group D Ads have low or no oncogenic potential except for Ad9 which can cause mammary tumours in female rats.

1.1.1 Viral structure and genome organization

Adenoviruses are icosahedral particles of ~70-100 nm. The adenovirus genome is ~36kB of linear dsDNA, which has inverted terminal repeat (ITR) sequences containing the origins of DNA replication (Figure 1-1). The genome is divided into early and late regions based on the transcription units being expressed before or after viral DNA replication (Shenk, 2001). Adenoviruses contain 5 early transcription units that are transcribed by RNA polymerase II and expressed prior to viral DNA synthesis: early region 1A (E1A), E1B, E2, E3 and E4. A single late unit is subdivided into 5 groups of late mRNAs (L1 to L5) and encodes proteins involved in viral particle formation (Shenk, 2001). All transcription units produce multiple proteins or open reading frames (orfs) due to alternative splicing (Nevins, 1987).

1.1.2 Infectious Cycle

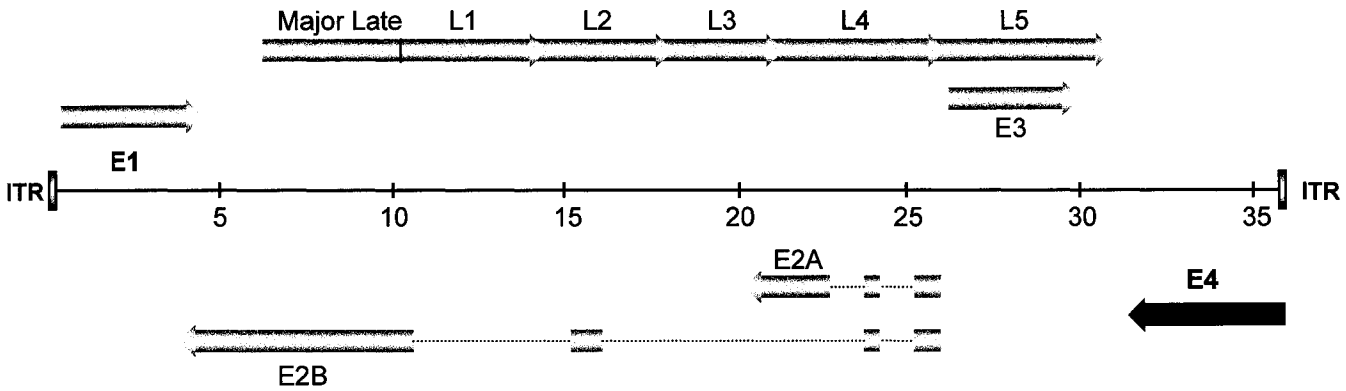
The infectious cycle is divided into early and late phases separated by the event of viral DNA replication. Adsorption of virus to the cell membrane is mediated by the viral fiber protein which attaches to a 46kD cellular protein of the Ig super-family that has been termed coxsackie and adenovirus receptor (CAR) (Bergelson et al., 1997). Adenovirus-receptor complexes are then internalized into endocytic vesicles. Upon release into the cytosol, outer viral capsid proteins are shed yielding a sub-viral particle that enters the nucleus through nuclear pores. In the nucleus the viral DNA is released as a chromosomal type structure that becomes coated with cellular histones. The virus uses the host's transcriptional and translational machinery to replicate itself and express viral

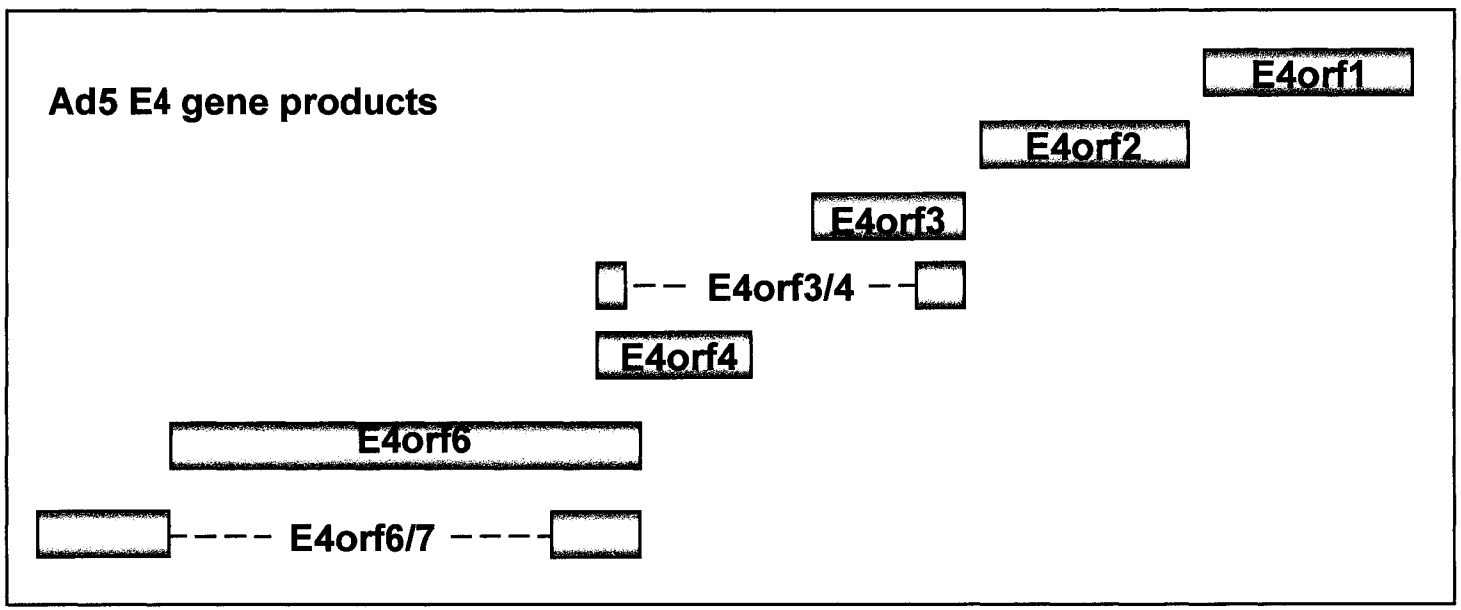
Figure 1-1 Genomic Organization of Ad5 and the E4 transcription unit

(Adapted from Täuber and Dobner, 2001)

Shown above is the linear dsDNA genome of Ad5, indicated in the center as a line with inverted terminal repeats (ITR) at each end. Lengths are marked in kb. Early transcription units are shown relative to their position and orientation in the Ad5 genome. Early genes (E1-E4) are indicated by grey bars and the dotted lines denote introns. Late genes are produced from the major late promoter. Shown below is the E4 unit which is controlled by the E4 promoter and produces a transcript that is differentially spliced yielding seven known proteins or open reading frames (orfs) including the E4orf4 protein

Ad5 Genome Organization





genes. The replicative cycle takes approximately 24 hours in cultured HeLa cells producing 10^4 virions /cell (ref).

1.1.3 Adenovirus gene expression

Early viral gene expression allows the virus to accomplish four main tasks before beginning viral replication. First the virus must force the cell into S phase in order to gain access to the DNA replication apparatus of the host cell. Secondly, the virus must block the apoptotic response of the cell due to this unscheduled DNA synthesis. Next, the immune response of the host, directed at infected cells, must be neutralized. Finally, the virus must produce viral proteins required for viral replication.

E1A proteins are essential in early adenovirus infection and are required to activate viral transcription and induce S-phase allowing viral DNA synthesis (Boulanger and Blair, 1991). The major E1A mRNAs encode proteins of 289 and 243 residues (298R and 243R), which are identical except for a 46 amino acid region termed conserved region 3 (CR3) contained in the 289R product. The CR3 region of 289R allows it to transactivate all early viral promoters and the 243R protein transactivates only the E2 promoter (Nevins, 1981). E1A proteins do not interact with DNA directly, but stimulate viral gene transactivation and DNA synthesis by interacting with several cellular proteins, including p300/CBP and pRB family proteins (Sang et al., 2002).

E1A over-expression stimulates p53 stabilization and subsequent apoptosis in the absence of viral E1B proteins responsible for blocking E1A-dependent apoptosis (Debbas and White, 1993; Lowe and Ruley, 1993). Apoptosis, or programmed cell death is a genetically controlled mechanism of cell suicide used by multi-cellular organisms to

remove unneeded, damaged or infected cells (Kerr et al., 1972; Riedl and Shi, 2004). E1B-19K is a functional analog of the cellular anti-apoptotic protein Bcl-2, and it acts to inhibit or delay E1A-induced apoptosis (Chiou et al., 1994; Boulakia et al., 1996). E1B-55K binds p53 and acts as a repressor of p53-dependent transcription (Yew, 1994; Teodoro et al., 1997). In addition, E4orf6 and E1B55K proteins function to down regulate E1A-induced p53 stabilization by promoting p53 ubiquitination and degradation via the 26s proteosome (Blanchette et al., 2004; Querido et al., 2001; Wienzek et al., 2000).

The E2 region encodes three proteins, including the adenovirus DNA polymerase, required for viral DNA replication (Shenk, 2001). Proteins produced from the E3 region are involved mainly in subverting the host's immune response except for E3-11.6K, which is involved in promoting the release of virus from infected cells {Wold et al., 1994; Tollefson et al., 1996; Tollefson et al., 1996). The E4 region encodes seven proteins (E4orf1, E4orf3, E4orf3/4, E4orf4, E4orf6, E4orf6/7 and E4orf7) that have various functions in furthering both the early and late stages of adenovirus infection (Tauber and Dobner, 2001). E4orf4 will be discussed in greater detail in section 1.3.

After viral replication, genes from the late region are expressed to allow the production of viral packaging proteins. Once viral genomes are packaged, virions lyse and exit the cell by a poorly characterized mechanism that likely involves E4orf4 and E3-11.6K proteins (Tollefson et al., 2003; Doronin et al., 2003; Marcellus et al., 1998)

1.3 E4orf4

1.3.1 Structure of E4orf4

E4orf4 is a 114 residue protein that shares no extensive sequence homology with any known protein. E4orf4 contains a highly basic sequence (in Ad2, R^{66}AKRRDRRRR^{75}; consensus RxKRRxRRRR); however, it is still unclear if this sequence serves as a nuclear targeting or retention signal (Miron et al., 2004). A proline-rich motif within E4orf4, P^{4}ALPAPP^{10}, conforms to the Src SH3 binding consensus sequence (PXXPXXφ, where φ is a hydrophobic residue); however, deletion of this motif does not prevent E4orf4 binding to Src kinases (Champagne et al., 2004). Apart from small regions at the amino and carboxy termini, deletion of even short portions of the central region of E4orf4 yields products that are generally non-functional and often unstable suggesting that the central core of the E4orf4 protein contains structure of functional importance (Marcellus et al., 2000). E4orf4 mRNA is produced from the E4 promoter early after infection, however E4orf4 persists at high levels even late in infection as it is highly stable (Boivin et al., 1999).

1.3.2 Functions of E4orf4

1.3.2.1 E4orf4 and early viral gene expression

Early work on E4orf4 revealed its involvement in down-regulating cellular transcription factors required for early viral gene expression. Expression of E1A and dibutyryl cyclic AMP (cAMP) synergistically induced *c-fos* and *junB* transcription as well as *c-fos* mRNA translation (Engel et al., 1988; Müller et al., 1989). c-Fos and JunB associate to form the transcription factor AP-1, which binds to AP-1 sites in early viral

promoters to activate gene expression (Engel et al., 1988). This transcriptional activation is followed by a down-regulation of AP-1 DNA binding activity and early viral gene expression. This effect was mapped to E4orf4, which inhibits *junB* transcription and induces the hypo-phosphorylation of c-Fos and E1A proteins (Muller et al., 1992; Whalen et al., 1997).

E4orf4 immunoprecipitates were shown to contain the Bα, A and C subunits of protein phosphatase 2A (PP2A), a trimeric serine/threonine phosphatase (Kleinberger and Shenk, 1993). The E4orf4-PP2A complex exhibited phosphatase activity *in vitro* and it was found that the E4orf4-mediated repression of *junB* transcription could be reversed in the presence of 5 nm okadiac acid (OA) a non-specific inhibitor of PP2A (Kleinberger and Shenk 1993; Cohen et al., 1990). While it cannot be ruled out that the E4orf4-PP2A complex directly promotes the dephosphorylation of E1A and c-Fos, the kinetics with which these proteins become hypo-phosphorylated in the presence of E4orf4 suggest that the effect may be induced by the inhibition of a kinase (Muller et al., 1992). Kinases reported to be inactivated by PP2A include mitogen activated protein kinases (MAPKs) and p70-S6K (Millward et al., 1999). An alternative hypothesis would be that E4orf4 inhibits PP2A phosphatase activity which could be required for dephosphorylation and activation of a kinase that promotes phosphorylation of E1A and c-Fos. Kinases that are activated by PP2A include, the Cdc2 regulator Wee1 (Millward et al., 1999).

E4orf4 expression in transient transfection experiments negatively regulates E2F-1 transactivation of the viral E2 promoter and E2 mRNA expression during virus growth (Mannervik et al., 1999). E4orf4 also inhibits transactivation of the E4 region, resulting in the down-regulation of its own expression (Bondesson et al., 1996). E4orf4 down-

regulation of E4 expression was found to be inhibited by okadaic acid, suggesting that the interaction between E4orf4 and PP2A may be involved (Bondesson et al., 1996). The down-regulation of E4 expression may serve to limit production of E4orf4 and possibly other toxic viral genes (see section 1.3.3).

1.3.2.2 E4orf4 and RNA splicing

E4orf4 functions in alternative RNA splicing to promote the switch from early to late viral gene expression (Kanopka et al., 1998). SR (serine-arginine) proteins are a family of RNA splicing factors that must be hyperphosphorylated to function in splicesome assembly and splicing catalysis (Zahler et al., 1992). E4orf4 expression correlates with the dephosphorylation of SR proteins, converting their splicing properties to that detected in late adenovirus infection (Kanopka et al., 1996; Kanopka et al., 1998). E4orf4 binds, either directly or indirectly, to a subset of hyper-phosphorylated SR proteins, and E4orf4 mutants that fail to interact with the B subunit of PP2A fail to activate late pattern splicing (Estmer_Nilsson et al., 2001). This dephosphorylation is presumably via the interaction of E4orf4 with protein phosphatase 2A (PP2A) since okadaic acid is able to abrogate the effect of E4orf4 on SR dephosphorylation (Kanopka et al., 1998).

1.3.2.3 E4orf4 and mTOR

The mammalian target of rapamycin (mTOR) protein controls the translation initiation of mRNAs required for cell growth. In response to nutrient and growth factor signaling phosphatidylinositol-3 kinase (PI3K) is activated and can then phosphorylate

phosphatidylinositol -4,5 bisphosphate [PtdIns(4,5)P2] to yield the lipid second messenger phosphatidylinositol -3,4,5 triphosphate [PtdIns(3,4,5)P3], which recruits protein kinase B (PKB) to membranes. Pyruvate dehydrogenase kinase -1 and -2 (PDK1 and PDK2) activate PKB which then phosphorylates and activates mTOR. mTOR phosphorylates 4E-BP (eukaryotic initiation factor 4E-binding protein 1) at inhibitory sites, promoting the release of eukaryotic initiation factor 4E (eIF4E) which stimulates translation. mTOR also phosphorylates and activates p70-S6K, which phosphorylates the 40s ribosomal protein S6 and promotes translation (Tee and Blenis, 2005).

It was recently found that E4orf4 and E4orf1 cooperate to mimic nutrient and growth factor signaling leading to activation of the mTOR pathway, thus allowing viral replication to occur in the absence of nutrients (O'Shea et al., 2005). E4orf1 mimics growth factors to stimulate PI3K activation and PKB phosphorylation leading to TOR activation. In contrast E4orf4 acts in the same pathway as glucose, independently of PKB, to activate mTOR and mediate the bulk of p70-S6K phosphorylation. E4orf4 mutants that were unable to bind Bα were unable to cooperate with E4orf1 to induce phosphorylation of p70-S6K, suggesting that the E4orf4-PP2A interaction is involved in mediating mTOR activation.

1.3.3 Discovery of E4orf4-mediated p53-independent cell death

By infecting p53-positive or p53-null cell lines with adenovirus mutants lacking the E1B region, it was found that virus expressing E1A-289R could promote cell death regardless of p53 status, while E1A-243R caused death only in the presence of p53 (Teodoro et al., 1995). Since E1A-induced apoptosis relies on functional p53, it was concluded that one of the early genes trans-activated by E1A-289R was responsible for

the p53-independent cell death (Teodoro et al., 1995). Using viral mutants, this death function was mapped to the E4orf4 protein and it was subsequently shown that E4orf4 was sufficient to mediate p53-independent cell death when expressed alone (Marcellus et al., 1998; Marcellus et al., 1996; Shtrichman and Kleinberger, 1998). E4orf4-induced cell death also appears to be specific to tumour cell lines with no detectable toxicity in normal primary cells (Shtrichman et al., 1999; Marcellus et al., unpublished data). One possible reason suggested for this specificity is that since E4orf4 generally down-regulates viral-induced signal transduction, E4orf4 may be antagonistic to growth signals present in cancer cell lines and these conflicting signals may lead to death (Shtrichman and Kleinberger, 1998).

1.3.3.1 Features of Apoptosis

Apoptotic cell death is characterized by shrinkage and rounding of cells, disruption of the cytoskeleton, chromatin condensation, cleavage of DNA to nucleosome-sized fragments, cytoplasmic vacuolization and membrane blebbing, and in the final stages, fragmentation of the cell into membrane-bound apoptotic bodies that are engulfed by neighboring cells (Saraste and Pulkki, 2000). In this way cells containing genotoxic damage, imbalances in growth such as in cancer cells, or those challenged by virus infection are eliminated from the host.

Common to most, but not all, apoptotic pathways is the activation of caspases (Salvesen and Dixit, 1999). Caspases are cysteine proteases that can be activated directly, as with tumour necrosis factor receptor (TNFR) and Fas which, after ligand binding, form signaling complexes that bind and activate initiator caspases (Bratton et al,

2000). With many initiators, including p53, activation depends on a signal from an upstream checkpoint regulated by oligomerization of the Bcl-2 family of integral membrane proteins (Corey and Adams, 2002). Oligomerization occurs via Bcl-2 homology (BH) domains. Homo-oligomerization of Bax promotes caspase activation, which is prevented by Bcl-2 and similar proteins that hetero-oligomerize with Bax. Bad triggers apoptosis by binding Bcl-2, thus preventing its hetero-oligomerization with Bax. Cells are maintained by 'survival factors' and the positive balance of death suppressors (Cory and Adams, 2002). Bcl-2 family members are components of membrane complexes in mitochondria and the endoplasmic reticulum and may function in channels that regulate release of initiators of caspases, such as cytochrome C. Activation of the caspase cascade results in cleavage and activation of enzymes that dismantle cellular components and kill the cell.

1.3.3.2 Features of E4orf4-mediated p53-independent cell death in transformed cells

E4orf4 generally takes 24-48 hours to kill mammalian cancer cell lines. The features of E4orf4 induced cell death are complex and appear to be highly cell line-dependent. In different cell lines E4orf4 can induce some of the common features of cells undergoing apoptosis. In CHO (Chinese hamster ovary) cells and in p53-null H1299 (human non-small cell lung carcinoma) cells, E4orf4-mediated cell death displays some apoptotic features such as phosphatidyl serine flip onto the outer plasma membrane, membrane blebbing, chromatin condensation and DNA degradation (Lavoie et al., 1998; Teodoro et al., 1995; Marcellus et al., 1998). Caspase cleavage is not induced by E4orf4 in H1299 cells or human 293T (transformed with Ad E1A, E1B and SV40 large T

antigen) cells (Robert et al., 2002; Symborsky et al., unpublished data). Furthermore, E4orf4-induced cell death in H1299 or CHO cells is not inhibited by the pan-caspase inhibitor zVAD-fmk (Lavoie et al., 1998; Symborsky et al., unpublished data).

These findings are in contrast to another report where H1299 or 293T cell lines were transfected with plasmid DNA expressing E4orf4 (Livne et al., 2001). In 293T cells, E4orf4-induced apoptosis was diminished by over-expression of dominant-negative mutants of caspase-8 or the death receptor adaptor protein FADD, but not a dominant-negative caspase-9 mutant (Livne et al., 2001). These results suggest that E4orf4 could function through a caspase-8 pathway similar to death receptors. CrmA, an inhibitor of caspase-8 cleavage, inhibited nuclear condensation and increased 293T cell survival; however, in H1299 cells CrmA did not affect E4orf4-mediated cell death, suggesting that caspase-8 cleavage could be required for morphological features of apoptosis but not for E4orf4-mediate cell death in the H1299 cell line (Livne et al., 2001). In disagreement with the study by Livne *et al.*, Robert and colleagues found no caspase cleavage in E4orf4-expressing 293T cells and no inhibition of nuclear condensation upon treatment with either of the pan-caspase inhibitors zVAD.fmk or BokD.fmk (Robert et al., 2002). The reason for the discrepancy in results is unclear and may have been due to clonal differences in cell lines or particular experimental conditions.

In C-33A cells, over-expression of E4orf4-GFP promoted the release of cytochrome C and apoptosis inducing factor (AIF), a mediator of caspase-independent cell death (Robert et al., 2002). While Bcl2 over-expression was able to inhibit both cytochrome C and AIF release in C-33A, it did not diminish E4orf4-induced cell death. Therefore, these factors are not likely to be involved in the mechanism of E4orf4 (Robert

et al., 2002). Another group observed release of cytochrome C in H1299 and 293T cells upon E4orf4 expression (Livne et al., 2001). This effect differs from another study where no cytochrome C or AIF release was observed in E4orf4 expressing-H1299 cells (Symborsky et al., unpublished data).

While E4orf4 expression elicits variable features of apoptosis in the various cell lines tested, it also displays features not associated with apoptotic cell death. In contrast to p53-overexpression, which exhibits classic features of apoptosis, H1299 cells over-expressing E4orf4 increase in cell volume rather than condense and cells are delayed in uptake of the vital dye, trypan blue (Li and Branton, unpublished data). Furthermore, the nuclear condensation that is observed in E4orf4-expressing cells is visually different from that observed for apoptotic inducers such as p53, with E4orf4-expressing cells showing less compact nuclear condensation. Based on the variable cell line-dependent phenotypes we believe that apoptosis is not the primary mechanism being used by E4orf4 and now believe that E4orf4 must kill transformed cells by some alternate pathway.

1.3.4 E4orf4 point mutant studies

As discussed above, E4orf4 binds to the Bα subunit of PP2A and some of its biological effects appear to involve PP2A. To determine the role of the E4orf4- Bα interaction in E4orf4 killing the ability of E4orf4 mutant proteins to bind the Bα subunit and to induce cell killing were compared. Series of point mutations were generated in E4orf4, either randomly (Shtrichman et al., 1999), or by selective mutagenesis affecting residues that are highly conserved in all adenovirus serotypes (Marcellus et al., 2000). Both studies indicated a high degree of correlation between binding to the Bα subunit and

induction of cell death. That is, mutants that interact very poorly or not at all to Bα are highly defective for killing.

One study, which employed a much larger array of mutants, identified two mutant classes. Class I mutants (mapping largely between residues 51-89) fail to bind Bα and are defective for cell killing (50-80% cell survival) (Marcellus et al., 2000; Champagne et al., 2004). Class II mutants can interact with Bα at fairly normal levels, but similar to Class I mutants are also defective for killing (Marcellus et al., 2000). The results with Class I mutants suggest that the absence of Bα interaction is sufficient to relieve most of E4orf4-mediated cell death, however since cell death was not completely prevented, a B-independent mechanism of cell death may also be involved. The existence of Class II mutants suggests that Bα interaction is not sufficient and the residues affected in these mutants may be involved in an interaction with some other cellular protein required for cell death. Alternatively, it is possible that although binding takes place, it is non-functional in terms of eliciting appropriate effects on PP2A. Further support for the importance of the Bα subunit was obtained in a study showing that expression of anti-sense RNA specific for Bα transcripts appeared to inhibit E4orf4 cell killing (Shtrichman et al., 1999). These results indicated that binding to the Bα subunit contributes to E4orf4-mediated cell death.

1.3.5 Specificity of E4orf4-B subunit interaction

As multiple B subunits exist in four families that share little homology, it was of interest to determine the specificity of the E4orf4 interaction. In one study, E4orf4 binding was detected with members of the B'(B56) family of subunits, although mutant analysis indicated that such binding was not involved in E4orf4-mediated cell killing

(Shtrichman et al., 2000). Our group has also analyzed B subunit binding, but with different results. Although high levels of binding of E4orf4 were detected with all four highly related members of the B(B55) family (Bα , Bβ, Bδ, and Bγ), none was detected with any members of the B'(B56), B" or B"' subunit families, which are largely unrelated to B55 proteins (Marcellus and Branton, unpublished data). Although this issue remains unresolved there is a general consensus that it is the interaction of the B55 subunit that is important for E4orf4-mediated cell death.

1.3.6 E4orf4 and modulation of PP2A substrate phosphorylation

Through its interaction with the B subunit, E4orf4 may create a novel type of PP2A complex with modified substrate specificity. It has generally been assumed that E4orf4 enhances the dephosphorylation of proteins, due to the observed hypo-phosphorylation of AP-1, E1A and SR proteins. However, the hypo-phosphorylation of these proteins is not believed to result from a direct dephosphorylation event mediated by PP2A, but rather a down-regulation of the kinase.

Studies were done to directly address the effect of E4orf4 on PP2A activity and it was found that E4orf4 could inhibit the ability of purified Bα–containing PP2A complexes to dephosphorylate phosphorylase *a* and histone H1 *in vitro* (Brignole and Branton, unpublished data) In addition, two substrates of PP2A, 4EBP-1 and p70-S6K (Bα specific) were hyper-phosphorylated in E4orf4-expressing cells (Brignole and Branton, unpublished data). E4orf4 does not change the ability of PP2A to dephosphorylate a PP2A peptide substrate *in vitro* suggesting that E4orf4 may modulate the phosphorylation of selected PP2A substrates (Marcellus et al., 2000; Roopchand et al., 2001). If E4orf4 required the phosphatase activity of PP2A to mediate cell death,

treatment of E4orf4 with low levels of PP2A inhibitors, such as okadaic acid or I_1^{PP2A}, might be expected to show some relief of killing. However, while each inhibitor alone did not affect cell death, they enhanced E4orf4 killing, suggesting that PP2A activity was not required for the induction of cell death by E4orf4 (Brignole and Branton, unpublished data).

1.3.7 E4orf4 and induction of extranuclear apoptosis

Extranuclear apoptosis or anoikis generally occurs when cells lose integrin-mediated attachments to the extracellular matrix (ECM). These cell-ECM attachments promote the assembly of signaling molecules such as Src family kinases and focal adhesion kinase (FAK) to focal adhesion sites, where they then activate downstream survival pathways (Frisch and Ruoslahti, 1997; Giancotti, 1997). Extranuclear apoptosis occurs in three steps, the first being the release stage, which involves the rounding of cells due to reorganization of focal adhesions and actin, after which membrane blebbing and finally condensation into apoptotic bodies occurs (Mills et al., 1999).

E4orf4 can initiate caspase-independent extranuclear apoptosis through a dysregulation of Src family kinases (Lavoie et al., 2000). While E4orf4-expressing cells are able to bind to ECM proteins, E4orf4 appears to interfere with downstream signals that promote cell spreading and survival. About 30% of E4orf4 is normally associated with the cytoskeleton; however, in the presence of activated c-Src or v-Src this level increases two- to three-fold and is accompanied by increased membrane blebbing and nuclear condensation, which can be decreased by PP2, a chemical inhibitor specific to Src family kinases (Lavoie et al., 2000). E4orf4 does not affect the *in vitro* kinase activity of c-Src, but appears to modulate Src-dependent tyrosine phosphorylation of specific

substrates with some having higher (FAK and paxillin) and others lower (cortactin) levels of tyrosine phosphorylation (Lavoie et al., 2000). Expression of E4orf4 also correlates with a dramatic re-localization of tyrosine phosphorylated proteins, actin fibers and cortactin to the cell periphery (Lavoie et al., 2000).

1.3.7.1 Mapping of E4orf4-Src interaction domains

E4orf4 can associate and co-localize with Src family kinases and these complexes also contain the PP2A catalytic subunit (Lavoie et al., 2000; Champagne et al., 2004). FLAG-tagged E4orf4 interacts with c-Src via its SH1 kinase domain, but not with SH2 or SH3, as determined using GST-fusions of the SH domains and Src proteins deleted for each of the SH domains (Champagne et al., 2004). Since these experiments were done with cell lysates and recombinant GST fusions, it is not certain whether the E4orf4-Src interaction is direct.

Mutation of the R-rich region of E4orf4 to alanine residues ($Arg^{69/70/72/73/74/75}$ or 6R-A) completely abolished binding to the SH1 kinase domain of Src as well as endogenous c-Src. 6R-A did not completely prevent binding to exogenous PP2A-Bα, but decreased it by 40% relative to wild-type E4orf4 binding (Champagne et al., 2004). In contrast the Class I E4orf4 mutant R81AF84A, which does not interact with the Bα subunit of PP2A, could efficiently interact with Src (Champagne et al., 2004). A GFP fusion of residues 62-79 of E4orf4 (GFP-E4orf4-[62-79]) was sufficient to interact with the GST-tagged kinase domain of Src, however GFP-E4orf4-[62-95], which contains residues important for B binding, was more efficient suggesting that the residues downstream of the R-rich motif (80-95) stabilize the interaction (Champagne et al.,

2004). The results suggest that E4orf4 can interact with Src independently of its interaction with PP2A-Bα using distinct, but overlapping regions.

1.3.7.2 E4orf4 tyrosine phosphorylation and localization

E4orf4 contains eight tyrosine residues, two of which (Y42 and Y59) reside in a motif that resembles the consensus sequence for phosphorylation by Src kinases (EEEIY[G/E]EFD). GST-c-Src can mediate the phosphorylation of recombinant E4orf4 *in vitro* and the specificity of phosphorylation was confirmed using a phosphospecific E4orf4 (Tyr^{42}) antibody (Champagne et al., 2004). Single phenylalanine substitutions of three of these tyrosines residues Y26F, Y42F and Y59F were found to reduce Src-induced phosphorylation of E4orf4 by 25%, 50% and 25%, respectively and an E4orf4 mutant harboring all three of these substitutions (E4orf4-Y3F) removed almost all of the Src-induced phosphorylation of E4orf4 (Gingras et al., 2002).

E4orf4 tyrosine phosphorylation was not required for its interaction with Src, however it was required for E4orf4-Src complex to interact with the Src substrates p62doc and cortactin and modulate Src-induced phosphoryation of these proteins (Gingras et al., 2002). Comparing wild type E4orf4, E4orf4-Y3F and E4orf4-Y42E (mimicking constitutive phosphorylation) it was shown that Src-mediated tyrosine phosphorylation was positively correlated to the stable accumulation of E4orf4 in the cytoplasm, it's translocation to the plasma membrane, induction of membrane blebbing, DNA condensation and cell death in 393T cells (Gingras et al., 2002).

The R-rich region of E4orf4 (residues 66-75) was found to be important for the nucleolar and nuclear localization of E4orf4 in cells (Miron et al., 2004). Mutation of the

arginine residues to alanines resulted in loss of E4orf4 targeting to both nuclei and nucleoli as well as a 39% reduction in E4orf4-killing as measured by colony survival assay (Miron et al., 2004). These results suggest that the nuclear/nucleolar targeting of E4orf4 contributes to its death function.

1.3.7.3 E4orf4 mediates cell death by both cytoplasmic and nuclear pathways

While the phosphorylation of E4orf4 by Src appears to be important for transducing the cytoplasmic/membrane functions of E4orf4 leading to cell death, the E4orf4-Y3F mutant does not completely relieve cell death (22% cell survival vs. 43% for wt E4orf4 and E4orf4-Y3F mutant, respectively), suggesting the existence of another death pathway (Gingras et al., 2002). This issue was addressed in another study where E4orf4-GFP constructs were tagged with a CAAX sequence to retain E4orf4 at the cell membrane or a nuclear localization sequence (NLS) to sequester E4orf4 in the nucleus (Robert et al., 2002). E4orf4-GFP and E4orf4 (Y3-F)-GFP each carrying either the CAAX or NLS targeting sequences were tested for membrane blebbing and nuclear condensation in 293, 293T, and C-33A cell lines. E4orf4 (Y3F)-GFP-CAAX was unable to induce cell death as efficiently as E4orf4-GFP-CAAX, demonstrating that tyrosine phosphorylation was important to induce cell death through the cytoplasmic pathway. In contrast, E4orf4 (Y3F)-GFP-NLS and E4orf4-GFP-NLS were able to induce cell death to similar degrees, although the death response was delayed in comparison to E4orf4-GFP or E4orf4-GFP-CAAX, demonstrating that a E4orf4 nuclear Src-independent death signal does not require tyrosine phosphorylation of E4orf4 (Robert et al., 2002).

Therefore, E4orf4 appears to mediate two distinct death pathways; a Src-regulated pathway that requires E4orf4 tyrosine phosphorylation and accumulation in the membrane, and a nuclear (Src-independent) pathway that results from E4orf4 accumulation in the nucleus. The current belief is that the contribution of either pathway will be determined by the availability of Src-specific targets that are modulated by the E4orf4-Src complex as well as the activity of the signaling pathways involved in a given transformed cell type (Robert et al., 2002).

1.4 Protein phosphatase 2A (PP2A)

1.4.1 Classification of phosphatases

Reversible phosphorylation catalyzed by protein kinases and phosphatases is an essential post-translational mechanism used in many cellular processes. Eukaryotic phosphatases are designated into three families, phosphoprotein phosphatase P (PPP), phosphoprotein phosphatase M (PPM) and phospho-tyrosine phosphatases (PTP), based on the structure of their catalytic domains. The PPP family is comprised of serine/threonine phosphatases, PP1, PP2A, PP2B, PP4, PP5, PP6 and PP7 (Cohen et al., 1990; Chen et al., 1994; Huang et al., 1997). The PPM family includes the PP2C subfamily of serine/threonine phosphatases, which are stimulated by the presence of Mg^{2+} ions. The PTP family encompasses tyrosine phosphatases and dual specificity phosphatases that can dephosphorylate phospho-tyrosine alone or phospho-serine and –threonine residues of proteins, respectively.

1.4.2 PP2A Organization

PP2A is a trimeric serine/threonine phosphatase that has been highly conserved across eukaryotic species. PP2A accounts for up to 1% of the total protein in the cell and is the major phosphatase in many tissues and cells (Ruediger et al., 1991; Sontag, 2001). PP2A has pleitropic functions, being implicated in the regulation of cellular metabolism, DNA replication, RNA splicing, translation, cell cycle, cell morphogenesis, development and transformation by polyoma and SV40 viruses (Janssens and Goris, 2001). The ability of PP2A to be involved in such varied processes can be explained by its structural plasticity. In mammalian cells PP2A trimers consists of a core dimer (Kremmer et al., 1997), composed of a catalytic C subunit and a structural A subunit that is associated with one of at least 20 known regulatory B subunit (Janssens and Goris, 2001) (Table 1-2).

Table 1-2 PP2A subunits in mammalian cells and Saccharomyces cerevisiae

	Mammalian	*Saccharomyces cerevisiae*
Structural	A (α and β)	*TPD3*
Regulatory	B (α, β, δ, γ)	*CDC55*
	B′ (α, β, δ, γ, ε)	*RTS1*
	B′′ (PR72/PR130, PR59, PR48)	-
	B′′′ (PR93/SG2NA, PR110/Striatin)	-
Catalytic	C (α and β)	*PPH21, PPH22*

1.4.2.1 Structural A Subunit

The 65 kD A subunit in higher eukaryotic cells exists in two isoforms (α and β) that share 86% sequence identity with the β isoform being much less abundant than α (Hendrix et al., 1993). The crystal structure of A has been solved and it forms a hook-shaped protein composed entirely of 15 tandem internal repeats of a 39 amino sequence termed a HEAT (huntingtin-elongation factor-A subunit-TOR kinase) motif (Groves et al., 1999; Hemmings et al., 1990). HEAT motifs are believed to be involved in protein-protein interactions (Groves et al., 1999). The A subunit forms a platform to which the B and C subunits bind. The catalytic C subunit binds to HEAT repeats 11-15, whereas B subunits bind to repeats 1-10 (Ruediger et al., 1992; Ruediger et al., 1994). The A and C subunits interact to form PP2A core dimers ($PP2A_{AC}$); however, no complexes between A and B in the absence of C have been reported. The AC (core) dimer appears to lack substrate specificity (Kremmer et al., 1997). The Aα and Aβ subunits may function as tumor suppressors as they are frequently mutated in melanomas, lung and breast carcinomas (Ruediger et al., 2001; Ruediger et al., 2001; Janssens et al., 2005; Wang et al., 1998).

1.4.2.2 Regulatory B subunits

About twenty mammalian B regulatory subunits have been cloned and grouped into four largely unrelated families : B/B55/PR55, B′/B56/PR61, B″/PR72 and B‴. The different classes of B subunits share little or no homology yet they interact via the same or overlapping sites within the A subunit of the AC dimer such that the binding of the different B subunits to the AC dimer is mutually exclusive (Ruediger et al., 1992;

Ruediger et al., 1994). The B class has four members of about 55kD: Bα, Bβ, Bδ and Bγ (Strack et al., 1999; Mayer et al., 1991; Pallas et al., 1992; Zolnierowicz et al., 1994). The B' class contains at least 10 isoforms expressed from 5 genes, α, β, δ, ε and γ (McCright and Virshup, 1995; McCright et al., 1996; Csortos et al., 1996; Tanabe et al., 1996; Tehrani et al., 1996; Zolnierowicz et al., 1996). The B" class contains at least three isoforms PR72/130, PR48 and PR59 (Yan et al., 2000; Voorhoeve et al., 1999; Hendrix et al., 1993). Two additional B-type subunits, S/G_2 nuclear autoantigen and striatin, have been identified and represent a putative B"' (PR93/PR110) family (Moreno et al., 2000). B subunits function in defining PP2A substrate specificity, intracellular localization and tissue specificity (Janssens and Goris, 2001). There is little known about how the various B subunits affect the activity of PP2A.

The Bα and Bδ subunits are ubiquitously expressed in tissues while Bβ and Bγ expression is enriched in the brain (Janssens and Goris, 2001). There is ~90% identity among the B family subunits. B family subunits contain seven WD40 repeat motifs, which are believed to function as protein-protein interaction domains (Smith et al., 1999 ; Yu et al., 2000). WD40 repeats are loosely defined sequences of about 40 amino acids that often end in tryptophan and aspartic acid (Smith et al., 1999; Yu et al., 2000). There is no crystal structure available for B subunits; however, based on similarity to its closest structural homolog, the Gβ1 subunit, the seven WD40 repeats of the B subunits are predicted to fold into a β-propeller structure (Strack et al., 2002).

1.4.2.3 Catalytic C subunit

Mammalian cells possess two 36 kDa catalytic subunits ($PP2A_{C\alpha}$ and $PP2A_{C\beta}$), which are the product of two distinct genes sharing 98% identity (Stone et al., 1987 ; Arino et al., 1988). Both are ubiquitously expressed, however $PP2A_{C\alpha}$ is about ten times more abundant than $PP2A_{C\beta}$ (Khew-Goodall and Hemmings, 1988). A crystal structure has not yet been obtained for the C subunit and the precise mechanism by which the activity of $PP2A_C$ is modulated by the B regulatory subunits is unknown. $PP2A_C$ is regulated by both phosphorylation and methylation. $PP2A_C$ has a highly conserved C-terminal $T^{304}PDYFL^{309}$ motif, which contains a Y-307 phosphoryation site and a recognition site for carboxy-methylation on L-309 (Xie and Clarke, 1993; Xie and Clarke, 1994; Lee and Stock, 1993).

Phosphorylation of Y-307 results in PP2A inactivation and can be mediated *in vitro* and *in vivo* by over-expression of v-Src (Chen et al., 1992; Chen et al., 1994). Y-307 phosphorylation of $PP2A_C$ is also observed *in vivo* in response to stimulation of cells by epidermal growth factor, serum, insulin, TNFa, IL-1 (Srinivasan and Begum, 1994; Guy et al., 1995; Chen et al., 1994; Begum and Ragolia, 1996).

Methylation of proteins is a relatively rare event and is irreversible when occurring on amino groups but reversible on carboxyl groups (Clarke, 1985). Carboxy methylation of human $PP2A_C$ at L309 is mediated by LCMT-I (leucine carboxyl methyltransferase-I) (Lee and Stock, 1993; Xie and Clarke, 1994; De Baere et al., 1999). LCMT-II encodes a highly similar protein that has a ~300 amino acid stretch containing five Kelch-like motifs that are known to bind actin containing structures; however, whether it has methyltransferase activity against $PP2A_C$ is unknown (De Baere et al.,

1999). Removal of methyl groups at L309 is mediated by PP2A methylesterase-1 (PME-1), which was identified as a protein that interacted with catalytically inactive $PP2A_C$ mutants (Ogris et al., 1999).

Methylation of $PP2A_C$ may be important for regulating B subunit binding to the core dimer. PP2A trimers containing B´´´/striatin/SG2NA or B´/PR72 were found to be methylated (Moreno et al., 2000; De Baere et al., 1999). In the case of B/PR55α two studies have claimed that in order for B/PR55α to bind $PP2A_{AC}$ the L-309 residue had to be methylated, while another reported that B/PR55-containing trimers were sometimes methylated and sometimes not (Tolstykh et al., 2000; Bryant et al., 1999; De Baere et al., 1999).

1.4.3 PP2A in Saccharomyces cerevisiae

PP2A of *Saccharomyces cerevisiae* is very similar to the mammalian enzyme with respect to substrate specificity and sensitivity to inhibitors (Cohen et al., 1989). The catalytic subunit is encoded by two duplicated genes, *PPH21* and *PPH22* (Ronne et al., 1991). There is only one A subunit encoded by the *TPD3* gene (van Zyl et al., 1992). The B subunits are encoded by *CDC55* and *RTS1* representing the B and B' classes respectively (Healy et al., 1991; Shu et al., 1997).

The primary structure of the B and B' subunits of PP2A are highly conserved between yeast and mammals. There exists 53% amino acid identity (67% similarity) between mammalian Bα and Cdc55 and there is ~55% identity (66% similarity) between mammalian B' subunits and Rts1 (Zhao et al., 1997; Healy et al., 1991). Cdc55 and Rts1 have distinct roles in the cell and are not functionally interchangeable, consistent with

them representing different classes of regulatory B subunits (Zhao et al., 1997). The mammalian B'γ or B'α isoforms can rescue the temperature sensitivity of the *rts1* deletion strain, but the yeast *CDC55* gene cannot (Zhao et al., 1997). Likewise, *RTS1* cannot functionally replace *CDC55* (Zhao et al., 1997). None of the mammalian B subunits are able to rescue the cold sensitive phenotype of the *cdc55* deletion strain (Healy et al., 1991) and our unpublished data.

1.4.3.1 Tpd3

Like the mammalian A subunit, Tpd3 (tRNA production defect 3) is composed of 15 tandem HEAT motifs. Deletion of *TPD3* gives both cold sensitive and temperature sensitive phenotypes (van Zyl et al., 1992). At high temperature *TPD3* is required for tRNA production. At low temperatures *tpd3* strains fail are multi-budded and multi-nucleated indicating that Tpd3 is required for cytokinesis. This cold sensitive phenotype is similar to that observed in *cdc55* strains and implicates Tpd3 in regulating cell morphogenesis.

1.4.3.2 Cdc55

The *cdc55* mutant grows normally at 30°C, but at lower temperatures has a cold sensitive phenotype highly similar to *tpd3* strains, with >90% of cells becoming elongated with multiple buds and nuclei indicating defects in cytokinesis and septation (Healy et al., 1991). The *cdc55* strain is hypersensitive to nocodazole and benomyl, drugs that destabilize microtubule polymerization preventing mitotic spindle formation and cause the activation of the spindle assembly checkpoint. The spindle assembly checkpoint halts the cell cycle in response to the incomplete attachment of sister

chromatids to the spindle or spindle polymerization defects (Gardner and Burke, 2000). Since *cdc55* cells lose viability in the presence of spindle depolymerizing drugs, Cdc55 has been implicated in maintaining the spindle checkpoint (Minshull et al., 1996; Wang and Burke, 1997). However, another study has reported that *cdc55* strain has a normal arrest response in the presence of nocodazole for up to 6 hours with stable Pds1 and normal accumulation of Cln2 upon entry into the next cell cycle (Yang et al., 2000). The *cdc55* cells do however eventually lose viability, suggesting that the checkpoint may become non-functional later in these cells. Cdc55 is also involved in the regulation of Swe1 stability, as *cdc55* strains exhibit stabilized Swe1 protein and consequently higher levels of inhibitory Cdc28 phosphorylation (Yang et al., 2000).

1.4.3.3 Rts1

Rts1 (Rox three suppressor 1) is an 85 kDa protein that migrates as multiple bands between 90-95kDa due to phosphorylation as determined by ^{32}P labeling of yeast cells (Shu et al., 1997). A recent study indicated that Rts1 is an *in vivo* substrate for Cdc28 (Ubersax et al., 2003). *RTS1* was isolated as a multicopy suppressor of a *ROX3* gene mutation and also as a high copy suppressor of *hsp60-ts* mutant alleles (Shu and Hallberg, 1995; Evangelista et al., 1996). *ROX3*, a component of the RNA polymerase II holoenzyme, is involved in transcription of stress response genes and Hsp60 is a heat shock protein. These findings implicate Rts1 in the global stress and heat shock response pathways.

Rts1 also appears to be important for mitotic entry. Strains deleted for *RTS1* are temperature sensitive and at the restrictive temperature (37°C) *rts1* strains are delayed in

G2 as large budded cells with a 2N DNA content, undivided nuclei, decreased Clb2 mRNA levels and decreased Clb2-Cdc28 kinase activity. Over-expression of *CLB2* in *rts1* cells can suppress these cell cycle defects (Shu et al., 1997). Rts1 is also involved in maintaining the spindle checkpoint since the *rts1* strain is sensitive to both nocodazole and benomyl (Wu et al., 2000).

1.4.3.4 Pph21 and Pph22

The yeast *PPH21* and *PPH22* genes are over 90% identical and share 74% identity in amino acid sequence to the mammalian C subunit (Sneddon et al., 1990). Deletion of either *PPH21* or *PPH22* is not lethal, however the phosphatase activity in *pph21*Δ and *pph22*Δ strains are reduced by 51% and 33%, respectively (Sneddon et al., 1990). The *pph21*Δ *pph22*Δ double mutant strain grows very slowly and cells have a shrunken and pear shaped morphology with deregulated cytoskeleton (Sneddon et al., 1990). A *pph21 pph22 pph3* strain is inviable suggesting that the PP2A-related gene *PPH3* can provide some limited PP2A activity in the absence of *PPH21* and *PPH22* although Pph3 may not have the same *in vivo* functions as PP2A (Hoffmann et al., 1994 ; Ronne et al., 1991). *PPH21* and *PPH22* both have amino-terminal extensions of 60 and 68 amino acids, respectively; however, the function of these extensions is not known (Ronne et al., 1991).

Genetic studies suggest that PP2A functions in cell morphogenesis and is required during G2 for the activation of Clb-Cdc28 kinase complexes for mitotic entry. In one study *PPH21* and *PPH3* were disrupted and *PPH22* was expressed from a *GAL1* promoter to create strain H328 (Ronne et al., 1991). Depleting PP2A activity by growing

H328 in glucose-based medium resulted in cells producing abnormal elongated and pear-shaped buds suggesting that PP2A activity was required for proper bud formation (Ronne et al., 1991). In another study, a *pph21-102* strain was used that contains the temperature sensitive *pph21-102* allele on a centromeric plasmid in a *pph21 pph22 pph3* triple deletion background. At 37°C 40% of *pph21-102* cells have abnormal bud morphologies characterized by delocalized actin cortical patches, disorganized actin cables and diffuse chitin deposition. These cells also arrest in G2 with a 2N DNA content, undivided nuclei, a single microtubule organizing center (MOC) short mitotic spindles and fail to enter mitosis. Whether the PP2A activity responsible for the phenotypes observed in the *pph21-102* cells is Cdc55-directed, Rts1-directed or both cannot be discerned from these studies; however, a similar G2 arrest is observed in *rts1* deleted cells at high temperature suggesting that the G2 arrest may be a consequence of the inactivation of Rts1-containing trimers.

Binding of B subunits to the AC core in yeast is regulated by both phosphorylation and methylation of the C subunit (Evans and Hemmings, 2000;Wei et al., 2001; Wu et al., 2000). T364 and Y367 of Pph21 correspond to the conserved T304 and Y307 residues of mammalian $PP2A_C$, which are targets of phosphorylation (Wei et al., 2001). In the H328 strain the galactose-inducible wild type *PPH21* was replaced with *PPH21* alleles containing negatively charged, acidic amino acids in place of T364 or Y367. The T364D or Y367E mutations were able to abrogate Pph21 binding to Cdc55 and greatly decrease its interaction with Tpd3 (Wei et al., 2001). Pph21 mutants with more conserved substitutions (T364A or Y367F) bound Cdc55 and Tpd3 as well as wild type Pph21 (Wei et al., 2001). These data suggest that the phosphoryation of T364 or

Y367 or the replacement of these amino acids with negatively charged residues can abrogate binding of C with the A and B subunits.

PPE1 encodes the yeast PP2A methylesterase and *PPM1* and *PPM2* encode the yeast homologues of mammalian LCMT-I and LCMT-II, respectively (Ogris et al., 1999; De Baere et al., 1999; Wu et al., 2000). C subunit methylation is enhanced by ~50% in the *ppe1* strain suggesting that in exponentially growing cells about half of the C subunit is in the unmethylated form (Wu et al., 2000). Deletion of *PPM1*, but not *PPM2*, significantly decreases Pph21 methylation *in vivo* as determined by C subunit methylation-specific antibodies (Wu et al., 2000; Wei et al., 2001). In addition, wild type and *ppm2* yeast extracts could catalyze the methylation of purified bovine AC dimer *in vitro* using S-[^{3}H] adenosyl methionine, but a *ppm1* strain could not, suggesting that Ppm1, but not Ppm2, has methyltransferase activity against the C subunits (Wu et al., 2000). Deletion of *PPM1* greatly reduced C subunit binding to Cdc55, Tpd3 and Rts1 in co-immunoprecipitation experiments, suggesting that in yeast C subunit methylation is important for forming both trimeric and dimeric PP2A complexes (Wu et al., 2000; Wei et al., 2001).

1.4.3.5 Abundance and localizations of yeast PP2A subunits

The mRNA and cellular protein levels of the yeast PP2A subunits do not vary significantly throughout the cell cycle (Gentry and Hallberg, 2002; Spellman et al., 1998). However, PP2A subunits vary in abundance relative to one another. The relative ratios of PP2A subunits were quantified and for every Tpd3 subunit there are about 8 Pph21 and Pph22 subunits, 0.25 Cdc55 subunits and 2.5 Rts1 subunits (Gentry and

Hallberg, 2002). By GFP-tagging PP2A genes, the localizations of the PP2A subunits throughout the mitotic cell cycle have been determined. Pph21 and Pph22 were uniformly distributed in the cytoplasm and had a more intense signal in the nucleus. Rts1 localizes to the nucleus and cytoplasm at all cell cycle stages, but localizes to the kinetochore and bud neck in a cell cycle-specific manner. Rts1 is localized to the kinetochore during the S and G2/M phases and then translocates to the bud neck upon exit from mitosis where it plays a role in septin organization during cytokinesis (Dobbelaere et al., 2003; Gentry and Hallberg, 2002). Cdc55 localizes to the nucleus and the periphery of vacuoles in all cell cycle stages. Cdc55 also showed cell-cycle specific distributions at the bud neck in post-telophase cells and at bud tips in small to large budded cells (Gentry and Hallberg, 2002). Tpd3 is in the cytoplasm and nucleus in all cell cycle stages, but is also directed to the kinetochore by Rts1 (from S phase to mitosis), to bud tips by Cdc55 (in small to large budded cells), and to the bud neck by both Cdc55 and Rts1 after mitotic exit (Gentry and Hallberg, 2002).

Using deletion mutants of individual PP2A subunits expressing a GFP-tagged PP2A subunit, it was determined that both Rts1 and Tpd3 are dependent on the B and C subunits to maintain the proper frequency and intensity of their localizations throughout the cell cycle. In contrast, Cdc55 was able to localize independently of the presence of the other PP2A subunits, albeit at lower frequency and intensity. Therefore, while trimer formation may be necessary to maintain Cdc55 localization, Cdc55 appears to contain sufficient targeting information that allows it to reach its proper destinations (Gentry and Hallberg, 2002). These findings suggest that Cdc55 may have functions outside the context of the PP2A holoenzyme.

1.5 The Cell Cycle

1.5.1 Overview

The eukaryotic cell division process involves intricate mechanisms to ensure precise duplication and segregation of genetic material to daughter cells. The cell cycle is divided into two main stages. Interphase comprises a gap 1 phase (G1) where cells grow in mass and size, an S (synthesis) phase where DNA is replicated and a gap 2 (G2) phase where DNA damage can be repaired and proteins required for the second stage, mitosis, are synthesized.

Mitosis is the phase where duplicated DNA is segregated into daughter cells and can be further sub-divided into prophase, metaphase, anaphase and telophase. At prophase chromosome condensation occurs and in mammalian cells the nuclear envelope breaks down; however, it remains intact in yeast cells. During metaphase sister chromatids, which are held together by a protein complex termed the cohesin complex, become attached to the mitotic spindle. Sister chromatids become attached to the spindle via the kinetochore complex at their centromeres. Anaphase entails the separation of sister chromatids and their movement to opposite poles of the dividing cell. Finally, once the sisters are segregated into daughter cells, telophase occurs involving the collapse of the mitotic spindle and nuclear division. After nuclear division, cytokinesis occurs which involves the final separation of cytoplasm yielding two daughter cells.

The transition from one phase of the cell cycle to the next is controlled by complex molecular machinery, at the heart of which lies the cyclin dependent kinases (CDKs) and associated cyclins. CDKs rely on association with one of the several cyclins for promoting kinase activity. Cyclin protein levels oscillate during the course of the cell

cycle and direct the activity of the CDKs during the different stages of the cell cycle. Protein complexes that execute the non-reversible degradation of cyclins and other cellular factors are responsible for driving the transitions from one phase of the cell cycle to the next. Cell cycle checkpoint proteins coordinate the arrest of the cell in response to environmental insults or errors in the cell division process. Cell cycle checkpoints serve as internal monitors of spindle assembly, spindle position, cell morphogenesis and DNA damage to ensure fidelity during cell division (Elledge, 1996).

While there are differences in the molecular details of the cell cycle among organisms, fundamental characteristics are conserved among all eukaryotes. As the present study deals with expression of E4orf4 in the yeast *Saccharomyces cerevisiae*, the following discussion of the cell cycle will be focused mainly on the mechanisms at work in budding yeast.

1.5.2 CDK Regulation

Cdc28 is the CDK responsible for regulation of the mitotic cell cycle in *S. cerevisiae*. The CDK involved in the mitotic phase of the cell cycle has also been called Cdc2 in most organisms and is equivalent to Cdk1. Cdc28, like other CDKs, is a proline-directed serine/threonine kinase that phosphorylates proteins with S/T-P motifs. By phosphorylating various substrates Cdc28 regulates the major events of the cell cycle. CDKs are themselves subject to regulation by their association with CDK inhibitors (CKIs), cyclins and by phosphorylation (Mendenhall and Hodge, 1998).

Monomeric Cdc28 is inactive as a kinase and must associate with a cyclin protein. Both activating and inactivating phosphorylations occur at residues conserved across eukaryotes. Phosphorylation of the conserved threonine in the T-loop of Cdc28 is

activating and stabilizes cyclin binding while phosphorylation of conserved threonine and tyrosine residues in the Cdc28 ATP-binding domain hinders phosphate transfer from ATP to substrates (Mendenhall and Hodge, 1998). The CKIs Far1 and Sic1 bind to Cln-Cdc28 and Clb-Cdc28 complexes, respectively and inhibit kinase activity by excluding substrates from the Cdc28 active site (Mendenhall and Hodge, 1998; Barberis et al., 2004).

1.5.2.1 Cyclins

Cdc28 protein levels are constant throughout the cell cycle; however, Cdc28 kinase activity oscillates and is directed by its association with one of the nine cyclins: Cln1, Cln2, Cln3, Clb1, Clb2, Clb3, Clb4, Clb5 and Clb6. Cdc28 associates with different cyclins during different phases of the cell cycle. The various cyclins have several overlapping redundant functions; however, some roles are definite.

Cln3 levels are fairly stable throughout most of the cell cycle, peaking in late G1. Cln3 is part of a size sensor that is involved in monitoring cell size and cell growth rate. Cln3-Cdc28 phosphorylates Whi5 (Rb homolog) promoting the release of the transcription factors SBF and MBF that drive the expression of Cln1, Cln2, Clb5 and Clb6 as well as ~200 genes involved in DNA synthesis and repair (Costanzo et al., 2004; de Bruin et al., 2004; Futcher, 2002).

Cln1/2–Cdc28 complexes peak at START and fall during S phase and are important for promoting bud emergence. Cln1/2-Cdc28 phosphorylates and inactivates the CKI, Sic1, as well as Cdh1/Hct1 (see section 1.6.2.3), which inhibit the activity of Clb-Cdc28 complexes. The Clb5/6-Cdc28 complexes initiate DNA synthesis, the Clb3/4-

Cdc28 complexes mediate early mitotic events, such as spindle formation, and finally the Clb1/2-Cdc28 complexes function in coordinating mid to late mitotic events (Futcher, 1996). Cyclins are regulated by proteolytic mechanisms that involve ubiquitination and degradation by the proteosome. This turnover allows the transition of different Cyclin-Cdc28 complexes during the various cell cycle phases (see section 1.6).

1.5.2.2 Activating Cdc28 Phosphorylation

Cdk1 is regulated by both activating and inhibitory phosphorylation. Even if the inhibitory phosphorylations are removed, the Cdc28-cyclin complex is not fully active until the activating phosphorylation occurs. Phosphorylation of T169 of Cdc28 in *S.cerevisiae* (T161 of Cdc2 in frog and mammalian cells and T167 in *S. pombe*) promotes kinase activation by stabilizing cyclin binding and is mediated by CDK-activating kinase (CAK). A non-phosphorylatable Cdc28-T169A mutant is inactive *in vitro* and does not support cell division *in vivo* (Deshaies and Kirschner, 1995; Lim et al., 1996). The CAK of yeast is encoded by the essential *CAK1* gene. Cak1 activity is constitutive throughout the cell cycle and as such Cdc28-T169 phosphorylation does not appear to be periodic (Espinoza et al., 1996; Sutton and Freiman, 1997). PP2C mediates the dephosphoryation of Cdc28-T169 (Cheng et al., 1999).

1.5.2.3 Inhibitory Cdc28 Phosphorylation

T18 and Y19 are inhibitory phosphorylation sites on Cdc28, equivalent to T14 and Y15 of mammalian Cdc2 (Mendenhall and Hodge, 1998). The phosphorylation of Y19 has been characterized to a much greater extent than T18. Swe1 and Mih1 are the

yeast homologs of mammalian Wee1 and Cdc25. Swe1 and Mih1 represent the dual specificity kinase and phosphatase, respectively, responsible for the phosphorylation and dephosphorylation of the Cdc28 T18 and Y19 residues (Booher et al., 1993; Russell et al., 1989). Swe1 acts specifically on mitotic Clb2-Cdc28 complexes (Booher et al., 1993).

Phosphorylation on Y19 delays entry into mitosis as evidenced by the phospho-mimic mutant Cdc28-Y19E (glutamic acid) that emulates a constitutively inactive Cdc28, and causes a G2 arrest (Sorger and Murray, 1992; Amon et al., 1992). The phosphorylation of Cdc28-Y19 is increased when cells are treated with hydroxyurea (HU) or UV irradiation suggesting that Y19 phosphorylation has a role in S phase arrest and the DNA damage checkpoint, respectively (Amon et al., 1992). However, phosphorylation of Y19 is not essential for arrest to occur because cells carrying the non-phosphorylatable Cdc28-Y19F allele can arrest, like wild type cells, in G2 in the event of DNA damage or in S phase due to incomplete DNA replication (Sorger et al., 1992 ; Amon et al., 1992). This situation differs from that in *S. pombe*, *Xenopus* and mammalian cells, where Y19 phosphorylation is essential for cells to arrest after activation of the DNA damage or replication checkpoints (Jin et al., 1996; Kharbanda et al., 1994). In *S. cerevisiae*, Y19 phosphorylation plays a role in the morphogenesis checkpoint, a mechanism that delay cells in G2 in response to actin cytoskeleton perturbations that delay bud formation, providing time for cells to complete bud formation prior to nuclear division and mitosis (Lew, 2003). This checkpoint regulates Swe1 stability, allowing it to accumulate and inhibit Cdc28 activity (Lew and Reed, 1993; Lew and Reed, 1995a; Lew and Reed, 1995b; McMillan et al., 1999).

1.5.3 PP2A and regulation of Swe1

In a *cdc55* deletion strain it was observed that the phosphorylation levels of Cdc28 in Y19 was higher in both cycling or nocozadole arrested cells than in the wild type strain, suggesting a role for PP2A in regulating Cdc28 phosphorylation (Minshull et al., 1996). Therefore Cdc55/PP2A could be regulating Cdc28 Y19 phosphorylation either by promoting the activity of Swe1 or decreasing the activity of Mih1.

It was found that the activity of Mih1 was not significantly affected in the *cdc55* strain, but Swe1 protein levels were stabilized and could thus account for the increase in Cdc28 Y19 phosphorylation (Yang et al., 2000). Swe1 can bind Cdc28 and inhibit Cdc28 activity independently of its kinase activity (McMillan et al., 1999). Since Swe1 is negatively regulated by the Cdc28 kinase, which phosphorylates Swe1 and promotes its degradation, Swe1 promotes its own accumulation by inhibiting Cdc28.

It is likely that the unregulated PP2A activity in the *cdc55* strain causes the excess Swe1 accumulation since in a *cdc55pph21pph22* deletion strain Swe1 turnover was comparable to that observed for the wild type strain (Yang et al., 2000). These findings suggest that regulatory B subunits can inhibit PP2A activity towards certain substrates as has been shown in other studies (Ferrigno et al., 1993; Kamibayashi et al., 1994; Price et al., 1999). Alternatively, the loss of Cdc55 may result in unregulated Rts1-PP2A activity against substrates normally acted upon by Cdc55-PP2A. Consistent with this possibility is the observation that over-expression of Rts1 in *cdc55* cells exacerbates the abnormal budding phenotype of the *cdc55* strain whereas an *rts1cdc55* strain showed no morphology defects. It will be interesting to see if an *rts1cdc55* has normal Swe1 turnover as this study has not been reported. Whether the target of the unregulated PP2A

activity is Swe1 or some other regulator of Swe1 is not known. Possibilities include Hsl1, Cla4 and Cdc5 (kinases that phosphorylates Swe1), and the SCF complex which is responsible for ubiquitinating and targeting Swe1 for degradation (Sakchaisri et al., 2004; McMillan et al., 2002).

1.6 Cell Cycle Transitions

1.6.1 The Ubiquitin-Proteasome Pathway

Irreversible protein degradation of cyclins and other cell cycle related proteins provides the means of ensuring that the cell cycle will proceed in a uni-directional manner. The degradation of proteins involved in the cell cycle is mediated by the ubiquitin-proteasome pathway. Ubiquitin, an essential 9kD protein of 76 residues, is transferred to target proteins, marking them for degradation via the 26s proteasome.

Three enzymes function to mediate the transfer of ubiquitin to target proteins: E1, E2 and E3. The ubiquitin-activating enzyme, E1, uses ATP to form a high-energy covalent thioester bond between its active site cysteine and the C-terminal glycine residue of ubiquitin. The activated ubiquitin is then transferred to the active site cysteine residue of the E2 ubiquitin-conjugating enzyme (UBC), forming a second thioester linkage. Finally in cooperation with an E3 ubiquitin ligase, E2/UBC catalyzes the formation of an amide isopeptide bond between the lysine residue of the substrate and the C-terminus of ubiquitin. Multiple rounds of ubiquitination results in the formation of a poly-ubiquitin chain, which is recognized by the 26S proteasome, and degradation of the substrate. The E3 enzyme determines which proteins will be ubiquitinated and in this way is believed to provide specificity to the process (Pickart, 2000). There are two main E3 ligases that target cyclins as well as other non-cyclin proteins for degradation. The anaphase

promoting complex/cyclosome (APC/C) functions in mitosis until the end of G1 while the SCF (Skp/Cullin/F-box) complex acts predominantly in G1, S and G2 phases (Vodermaier, 2004).

There are at least three known SCF complexes, SCF1, SCF2 and SCF3 that are defined by the presence of an associated cullin protein Cul1, Cul2 or Cul3. The SCF1 complex is involved mainly in cell cycle control and is composed of Cul1, a zinc binding ring-finger protein (Rbx1/Roc1/Hrt1), an adaptor protein (Skp1) and one of several F-box proteins that confers substrate specificity. The F-box domain of F-box proteins is required for Skp1 association. The ring-finger protein, Rbx1, recruits the E2/UBC, of which there are two with which SCF can interact; Cdc34/Ubc3 and Ubc4/UbcH4. Cul1 links Rbx1 and the E2/Ubc to the Skp1 adaptor protein and the F-box protein. SCF complexes are constitutively active, however phosphorylation of the degron sequence within a given SCF substrates is a prerequisite for F-box recognition and binding (Vodermaier, 2004).

1.6.2 The Anaphase Promoting Complex /Cyclosome

1.6.2.1 APC structure and core subunits

The APC specifically associates with the E2 enzymes Ubc4/UbcH5 or E2-C/UbcH10 in several organisms; however, the APC of budding yeast does not appear to require a specific E2 conjugating enzyme (Townsley and Ruderman, 1998). The core APC/C can be purified as a large 1500 kDa complex and is composed of to date thirteen and eleven subunits in yeast and mammalian cells, respectively (Castro et al., 2005). Functions have yet to be assigned to each of the APC subunits and it remains unclear how the subunits co-operate to function as an E3 ligase.

Compared to the SCF complex there is less known about the architecture of the APC/C, however some parallels can be drawn. The APC subunit, Apc11, is a zinc-binding ring finger protein, analogous to Rbx1 of SCF, and functions to recruit E2 (Gmachl et al., 2000; Leverson et al., 2000). Apc2 contains the cullin homology domain, like Cdc53 of the SCF complex, and together with Apc11 form the catalytic core of the APC. Cdc20, Hct1/Cdh1 and Ama1 are APC activators as well as substrate specificity factors, analogous to the F-box protein of SCF. In contrast to SCF, there appears to be no Skp1-like subunit in the APC and Cdc20 and Cdh1 do not interact with the cullin homolog Apc2 (Vodermaier, 2004).

1.6.2.2 Mitotic APC activators

There exist three APC activators in budding yeast, Cdc20, Hct1/Cdh1 and Ama1, which associate with the core APC machinery. Ama1 is specific to meiosis in *S. cerevisiae* and a homolog has not been identified in fission yeast or vertebrates (Cooper et al., 2000). Cdc20, Hct1 and Ama1 all contain WD repeats, which may mediate interactions with substrate proteins as well as other APC core subunits (Castro et al., 2005). Over-expression of Cdc20 or Hct1 can induce APC-dependent degradation of their respective substrates at all stages of the cell cycle, suggesting that they confer substrate specificity and limit the rate of protein degradation (Zachariae and Nasmyth, 1999). Several studies have demonstrated direct and specific interactions between Cdc20 or Hct1 and their respective substrates (Fang and Kirschner, 1998; Burton and Solomon, 2001; Pfleger et al., 2001; Schwab et al., 2001; Hilioti et al., 2001). Cdc20 and Hct1 are present in sub-stoichiometric quantities compared to the other APC subunits and their

activity is cell cycle regulated. APC^{Cdc20} activity is required for entry into anaphase and APC^{Hct1} is required for mitotic exit and the G1 phase.

1.6.2.3 APC^{Cdc20} and the metaphase to anaphase transition

Once DNA has been duplicated in S phase and sister chromatid pairs have been attached to the mitotic spindle, the cell must trigger the metaphase to anaphase transition, defined by the separation of sister chromatids and their subsequent migration to opposite poles of the dividing cell. APC^{Cdc20} mediates the metaphase to anaphase transition. Pds1 (securin) holds a cysteine protease, Esp1 (separase), inactive until all sister chromatids are properly attached to the mitotic spindle (Cohen-Fix et al., 1996; Ciosk et al., 1998). At that time APC^{Cdc20} targets Pds1 for ubiquitination and degradation, releasing Esp1, which goes on to degrade Scc1, a protein within the cohesin complex that holds sister chromatids together (Michaelis et al., 1997; Uhlmann et al., 1999). The cohesin complex consists of Scc1, Scc3, Smc1, and Smc3 and degradation of Scc1 causes complete dissolution of sister chromatid cohesion (Michaelis et al., 1997; Ciosk et al., 2000). Soluble and chromatin bound pools of Cdc5 exist; however, Cdc5-phosphorylated Scc1 within the cohesin complex is preferentially cleaved by Esp1 (Hornig and Uhlmann, 2004).

During the mitotic phase up until anaphase Cdc28/Clb2 activity is maintained at peak level allowing APC^{Cdc20} activation and phosphorylation of other substrates important for mitotic progression. Once sister chromatids have separated, the APC^{Cdc20} begins the process of Clb2 ubiquitination, thus lowering Cdk1 activity (Yeong et al., 2000). Activation of the second APC complex, APC^{Hct1}, is required to complete Clb2

degradation, putting the cell in a state of low CDK activity and allowing mitotic exit and G1 entry (Yeong, 2000).

1.6.2.4 Switching from APC^{Cdc20} to APC^{Hct1}: The FEAR and Mitotic Exit Networks

The FEAR (Cdc14 early anaphase release) network and Mitotic Exit Network (MEN) function to mediate the switch from APC^{Cdc20} to APC^{Hct1} by promoting the release of Cdc14, a serine/threonine phosphatase (Stegmeier and Amon, 2004).

Cdc14 is sequestered in the nucleolus by a protein complex termed RENT (regulator of nucleolar silencing and telophase) via its interaction with the Net1 (or Cfi1) protein. The FEAR network promotes the phosphorylation of Net1 and the initial transient release of Cdc14 into the nucleus during early anaphase {Stegmeier et al., 2002; Pereira et al., 2002; Yoshida and Toh-e, 2002). A non-proteolytic activity of free Esp1 induces the release of Cdc14 from Net1 by an unknown mechanism requiring a group of proteins (Spo12, Slk19, and Cdc5) that together with Esp1 comprise the FEAR network (Stegmeier et al., 2002; Sullivan and Uhlmann, 2003). Cdc5 can induce phosphorylation of Cdc14 and Net1; however, in the case of Net1 this effect seems to be by an indirect mechanism (Visintin et al., 2003; Yoshida and Toh-e, 2002; Shou et al., 2002). Azzam and colleagues have shown that the direct phosphorylation of Net1 by Cdk1 is necessary for Cdc14 release (Azzam et al., 2004). Thus Cdk1 appears to be involved in its own down-regulation by a negative feed-back mechanism. Besides its function to inactivate Cdk1, the FEAR network is also involved in regulating spindle stability, microtubule forces, and the partitioning of DNA and nucleoli (D'Amours and Amon, 2004).

The initial release of Cdc14 by the FEAR functions to positively regulate the MEN by a forward amplification mechanism. FEAR is distinct from the MEN since it cannot induce Cdk1 inactivation and mitotic exit as the MEN does (Stegmeier et al., 2002). MEN components include a GTPase, Tem1, the guanine nucleotide exchange factor (GEF) Lte1, the GTPase activating protein (GAP) heterodimer Bub1 and Bfa1, the scaffold protein Nud1, and the kinases, Cdc15, Dbf2-Mob1 and Cdc5 (Yeong et al., 2002). Inactive Tem1-GDP interacts with Bub2-Bfa1 at the spindle pole bodies within the mother cell, while Lte1 is sequestered in the cortex of the bud. As the spindle moves into the bud during nuclear division, Lte1 is able to activate Tem1 to its GTP form, thus triggering a signaling cascade. Cdc5 positively regulates the MEN by phosphoryating and inactivating Bub2-Bfa1, further promoting Tem1 activity. Tem1-mediated activation of Cdc15, Dbf2 and Mob1 results in further phosphorylation of Net1 and Cdc14, amplifying Cdc14 release from the nucleolus to the rest of the cell (D'Amours and Amon, 2004).

Sustained Cdc14 release by the MEN results in the dephosphorylation of several proteins, many which are believed to be substrates of Cdk1, thereby reversing the mitotic state of the cell. Importantly, Cdh1 is dephosphorylated, allowing its association with the APC complex and Sic1 is dephosphorylated allowing its accumulation and inhibition of CDKs (Visintin et al., 1998; Jaspersen et al., 1999).

1.6.2.5 APC^{Hct1} and mitotic exit

APC^{Hct1} completes the destruction of Clb2 initiated by APC^{Cdc20}, shutting off Cdk1 activity and resetting the cell cycle (Noton and Diffley, 2000). By initiating the destruction of Clb2 and allowing the activation of Hct1, APC^{Cdc20} promotes is own

demise, as Cdc20 is also a substrate for APC^{Hct1} (Shirayama et al., 1998). This effect ensures that the first APC complex is shut off and contributes to the unidirectional nature of the cell cycle. APC^{Hct1} also mediates the degradation of Cdc5 (Shirayama et al., 1998).

APC^{Hct1} is shut off at the G1/S transition when Hct1p becomes phosphorylated and inactivated, predominantly by Clb5-Cdc28 (Jaquenoud et al., 2002). Phosphorylation not only prevents binding of Hct1 to the APC (Zachariae et al., 1998; Jaspersen et al., 1999), but leads to inactivation of Hct1 by nuclear export (Jaquenoud et al., 2002). Hypo-phosphorylated Hct1p is nuclear during the G1 phase, but redistributes to the cytoplasm between S phase and the end of mitosis upon phosphorylation by Clb5-Cdc28 (Jaquenoud et al., 2002). In this way APC^{Hct1} activity is restricted to the points in the cell cycle when CDK activity is low.

1.6.2.6 APC Phosphorylation

APC activity is regulated by the phosphorylation of its core subunits. The phosphorylation of some APC subunits may have activating effects, whereas the phosphorylation of others can be inhibitory. The phosphorylation state of the core subunits does not appear to affect the formation of the core APC complex (Peters et al., 1996). Phosphorylation by Clb2-Cdc28 and Cdc5 is activating towards the APC (Charles et al., 1998; Rudner and Murray, 2000). cAMP-dependent protein kinase (PKA) phosphorylates APC subunits of *S. pombe* and mammalian cells and inhibits APC activity (Kotani et al., 1998; Kotani et al., 1999). The RAS/protein kinase A (PKA) signaling pathway in budding yeast is also inhibitory to the APC (Bolte et al., 2003; Irniger et al., 2000).

The APC core subunits Apc1, Cdc27, Cdc16, Cdc23 and Apc9 (which is present only in budding yeast) are phosphorylated during mitosis in frogs, fission yeast, budding yeast and mammalian cells (Kotani et al., 1999; Rudner and Murray, 2000). The budding yeast APC subunits Cdc16, Cdc23 and Cdc27 were shown to be phosphorylated *in vitro* by purified recombinant Cdc28-Clb2 in the presence of γ^{32}P-ATP as well as *in vivo* by labelling of synchronized yeast cells with $^{32}PO_4$ (Rudner and Murray, 2000). The APC immunoprecipitates from a *cdc5-1* strain demonstrated that the phosphorylation of these subunits was due to mainly to Cdc28 and not co-precipitating Cdc5 (Rudner and Murray, 2000). Nonetheless, using *cdc28-1N* and *cdc5-1* kinase defective mutant strains it was demonstrated that both Cdc28 and Cdc5 can contribute to the *in vivo* phosphorylation of Cdc16, Cdc23 and Cdc27 (Rudner and Murray, 2000).

To confirm further that Cdc28 can phosphorylate these APC subunits *in vivo*, all the potential Cdc28 phosphorylation sites within Cdc16, Cdc23 and Cdc27 were mutated to alanines and *in vivo* labeling of these phospho-mutant strains with $^{32}PO_4$ demonstrated that the individual APC mutant proteins were resistant to phosphoryation and a triple mutant strain could not be phosphorylated at all. It appears that Cdc28, and not Cdc5, is responsible for the direct phosphorylation of APC subunits, nonetheless, Cdc5 is important for APC activity since APC immunoprecipitates from *cdc5-1* cells grown at the non-permissive temperature have a decreased ability to ubiquitinate cyclin B *in vitro* (Charles et al., 1998). As Cdc5 contributes to APC subunit phosphorylation *in vivo* and APC activity *in vitro*, it has been suggested that Cdc5 may phosphorylate Apc1, -2, -4, -5 *in vivo* (Cdc5 substrates *in vitro*) and affect the phosphorylation of the Cdc28 APC

targets or Cdc5 may modulate the activity or localization of Clb/Cdc28 complexes (Rudner and Murray, 2000).

1.7 Thesis Proposal

To better understand the mechanism of E4orf4 induced cell death, I have used the yeast *Saccharomyces cerevisiae* as a model system. *S. cerevisiae* offers several advantages for such studies in that it is highly amenable to genetic manipulation and many cellular processes are well conserved between yeast and mammalian cells. With this in mind the following studies set out to first establish that the E4orf4 protein was toxic in yeast and that the molecular interactions with PP2A were conserved. The yeast system also had the potential to provide the first direct genetic evidence that the B subunit of PP2A was absolutely required for E4orf4-mediated cell death. Secondly, using the yeast system as a tool to study E4orf4 killing, we intended to elucidate a probable mechanism for E4orf4-mediated cell death, which could be translated back to mammalian cells. Two main approaches were taken. The first involved building on an observation made in the second chapter of this work, specifically the modulation of mitosis by E4orf4. The study of the cell cycle effects of E4orf4 has revealed a novel role for Cdc55-PP2A in mitotic regulation. Through the combined efforts of the yeast community, all the non-essential genes have been systematically deleted and arrayed for use on a robotic platform for synthetic gene analysis (SGA). In our second approach, we made use of these tools with some modification, in an attempt to find downstream effectors of E4orf4's killing mechanism. The logic was that if a downstream target of the E4orf4 death pathway was deleted, cells should be able to tolerate E4orf4 expression. Success by this method required that there was a single predominant target of E4orf4 or the E4orf4-

PP2A complex and that this protein be encoded by a non-essential gene.

Chapter 2: Toxicity of Human Adenovirus E4orf4 Protein in *Saccharomyces cerevisiae* Results from Interactions with the Cdc55 Regulatory B Subunit of PP2A

2.1 Introduction

The high degree of conservation of the PP2A enzyme from mammals to yeast suggested that *Saccharomyces cerevisiae* could provide a genetically tractable system in which to study the molecular mechanism of the E4orf4-PP2A complex. As a case in point, although yeast do not undergo apoptosis, the mammalian pro-apoptotic protein Bax is lethal to yeast and genetic screening in yeast successfully identified proteins involved in the mechanism of Bax-induced cell death in mammalian cells (Greenhalf et al., 1996; Xu and Reed, 1998; Matsuyama et al., 1998; Marzo et al., 1998). These studies demonstrated that the core machinery involved in Bax-induced cell death was conserved between mammalian cells and yeast. Such studies suggested that yeast could be a powerful tool for defining the mechanism of E4orf4-induced cell death. In addition, experiments in a yeast system would allow a direct genetic analysis of the requirement of E4orf4 for the B regulatory subunit, as the work thus far in mammalian systems had been largely correlative. For yeast to represent a feasible system in which to conduct such studies, it was hoped that E4orf4 would elicit a significant biological effect. In addition, it was of interest to determine if the E4orf4-B subunit interactions were conserved.

We subcloned the E4orf4 gene into a galactose-inducible yeast vector to test the effects of E4orf4 expression in yeast. The results of the present study show that E4orf4 is lethal to yeast with cells accumulating in mitosis and having high levels of Cdk1 activity. While E4orf4-induced cell death in mammalian cancer cells has been correlated with its interaction with the Bα subunit, here we have used a genetic approach to demonstrate that the E4orf4-induced cell death is largely dependent on the presence of *CDC55,* encoding the regulatory B55/B class subunit, but not *RTS1*, the B56/B′ class subunit. In

addition, the molecular interactions between E4orf4 and PP2A were conserved in yeast and the interaction with the PP2A holoenzyme was entirely dependent on the interaction of E4orf4 with Cdc55. Nevertheless, some growth inhibition was also detected in the absence of E4orf4-Cdc55 interactions, suggesting that E4orf4 may possess a second Cdc55-independent mechanism of toxicity.

2.2 Materials and Methods

2.2.1 Yeast strains, media and culture conditions.

All studies were performed using haploid *S. cerevisiae* strains derived from the SEY6210 diploid strain (*leu2-3 112 ura3-52 his3-Δ200 lys2-801 trp-Δ901 suc2-Δ9*). The BY4742 strain (*MATα his3D1 leu2DO lys2DO ura3DO*) and the EGY48 strain (*MATα trp1 ura3 his3 leu2::plexAop-leu2*) were also used. MC75 (*MATα thr5 met*) and MC76 (*MAT*a *lys1 cry1*) strains were used to determine the mating type of SEY6210 haploids. Yeast transformations were performed using the one-step method of Chen *et al.* (Chen et al., 1992). Transformants were selected on synthetic minimal media containing the appropriate auxotrophic supplements.

2.2.2 Plasmids.

Wild type hemagglutinin-tagged E4orf4 (HA-E4orf4), HA-E4orf4 point mutants, HA-E311.6K and HA-Bax were subcloned from mammalian expression vectors into the high copy pYES2 vector (Invitrogen) under the control of the *GAL1* promoter. p424*GAL1*-FLAG-*CDC55* was constructed as follows. *FLAG* tagged-*CDC55* was obtained by PCR cloning from yeast genomic DNA using forward primer 5'-

GGGGTACCACTAGTATGGACTACAAAGACGATGACGATAAAATGGCACAAA ACAATTTTGATTTTAAATTC-3' and reverse primer 5'- AATCCCCTCGAGTTATA ATGCGGAAAAAATGAATAAATTATTAG-3', followed by restriction enzyme digest (*Spe*I and *Kpn*I) and subcloning into the p424*GAL1* (2μ, TRP selection) vector purchased from the American Type Culture Collection (ATCC). Plasmids p424*GAL1*-FLAG-*PPH21* and p424*GAL1*-FLAG-*PPH22* were constructed in a similar manner. FLAG-tagged *PPH21* was obtained by PCR using forward primer 5-CGGGATCCATGGACTACAAAGACGATGACGATAAAATGGATACAGATTTAGA TGTGCC-3' and reverse primer 5'-AATCCCCTCGAGTCATAAAAAGTAATCTGG CGTC-3' followed by restriction enzyme digestion (*Xho*I and *Bam*HI) and subcloning. Forward primer 5'-CGGGATCCATGGACTACAAAGACGATGACGATAAAATG GATATGGAAATTGATGACCC-3' and reverse primer 5'-GGAATTCTTATAAGA AATAATCCGGTGTC-3' were used to generate FLAG-tagged *PPH22* PCR product which was digested with *Bam*HI and *Eco*RI and subcloned into the p424*GAL1* vector. The YEP352 and YEP352-*RTS1*-HA_3 plasmids were kindly provided by Richard Hallberg.

2.2.3 Gene Disruptions.

Gene disruptions were performed on the SEY6210 diploid strain, followed by tetrad dissection after sporulation. *CDC55* and *RTS1* deletions were generated by the PCR-based disruption method using the kanMX2 module, as described in Wach *et al.* using the pFA6-kanMX2 plasmid as template (Wach et al., 1994). The *cdc55::kanMX2* disruption in SEY6210 was created using the 5'-disruption primer

5'CCTCATAAAATCTAGCCAACATATCGAGGTCAAACTGGAGAG**GATATCAA GCTTGCCTCG**-3' and the 3'-disruption primer 5'-GAATTCAAGTTCAATTTAAA TTTCAATTAAAACAGTAGTAGTATGTGGGGAAGATATGG**GGTCGACACTGG ATGGCGGC**-3'. The *rts1::kanMX2* disruption was also produced in the SEY6210 strain using the 5'-disruption primer 5'-CAATATGATGCTTGGTTTCAAGCAAAGA TTAATAAAGAAGAC**GATATCAAGCTTGCCTCG**-3' and the 3'-disruption primer 5'-CTTCGAGCTTGTAATGAATTGCTGTTTCACTGTATCTCGCT**GGTCGACAC TGGATGGCGG**. Bold sequences correspond to the kanMX2 module and the remainder to the 5' or 3' region of the deleted gene, as indicated. PCR products were transformed into diploid SEY6210 yeast using the high efficiency method of Gietz *et al.* (Gietz et al., 1995) and transformants were selected on YEPD plates containing 200 μg/ml geneticin (GIBCO). Disruptions were confirmed by PCR diagnostics.

2.2.4 Western Blot analysis.

Whole cell extracts were prepared by resuspending cells in yeast lysis buffer (25mM TrisCl pH 7.4, containing 125mM NaCl, 2.5mM EDTA, 1% Triton X-100 and protease inhibitors) followed by vortexing with acid-washed glass beads (Sigma). Alternatively, whole cell extracts were also prepared using Yeast Protein Extraction Reagent (Pierce). Protein was quantified by Bio-Rad protein assay reagent and 15 μg of total protein per sample was resolved by SDS-PAGE. Separated protein was transferred to PVDF membranes (Millipore) and immunoblotted with the indicated antibodies. Mouse monoclonal anti-HA antibody (HA.11, BAbCO) and mouse monoclonal M2 Flag antibody (Kodak/Sigma-Aldrich) were both used at 1/1000 dilution. The anti-Tpd3 rabbit polyclonal antiserum was a kind gift from Dr. James Broach (Princeton University) and

used at 1/2000 dilution. Membranes were incubated with secondary antibody linked to horse radish peroxidase (Jackson ImmunoResearch) at 1/10 000 dilution, followed by ECL detection (NEN Life Science Products).

2.2.5 Co-immunoprecipitation and phosphatase assays.

Yeast transformed with the indicated plasmid DNAs were grown in yeast nitrogen base (YNB) medium containing 2% (wt/v) glucose, transferred to glycerol (2%)-based medium, followed by induction of gene expression in YNB galactose (2%) for 24 hours. Cells were harvested and resuspended in yeast lysis buffer. Cells were lysed with acid washed glass beads (Sigma) and precleared cell lysates were immunoprecipitated with either HA (HA.11, BabCO) or FLAG (Kodak/Sigma-Aldrich) monoclonal antibody, followed by incubation with Protein G Sepharose beads (50% slurry in lysis buffer). Immunoprecipitates were washed five times with lysis buffer and then eluted by boiling in sample buffer. Samples were resolved by SDS-PAGE using gels containing 10% or 12% polyacrylamide and analyzed by western blotting using the appropriate antibody. For phosphatase assays, the precipitates were washed five times in lysis buffer and once in Ser/Thr phosphatase buffer (50mM Tris-HCl, pH 7, 0.1mM $CaCl_2$) and activity against a synthetic phosphopeptide substrate (RKpTIRR) was assayed using a malachite green-based assay kit (Upstate Biotechnology).

2.2.6 Spotting Assays and Colony Formation assay.

For spotting assays, yeast cells were grown in liquid YNB glucose (2% wt/vol)-based medium followed by YNB containing 2% glycerol or 2% raffinose in the case of the *rts1* deletion strain. Equal numbers of cells were serially diluted and spotted onto

YNB agar plates containing 2% galactose and 1% glycerol / 1% raffinose to induce gene expression from the *GAL1* promoter or onto YNB glucose (2%) control plates. Photographs were taken on the indicated day post-galactose induction. For colony formation assays, SEY6210 wild-type yeast containing either vector alone, pYES2-HA-E4orf4 or pYES2-HA-Bax plasmid were grown in YNB glucose liquid medium, then transferred to glycerol-containing medium. Cells were cultured in galactose medium to induce protein expression. At the indicated times post-induction, approximately 300 cells were plated in triplicate on medium containing 2% glucose and incubated at 30°C. Colonies were counted 48 hours post-incubation.

2.2.7 FACS analysis.

Wild type yeast transformed with pYES2-HAE4orf4 or vector alone were collected 24 hours after galactose induction. Cells were prepared for cell cycle analysis following the method outlined in Dien *et al.* (Dien et al., 1994). Propidium iodide stained cells were acquired on a FACScan instrument and analyzed with WinMDI software.

2.2.8 Cdk1 Kinase Assay.

Wild type or *cdc55* strains of yeast expressing vector or E4orf4 were induced in galactose medium for 24 hours. Cells were lysed as described above and 300 μg of whole cell extract was immunoprecipitated with 1μg of rabbit polyclonal Clb2 antibody (kindly provided by Doug Kellogg) and protein A Sepharose beads (50% slurry). Precipitates were washed five times in lysis buffer and one time in kinase assay buffer (50mM HEPES, pH 7.5, 1mM EGTA, 2mM $MgCl_2$, 1mM DTT). Precipitates were incubated for 30 minutes at 30°C in 20μl of reaction mix containing kinase assay buffer, 5μM cold

ATP, 10μg H1 histone (Sigma), and 10μCi γP^{32}-ATP (6000Ci/mmol specific activity). Kinase reactions were stopped by adding 10μl of 4x sample buffer followed by boiling for 5 minutes. Reactions were subject to SDS-PAGE containing 10% polyacrylamide and transferred to PVDF. Membranes were exposed to film (Kodak Biomax) to visualize the level of H1 phosphorylation.

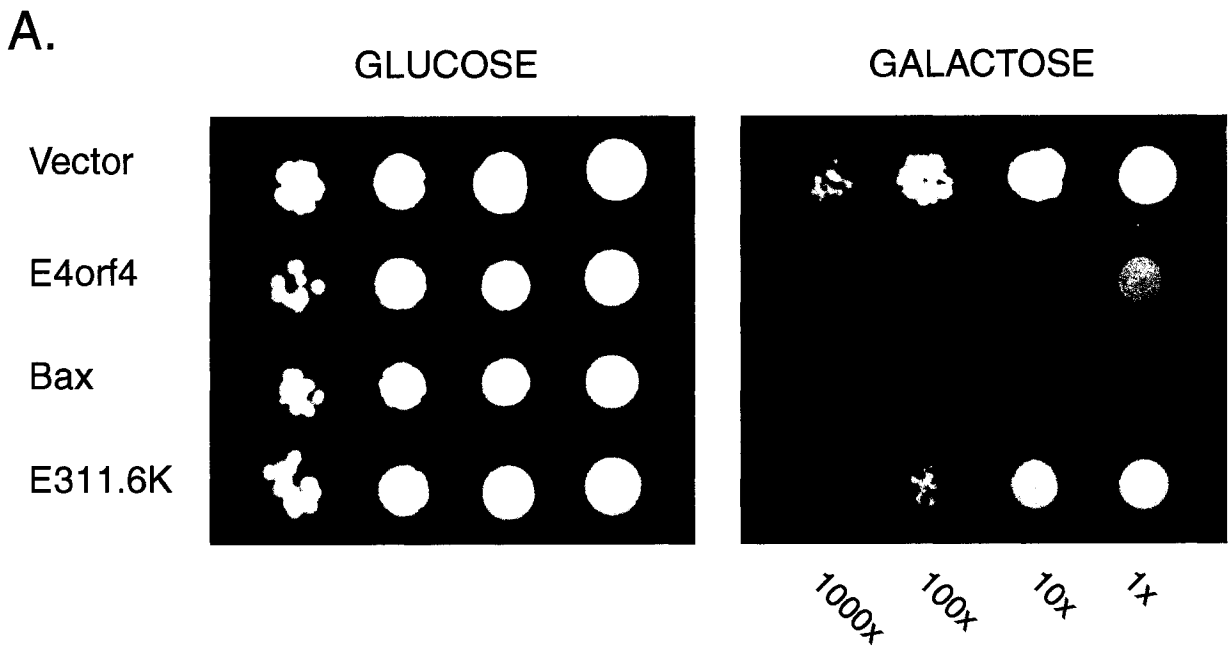
2.3 Results

2.3.1 Conditional expression of E4orf4 in yeast renders cells inviable and promotes an abnormal elongated morphology similar to *cdc55* strains.

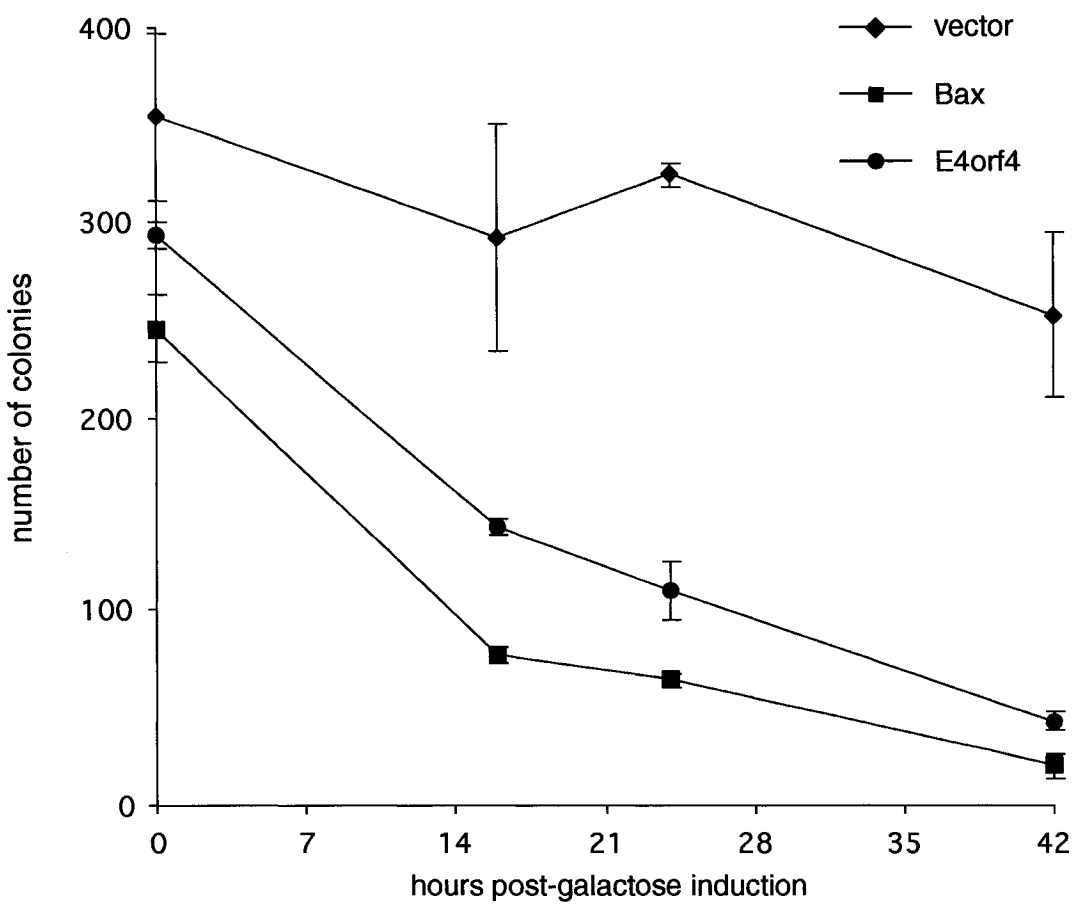
HA-tagged E4orf4 cDNA was subcloned into the episomal yeast vector pYES2 under the control of the *GAL1* promoter. The *GAL1* promoter is repressed in the presence of glucose and induced by galactose, thus allowing differential expression of E4orf4 based on the carbon source in the growth medium. To determine if E4orf4 expression had any effect on yeast colony growth, spotting assays were performed and the effects of E4orf4, E311.6K, Bax, or empty pYES2 vector, on cell growth were compared. Although the toxic effects of E311.6K are unknown, Bax is a cellular pro-apoptotic protein that has previously been shown to be lethal in yeast (Greenhalf et al., 1996; Zha et al., 1996). Figure 2-1B shows that all proteins were expressed at high levels. Two closely-migrating forms of E4orf4 were apparent, as seen in mammalian cells (Lavoie et al., 1998; Marcellus et al., 2000. The molecular basis for the presence of two E4orf4 species is not known. Figure 2-1A shows that E4orf4 expression significantly delayed colony growth compared to the vector control. The E311.6K protein had some minor effect, which became undetectable past day 3, and was not considered further. As demonstrated

Figure 2-1 Effect of E4orf4 protein expression on yeast colony growth

A. Wild type SEY6210 haploid yeast was transformed with control vector, HA-E4orf4, HA-Bax or HA-E311.6K containing plasmids. Equal numbers of cells were serially diluted and equal volumes were spotted onto either glucose control plates or galactose inducing plates. Pictures were taken after 3 days incubation at 30°C. **B**. Anti-HA immunoblot of yeast whole cell extracts. **C**. SEY6210 cells containing vector, E4orf4 or Bax were grown in glucose-based medium followed by glycerol-based medium and then protein expression was induced in galactose medium. At the indicated times post-galactose induction, approximately 300 cells were plated onto glucose-based medium in triplicate. The number of colonies formed after 2 days of incubation at 30°C was counted. Each time point is the average of the number of colonies counted on 3 plates.


A.
GLUCOSE
GALACTOSE
Vector
E4orf4
Bax
E311.6K
1000x
100x
10x
1x
B.
vector
E4orf4
E311.6K
Bax
kDa
28 -
18 -
anti-HA

C.


vector
Bax
E4orf4
number of colonies
400
300
200
100
0
0
7
14
21
28
35
42
hours post-galactose induction

previously, Bax expression resulted in a dramatic cessation of colony growth. E4orf4 expressed in the SEY6210 diploid strain also resulted in impaired growth similar to the haploid strain. To assess whether or not E4orf4 expression is lethal to yeast, a colony formation assay was performed. Cells were cultured in glucose-based medium, transferred to glycerol-containing medium for 2 hours and then finally expression of E4orf4 was induced in medium containing 2% galactose. At various times following the induction of E4orf4, Bax or the vector control, approximately 300 cells were plated onto glucose-based medium. The number of cells which remained viable and thus formed colonies on the plates were counted. Figure 2-1C shows that cells expressing E4orf4 formed significantly fewer colonies than the vector control and displayed toxicity similar to the positive control for cell death, Bax. Figure 2-2B shows that expression of E4orf4 induced an abnormal elongated morphology in about 25-35% of cells in the culture that was not apparent with cells expressing the empty plasmid vector alone (Figure 2-2A). This aberrant morphology was similar to that observed in *cdc55* strains at low temperatures, where over 90% of the cells were elongated (Figure 2-2C). This common morphological phenotype as well as the conservation of PP2A from yeast to mammalian systems suggested that E4orf4 may be targeting Cdc55, the yeast homolog of the mammalian Bα subunit, possibly indicating a molecular link between E4orf4 function in mammalian cells and yeast.

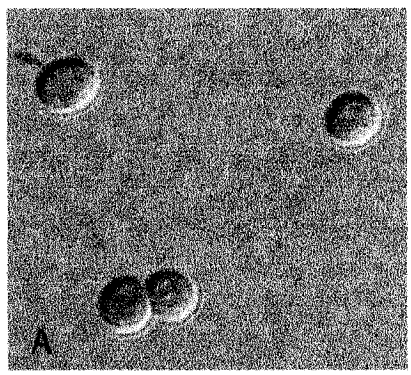
2.3.2 E4orf4 requires *CDC55* to elicit full toxicity in yeast.

Previous work using E4orf4 point mutants has correlated induction of cell death in mammalian tumor cell lines with binding of E4orf4 to the Bα subunit of PP2A

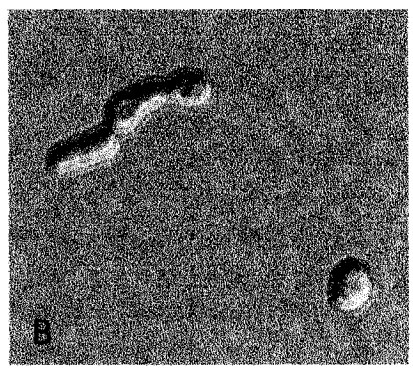
Figure 2-2 E4orf4 expression in wild type yeast confers an elongated cell morphology

Yeast transformed with **A**. vector alone or **B**. E4orf4 were cultured in galactose-based medium at 30°C. **C**. The *cdc55* strain was grown in galactose medium at 18°C. Live cells were mounted onto glass slides and viewed at 100x magnification with a light microcope (Axioplan, Ziess) using Nomarski DIC optics. Images were captured and visualized on a PC computer using a digital-output CCD camera and SPOT Software v2.2 (Diagnostic Instruments).

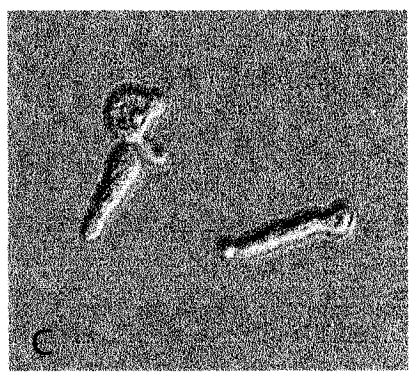
Vector - WT



E4orf4 - WT



cdc55



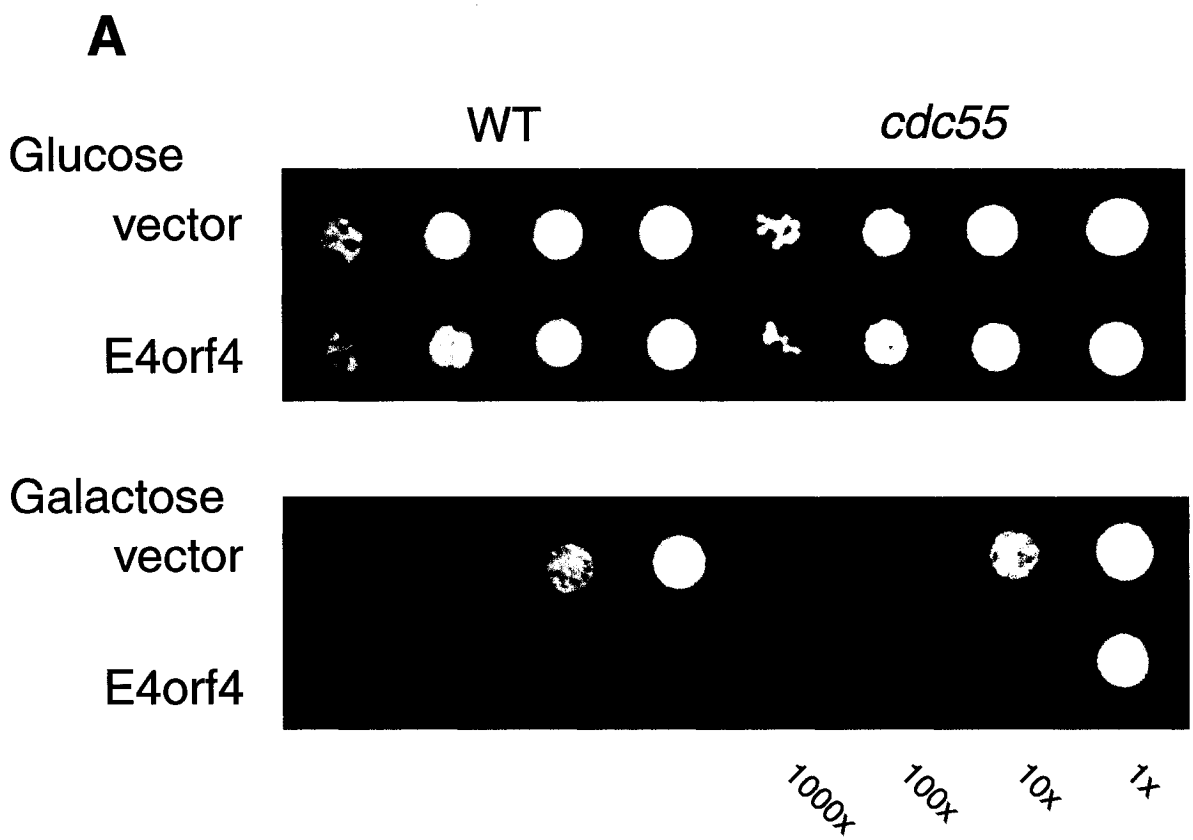
(Marcellus et al., 2000; Shtrichman et al., 1999); however, more direct genetic analyses in mammalian cells have not been possible due to the lack of appropriate 'knock-out' cell lines. To demonstrate such a direct requirement for the B subunit, a *cdc55* strain was created using standard protocols that allow replacement of the *CDC55* gene with a kanamycin cassette. Spotting assays were then performed using wild type yeast or the *cdc55* strain transformed with either empty vector or one expressing E4orf4. Figure 2-3B shows the expression of E4orf4 in these cells following western blotting of equal amounts of cell protein. Figure2-3A shows that in the absence of *CDC55*, the E4orf4-induced growth delay was greatly diminished. These results indicate that E4orf4 mediates much of its effect in yeast via the Cdc55 subunit; however, as illustrated in figure 2-3A, and as seen repeatedly, a slight degree of growth inhibition was nevertheless apparent in the *cdc55* cells. These results suggested that E4orf4 may affect growth via two mechanisms, one dependent upon and one independent of Cdc55 (see more below). In addition, the level of expression of E4orf4 in the *cdc55* strain was observed to be greater than in wild type cells. While the basis for this difference is unclear, it is possible that the cells attempt to down regulate E4orf4 expression when the Cdc55 target is present in an effort to reduce E4orf4-induced, Cdc55-dependent effects on growth.

2.3.3 E4orf4 requires Cdc55 to delay yeast cells in mitosis.

Genetic mutation studies have shown that PP2A subunits are involved in cell cycle regulation (Janssens and Goris, 2001). To determine if yeast cells over-expressing E4orf4 accumulate in a specific phase of the cell cycle, E4orf4- and vector-expressing

Figure 2-3 E4orf4 requires *CDC55* to induce the majority of its toxicity

Wild type and *cdc55* cells were transformed with vector or E4orf4. **A.** Yeast were serially diluted and spotted onto glucose- or galactose-based agar plates followed by incubation at 30°C. Photographs were taken at day 2 post-induction. **B.** Whole cell extracts prepared from yeast cells collected from the above plates were subject to SDS-PAGE immunoblot assay using anti-HA antibody. Lanes 1 and 3 represent empty vector DNA alone and lanes 2 and 4 represent E4orf4 expression.

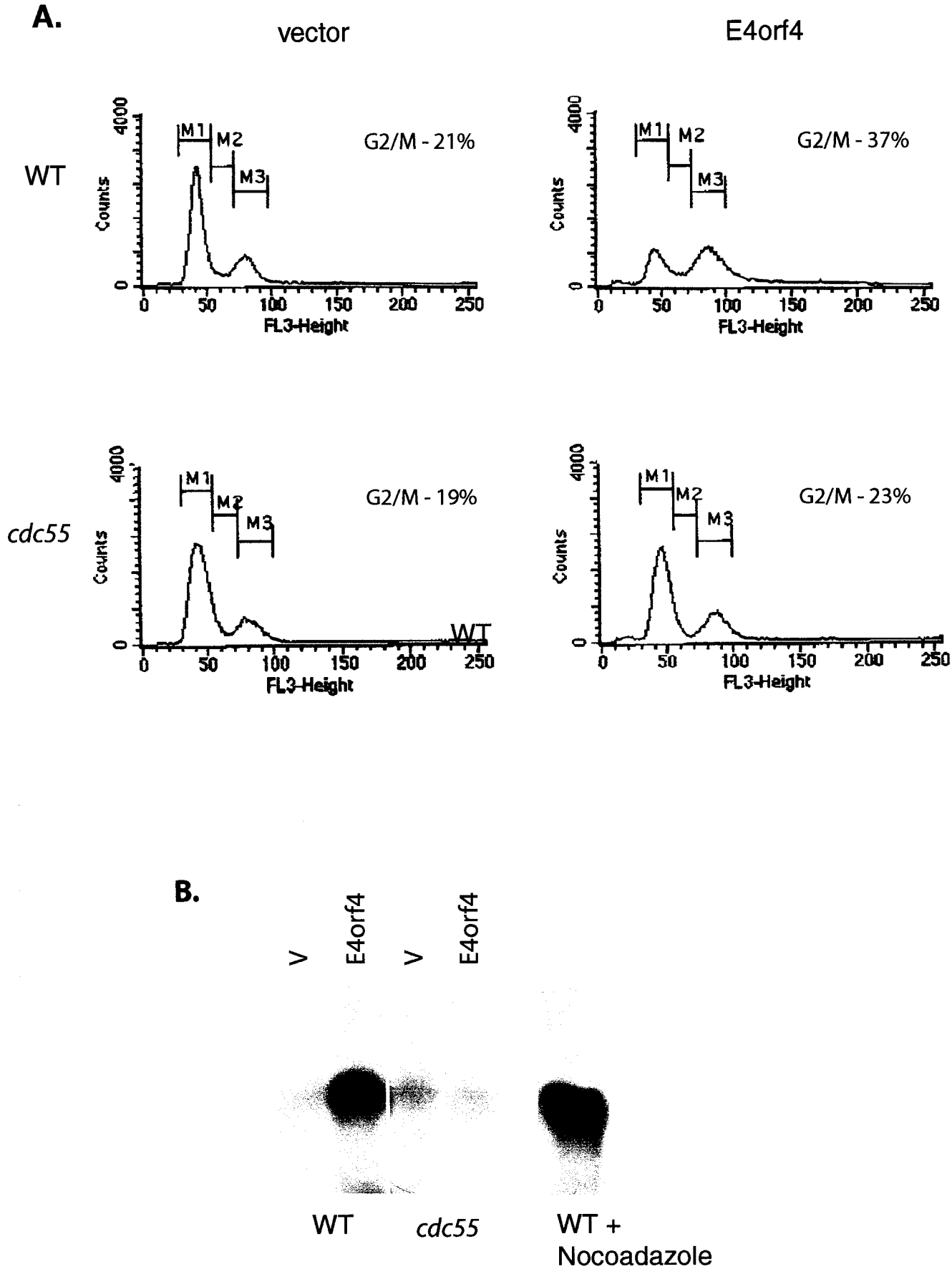

A
WT
cdc55
Glucose
vector
E4orf4
Galactose
vector
E4orf4
1000x
100x
10x
1x


B
WT
cdc55
anti-HA
1
2
3
4

yeast were analyzed by FACS at 24 hours post-induction in wild type and *cdc55* cells. In wild type cells E4orf4 induced a significantly increased population of cells having a 2N DNA content compared to the control suggesting that E4orf4 induces a growth arrest or growth delay during the G2 or M phases (Figure 2-4A). E4orf4 did not appear to have any effect on the cell cycle in the *cdc55* strain (Figure 2-4A). To differentiate whether E4orf4 was inducing a G2 or M arrest, we assayed the activity of Clb2-associated Cdc28. Cells arrested in mitosis maintain high levels of Cdc28-Clb2 kinase activity since mitotic cyclins are not degraded. If cells expressing E4orf4 were arrested or accumulating in mitosis, the Cdc28-Clb2 complex should remain active. To further investigate this possibility immunoprecipitation-kinase assays were performed. Wild type or *cdc55* yeast expressing vector or E4orf4 were lysed 24 hours post-galactose induction and the Cdc28-Clb2 complex was immunoprecipitated using anti-Clb2 antibody. The immunoprecipitates were then tested for their ability to phosphorylate H1 histone. As is evident from figure 2-4B, Clb2-immunoprecipitates from wild type yeast expressing E4orf4 phosphorylated H1 at much higher levels than Clb2 precipitates from cells expressing the vector DNA control. Thus, E4orf4 expression induced Cdc28-Clb2 kinase activity. As a positive control wild type cells containing vector DNA alone were treated with nocodazole to induce mitotic arrest and immunoprecipitates from these cells also displayed high levels of phosphorylated histone (Figure 2-4B). In contrast, E4orf4 did not show any significant effect on Cdc28-Clb2 kinase activity compared to the control in the *cdc55* strain as Clb2 immunoprecipitates from these extracts showed similar levels of H1 phosphorylation (Figure 2-4B).

Figure 2-4 Yeast expressing E4orf4 induces a mitotic delay/arrest and maintains Cdc28-Clb2 kinase activity only in the presence of *CDC55*

A. Cells expressing vector or E4orf4 were collected 24 hours post galactose induction, prepared for staining with propidium iodide, and subject to FACS analysis. M1 represents 1N DNA content (G1 cells), M2 denotes cells in S phase, and M3 represents 2N DNA content (G2/M cells). **B.** Whole cell extracts from wild type or *cdc55* cells expressing vector or E4orf4 were immunoprecipitated with Clb2 antibody. Whole cell extract from wild type cells treated with 10μg/ml of nocodazole for 3 hours was also subject immunoprecipitation with anti-Clb2 and served as a positive control for mitotic arrest. Kinase assays were performed to determine the activity of the Cdc28-Clb2 complex towards histone H1 *in vitro*. The lanes shown represent samples processed at the same time, run on the same gel and exposed to film for an identical period of time.


A.
vector
E4orf4
WT
cdc55
M1
M2
M3
G2/M - 21%
G2/M - 37%
G2/M - 19%
G2/M - 23%
Counts
FL3-Height
WT
B.
V
E4orf4
V
E4orf4
WT
cdc55
WT +
Nocoadazole

2.3.4 E4orf4 requires interaction with Cdc55 to recruit the PP2A holoenzyme.

To determine if the requirement for *CDC55* in the E4orf4-induced slow growth phenotype involves an interaction between E4orf4 and Cdc55, co-immunoprecipitation experiments were carried out. Cells lacking *CDC55* were co-transformed with HA-tagged E4orf4 and/or FLAG-tagged Cdc55. Whole cell extracts prepared at 24 hours post-galactose induction were immunoprecipitated with either anti-HA or anti-Flag antibodies, and following separation by SDS-PAGE and transfer, western blotting was performed using the appropriate antibodies. E4orf4 and Cdc55 were found to co-immunoprecipitate, indicating that these proteins associate in the same complex in yeast (Figure 2-5 panel 2, lane 4 and panel 4, lane 4). Previous studies in mammalian cells demonstrated that all three subunits of PP2A can be found in E4orf4 complexes (Kleinberger and Shenk, 1993). Western blotting analysis of anti-FLAG immunoprecipitates using a rabbit polyclonal serum against the Tpd3 A subunit of PP2A indicated that Tpd3 co-immunoprecipitated with FLAG-Cdc55 in the absence or presence of E4orf4 (Figure 2-5 panel 6, lanes 3 and 4, respectively); however, Tpd3 only co-immunoprecipitated with HA-E4orf4 in *cdc55* cells that expressed exogenous FLAG-Cdc55 (Figure 2-5 panel, compare lanes 2 and 4). These results indicated that E4orf4 interacts with Tpd3 through Cdc55.

To characterize the E4orf4-PP2A complex in yeast further, wild type cells containing vector alone or the HA-E4orf4 galactose-inducible plasmid were transformed with DNA vectors expressing either FLAG-tagged *PPH21* or *PPH22,* which encode the

Figure 2-5 E4orf4 requires Cdc55 to recruit the PP2A complex.

The *cdc55* strain was co-transformed with the indicated plasmids (pYES2, p424GAL1, pYes2-HA-E4orf4 or p424*GAL1*-FLAG-*CDC55*) and whole cell extracts were prepared 24 hours post-galactose induction. Extracts were incubated with anti-HA or anti-FLAG antibodies. Immunoprecipitates were separated by SDS-PAGE and subject to immunoblotting with anti-HA (panels 1, 4), anti-FLAG (panels 2, 5) or rabbit polyclonal anti-Tpd3 antibodies (panels 3, 6).

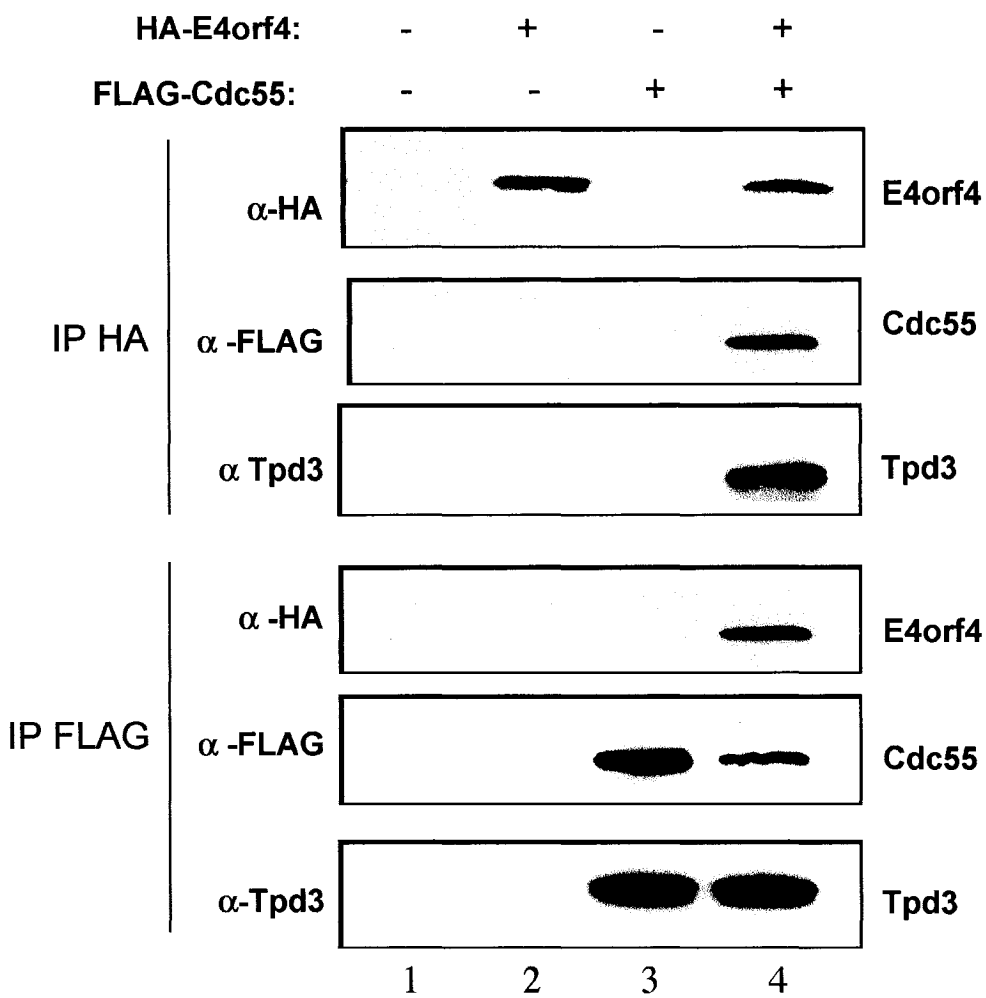
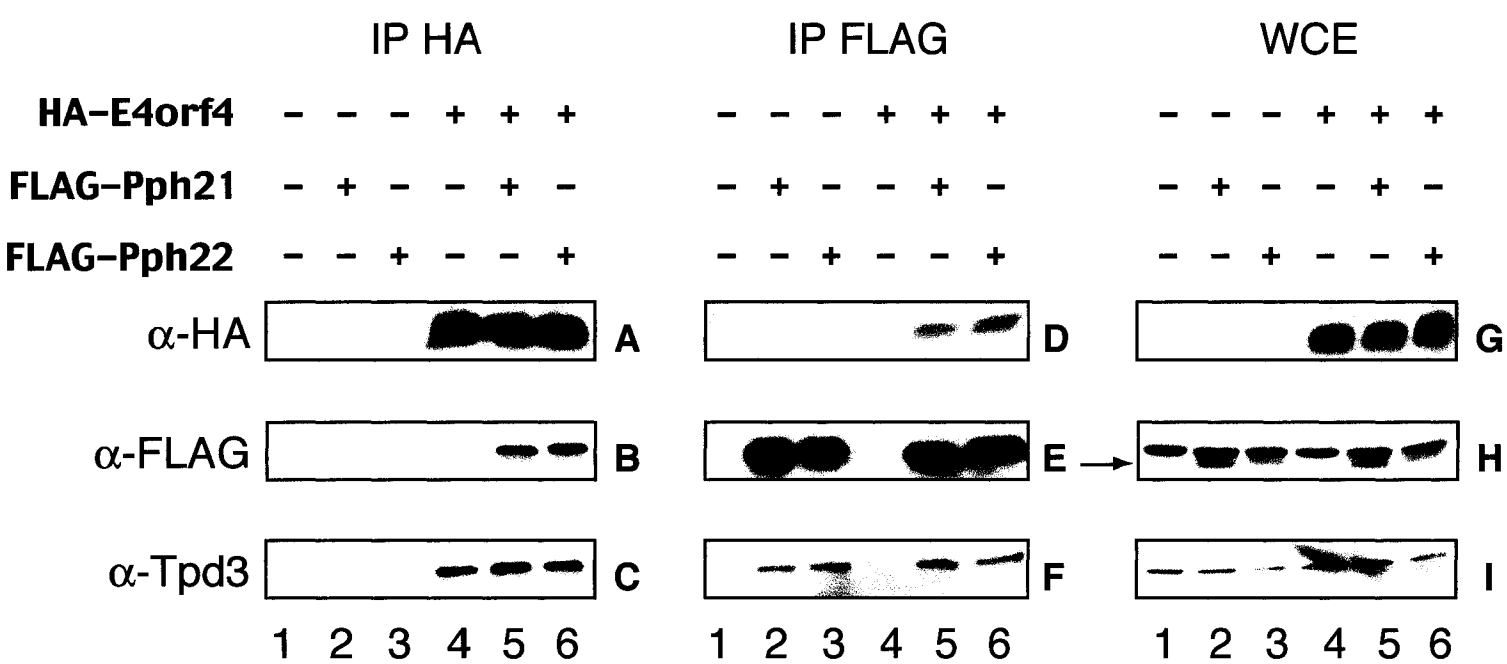

HA-E4orf4: - + - +
FLAG-Cdc55: - - + +
IP HA
α-HA
E4orf4
α -FLAG
Cdc55
α Tpd3
Tpd3
IP FLAG
α -HA
E4orf4
α -FLAG
Cdc55
α-Tpd3
Tpd3
1 2 3 4

Figure 2-6 E4orf4 can immunoprecipitate PP2A complexes containing both forms of the yeast catalytic subunit.

Wild type yeast was co-transformed with plasmids expressing the indicated proteins and cells were collected 24 hours post-galactose induction. Whole cell extracts (wce) were prepared and subject to immunoprecipitation with antibody against the FLAG or HA epitope. Proteins were separated by SDS PAGE followed by Western blotting with anti-HA (panels A, D), anti-FLAG (panels B, E), or anti-Tpd3 antibodies (panels C, F). Protein expression from wce was also determined (panels G, H, I).


IP HA
IP FLAG
WCE
HA-E4orf4 - - - + + +
FLAG-Pph21 - + - - + -
FLAG-Pph22 - - + - - +
α-HA
α-FLAG
α-Tpd3
A
B
C
D
E
F
G
H
I
1 2 3 4 5 6
1 2 3 4 5 6
1 2 3 4 5 6

PP2A catalytic C subunits of *S. cerevisiae*. These cells were cultured in the presence of galactose for 24 h to allow co-expression of E4orf4 and the FLAG-tagged C subunits. Immunoprecipitation with HA antibody followed by western blotting with anti-FLAG revealed that both Pph21 and Pph22 can be co-immunoprecipitated with E4orf4 (Figure 2-6B, lanes 5 and 6, respectively). In the reciprocal experiment, immunoprecipitation with FLAG-antibody and blotting against the HA epitope confirmed that E4orf4 formed complexes with both Pph21 and Pph22 in the presence of endogenous Cdc55 (Figure 2-6D, lanes 5 and 6, respectively). Figure 2-6C (lanes 4-6) again shows that endogenous Tpd3 associates with E4orf4 complexes. The association of Tpd3 with both Pph21 and Pph22 was also apparent both in the presence (Figure 2-6F, lanes 5 and 6) and absence (Figure 2-6F, lanes 2 and 3) of E4orf4. Thus the results shown in Figures 5 and 6 indicated that E4orf4 associates with the entire yeast PP2A holoenzyme as long as Cdc55 is present.

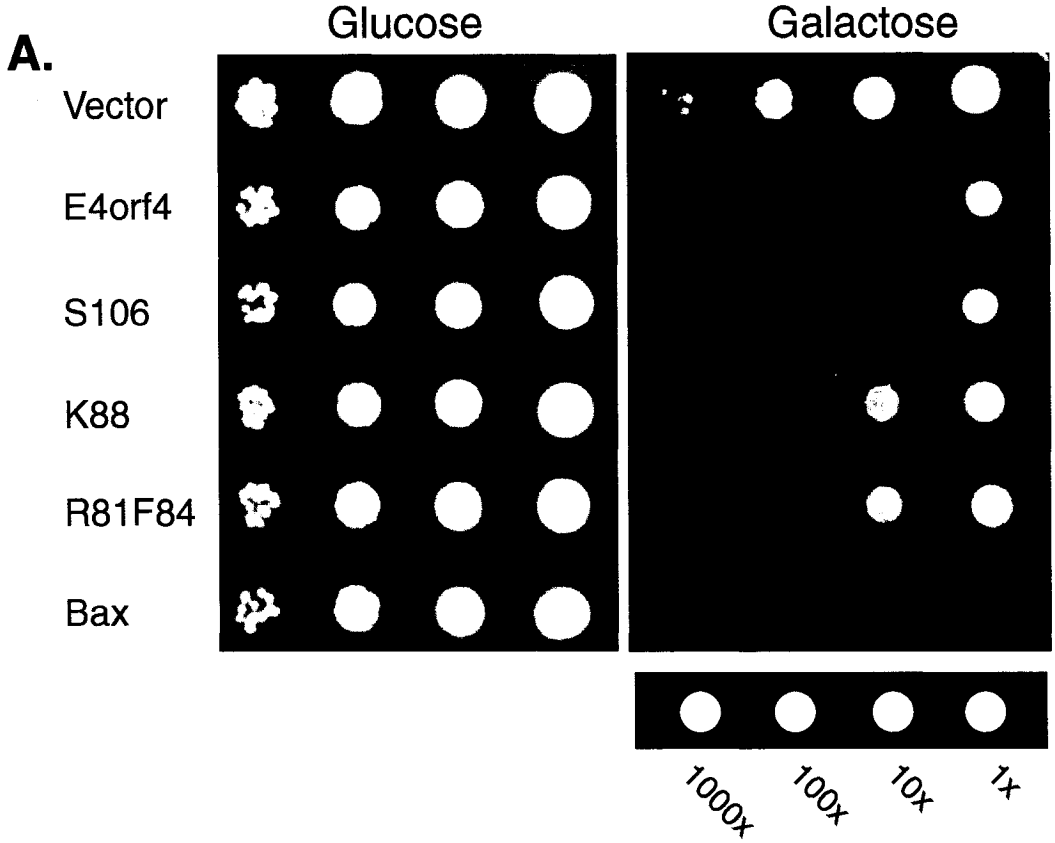
2.3.5 Correlation of Cdc55 binding, phosphatase activity and elongated cell morphology to E4orf4-induced killing using E4orf4 point mutants.

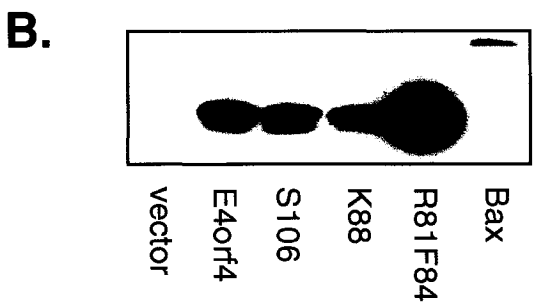
To correlate E4orf4-induced toxicity with binding to Cdc55 and associated phosphatase activity, use was made of certain E4orf4 point mutants. These mutants had previously been analyzed for the ability of their altered E4orf4 products to bind to the mammalian Bα subunit, to associate with PP2A activity, and to induce cell death in mammalian tumor cell lines (Marcellus et al., 2000). R81/F84 is a Class I mutant containing alanine residues in place of R81 and F84. R81F84 does not bind Bα, lacks associated phosphates activity, and is defective for killing human tumor cells. K88 contains an alanine residue in place of K88 and is a representative of Class II mutants,

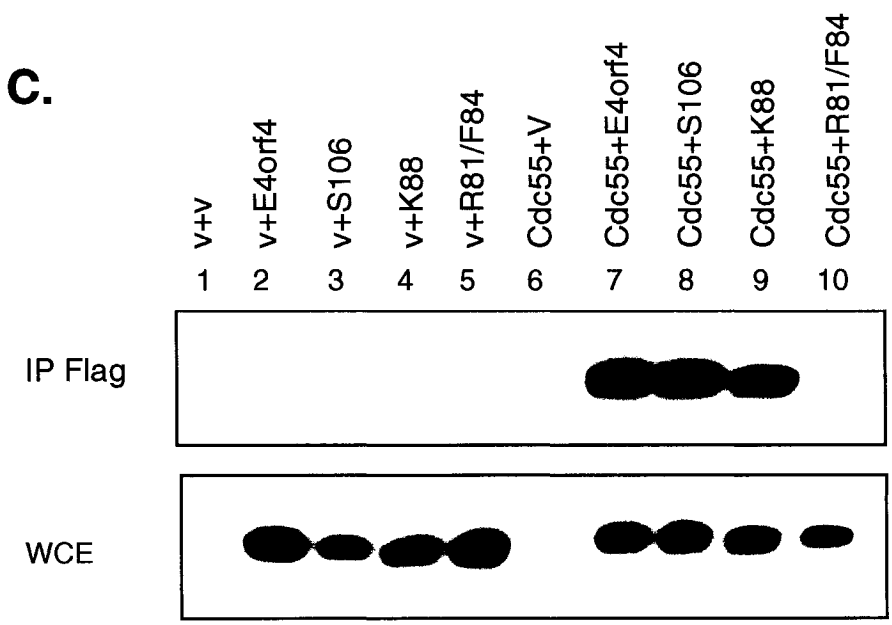
which bind Bα at reasonable levels, have associated phosphatase activity, but which are also defective for killing. S106 contains alanine in place of S106 and is a class III mutant with a profile identical to wild type E4orf4 and they are comparable with respect to binding Bα, associated phosphatase activity and cell killing. To determine the ability of these E4orf4 point mutants to interact with Cdc55 and to associate with phosphatase activity, co-immunoprecipitation experiments were performed in the *cdc55* strain transformed with vector DNAs expressing FLAG-*CDC55* and wild type HA-E4orf4 or the HA-E4orf4 point mutants. Immunoblotting with anti-HA antibodies confirmed that high levels of all mutants were expressed (Figure 2-7C, bottom panel). Immunoprecipitation with anti-FLAG antibodies and western blotting with anti-HA antibodies (Figure 2-7C, top panel) indicated that both the S106 and K88 mutant E4orf4 proteins co-precipitated with FLAG-*CDC55* (lanes 8 and 9) about as well as did wild type E4orf4 (lane 7). Binding of the R81/F84 E4orf4 protein (Figure 2-7C, lane 10) was undetectable. Thus the class I mutant R81/F84 was totally defective for binding of both yeast Cdc55 and the mammalian Bα subunit. Extracts were also immunoprecipitated with anti-HA antibodies and associated phosphatase activity was assayed by measuring phosphate release from a synthetic peptide containing a universal phospho-threonine phosphatase substrate site (Figure 2-7D left panel). Only basal levels of phosphatase activity were detected in HA-E4orf4-immunoprecipitates lacking the Cdc55 subunit. Wild type E4orf4 and S106 in cells reconstituted with Cdc55 displayed high levels of phosphatase activity. K88 was somewhat defective for phosphatase activity and R81F84 displayed low levels comparable to the vector controls (Figure 2-7D, left panel). The effect of E4orf4 on

Figure 2-7 Analysis of colony growth, Cdc55 binding and phosphatase activity using E4orf4 point mutants.

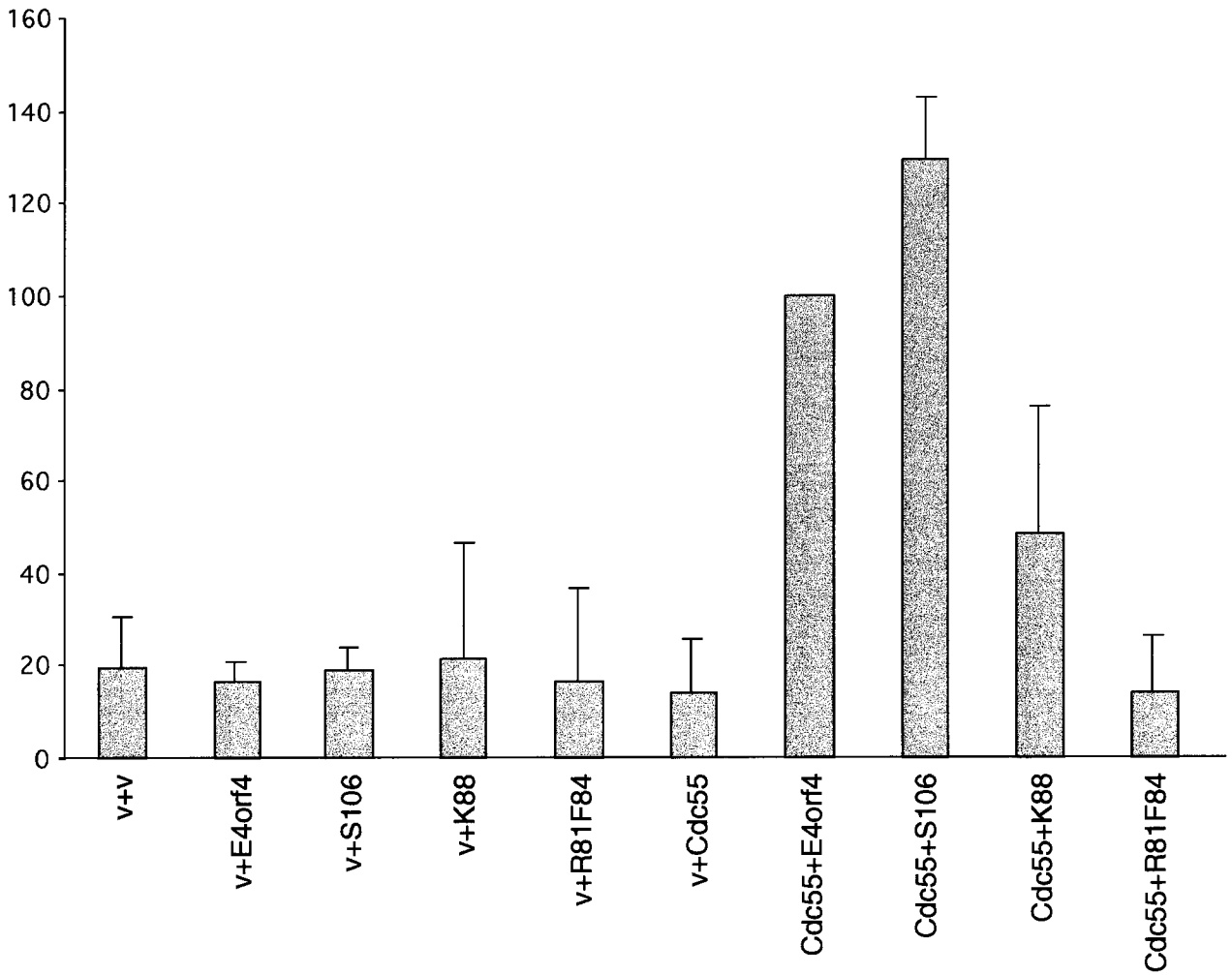
A.Yeast transformed with plasmids expressing the indicated HA-tagged proteins were spotted onto glucose- or galactose-based medium and incubated at 30°C. Pictures were taken after 3 days. **B**. Anti-HA western blot of whole cell extracts prepared from cells expressing the indicated proteins. **C**. Co-immunoprecipitation experiments using whole cell extracts prepared from the *cdc55* strain transformed with the indicated plasmids; CDC55 is Flag-tagged and E4orf4 is HA-tagged. **D**. (Left panel) Analysis of phosphatase activity associated with E4orf4 and E4orf4 mutants. *cdc55* cell extracts containing the indicated plasmids (CDC55 is FLAG-tagged, E4orf4 is HA-tagged) were subject to immunoprecipitation with HA antibody. Immunoprecipitates were assayed for PP2A activity as described in materials and methods. The results represent the average of three independent experiments. Values are relative to the results obtained with wild type E4orf4 and Cdc55 which was set at 100 (arbitrary standardized units of phosphatase activity). (Right Panel) Analysis of phosphatase activity associated with Cdc55 in the absence and presence of E4orf4. *cdc55* cell extracts expressing the empty vector and FLAG-Cdc55 or HA-E4orf4 and FLAG-Cdc55 were immunoprecipitated with FLAG antibody and assayed for phosphatase activity as described in materials and methods.

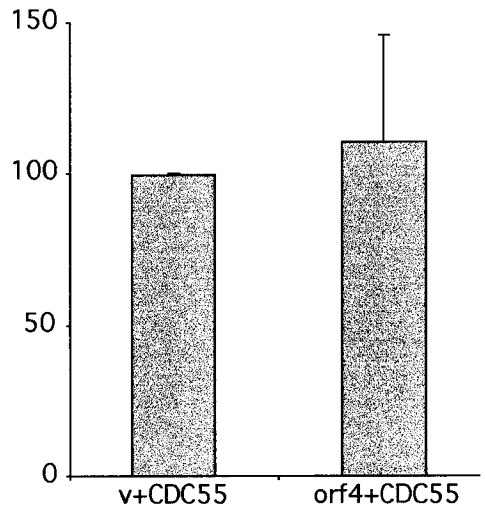

A.
Glucose
Galactose
Vector
E4orf4
S106
K88
R81F84
Bax
1000x
100x
10x
1x


B.
vector
E4orf4
S106
K88
R81F84
Bax


C.
v+v
v+E4orf4
v+S106
v+K88
v+R81/F84
Cdc55+V
Cdc55+E4orf4
Cdc55+S106
Cdc55+K88
Cdc55+R81/F84
1
2
3
4
5
6
7
8
9
10
IP Flag
WCE

D.


160
140
120
100
80
60
40
20
0
v+v
v+E4orf4
v+S106
v+K88
v+R81F84
v+Cdc55
Cdc55+E4orf4
Cdc55+S106
Cdc55+K88
Cdc55+R81F84


150
100
50
0
v+CDC55
orf4+CDC55

PP2A/Cdc55 phosphatase activity was also tested by assaying the phosphatase activity of FLAG-Cdc55 immunoprecipitates in the absence and presence of E4orf4 expression. We found that E4orf4 did not promote any significant change in the ability of PP2A/Cdc55 to dephosphorylate the peptide substrate (Figure 2-7 D, right panel).

These E4orf4 mutants were also tested in wild type haploid yeast for colony growth and cell morphology. Figure 2-7B shows an anti-HA blot of whole cell extract confirming that the mutant E4orf4 proteins were expressed at high levels. Figure 2-7A shows that following galactose induction, S106 induced toxicity as efficiently as did wild type. Both K88 (class II) and R81F84 (class I) yielded a reduced, although still detectable, effect on cell growth. In the case of K88, this effect could have resulted from interactions with Cdc55; however, such was unlikely to be the case with R81/F84 as it did not appear to interact with Cdc55. These results again suggested that E4orf4 possesses a second growth inhibitory function that is independent of interactions with Cdc55. To examine the effects of mutant E4orf4 proteins further, cells were collected from these plates and their morphologies were examined. As shown previously in Figure 2-2B, cells expressing wild type or S106 E4orf4 contained a high percentage (25-30%) of elongated cells. Those expressing the R81/F84 mutant were entirely of normal morphology and resembled those containing vector alone, as shown in Figure 2-2A. Cells expressing the K88 mutant also displayed largely normal morphology, although a few cells (≤1%) did have an elongated morphology. These data suggested that induction of the elongated morphology requires efficient and functional binding of E4orf4 to Cdc55.

2.3.6 E4orf4 does not require *RTS1* to induce toxicity in yeast and does not interact with Rts1

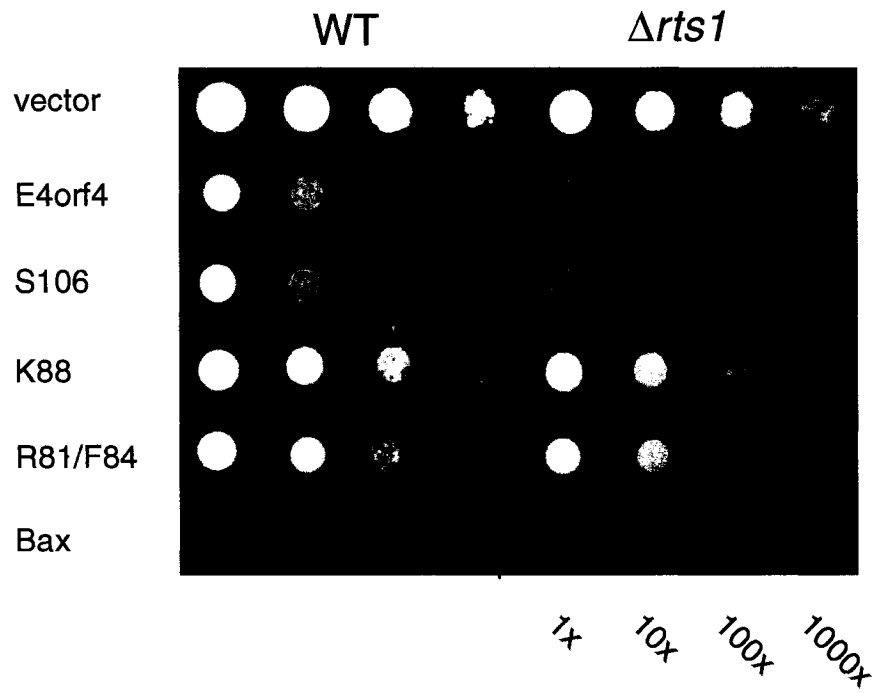
Budding yeast contain only one B/B55-like PP2A subunit (Cdc55) and one B'/B56-like species, Rts1 (Shu et al., 1997). The data presented in Figure 2-5 implied that E4orf4 does not interact with Rts1, as Tdp3 was found to co-immunoprecipitate with E4orf4 only in the presence of Cdc55. This observation suggested that Cdc55, and not Rts1, is required for the E4orf4-induced growth effect. To determine if Rts1 was required for E4orf4 function, wild type E4orf4 and E4orf4 point mutants were expressed in wild type yeast or in an *rts1* strain. Haploid wild type yeast or yeast containing an *RTS1* deletion was transformed with the indicated plasmid DNAs and spotting assays were performed. Figure 2-8B shows that all E4orf4 proteins were expressed at high levels. Figure 2-8A shows that wild type and S106 E4orf4 induced toxicity as well or better in the *rts1* strain compared to wild type cells, indicating that Rts1 is not required for E4orf4 function. Again the K88 and R81/F84 mutants induced a modest effect in both wild type and *rts1* cells. Thus, although the Cdc55 PP2A B subunit is required for E4orf4-induced slow growth, Rts1 is not.

We examined directly whether E4orf4 could interact with Rts1 (B′) by co-immunoprecipitation experiments. Yeast cells were co-transformed with plasmids expressing HA- or FLAG- epitope-tagged E4orf4, Cdc55 and Rts1. Cell extracts were prepared and immunoprecipitated with either anti-HA or anti-FLAG antibodies, and then analyzed by immunoblot. As previously shown in figure 5, HA-E4orf4 interacted with

Figure 2-8. The slow growth effect of E4orf4 in yeast specifically requires *CDC55* and not *RTS1*

A. Wild type or *RTS1* deleted cells were transformed with vector, E4orf4 or E4orf4 point mutants. Serial dilutions of cultured cells were spotted onto glucose or galactose-based agar plates. Shown is the galactose plate photographed on day 3 post-incubation at 30°C. **B.** Whole cell extracts were made and immunoblotted for the HA-epitope to confirm protein expression.

A.


WT
Δrts1
vector
E4orf4
S106
K88
R81/F84
Bax
1x
10x
100x
1000x

B.

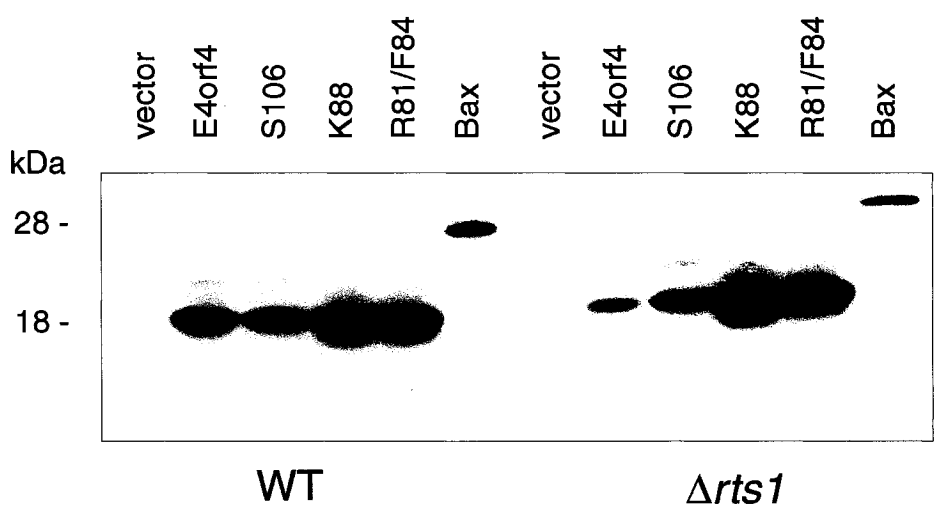
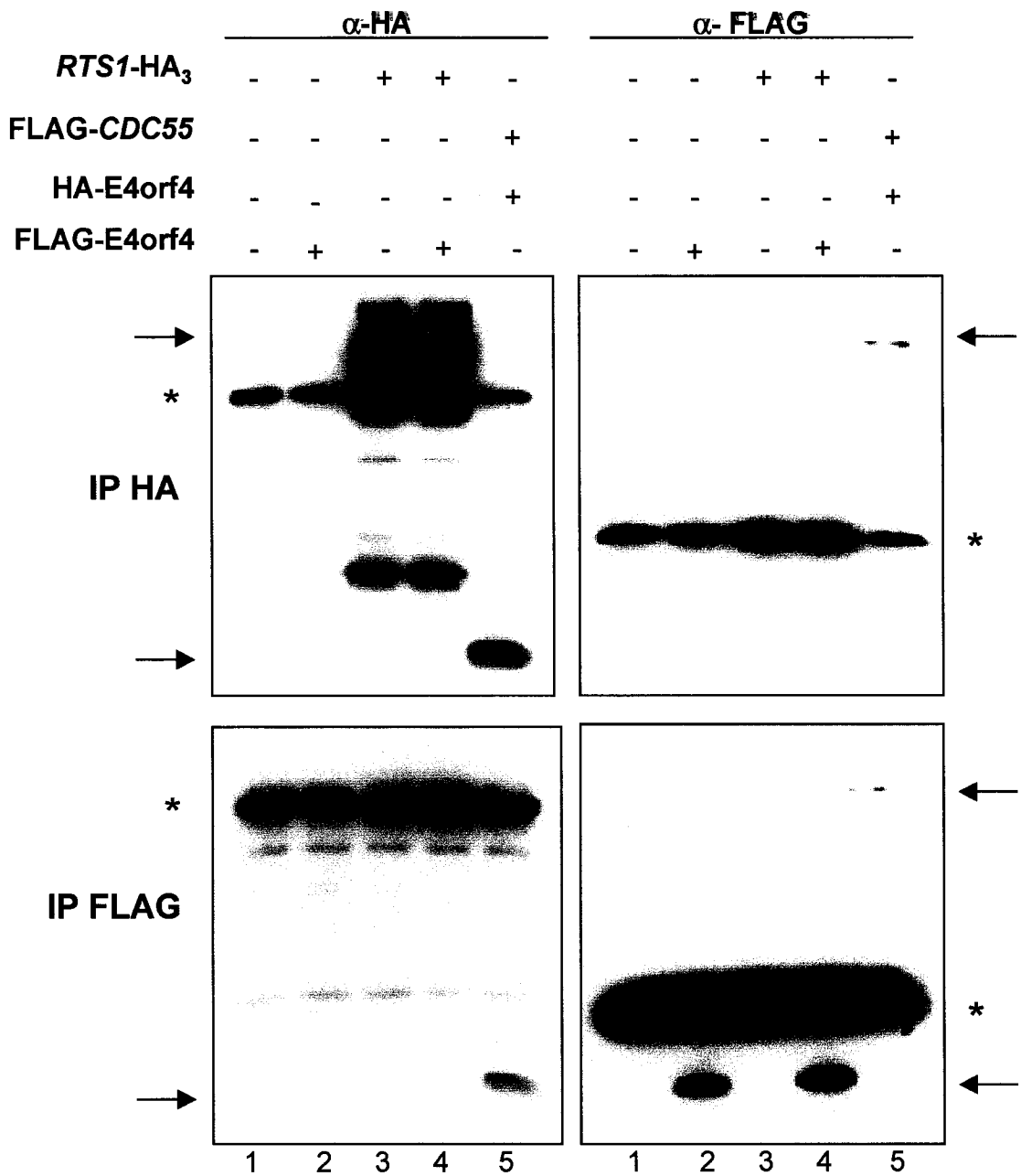

vector
E4orf4
S106
K88
R81/F84
Bax
vector
E4orf4
S106
K88
R81/F84
Bax
kDa
28 -
18 -
WT
Δrts1

Figure 2-9 E4orf4 specifically interacts with the Cdc55 regulatory subunit and not Rts1

Wild type yeast strains were transformed with plasmids encoding FLAG- or HA-E4orf4 and FLAG-*CDC55* or HA-*RTS1*. Cell lysates were immunoprecipitated with anti-FLAG or anti-HA antibodies followed by SDS-PAGE and western blotting with the indicated antibodies. * : indicates the heavy or light chain of the primary antibody.


α-HA
α- FLAG
RTS1-HA3
FLAG-CDC55
HA-E4orf4
FLAG-E4orf4
IP HA
IP FLAG
1 2 3 4 5
1 2 3 4 5

the FLAG-Cdc55 subunit in reciprocal co-immunoprecipitation experiments (Figure 2-9, lanes 5 of the lower left and upper right panels). In contrast, as seen in lanes 4 of these same panels, no interaction between HA-Rts1 and FLAG-E4orf4 was detected. These data demonstrate that E4orf4 interacts specifically with the Cdc55-containing pool of PP2A, and therefore any E4orf4-induced changes in PP2A activity or substrate specificity are presumably Cdc55-specific.

The cell cycle effects of E4orf4 were further studied in the W303 strain. To confirm that E4orf4 elicited a similar phenotype in this strain background we performed spotting assays, colony formation assays and examined cell bud morphology. E4orf4 was highly toxic in spotting assays and in colony survival assays almost all cells were unviable after 6 h of E4orf4 expression (see Appendix, Figure 1). Cells expressing control or E4orf4 plasmids were induced for 6 h and bud morphologies were examined. E4orf4 promoted an elongated phenotype in 25% of the cell population. E4orf4-expressing cells also showed a decrease in unbudded (indicative of G1), small budded and medium budded cells (indicative of S phase) and an increase in large budded cells (indicative of G2/M) (See Appendix, Figure 2). These results are consistent with E4orf4 promoting the accumulation of cells in mitosis.

2.4 Acknowledgements

We wish to thank James Broach, Doug Kellogg and Richard Hallberg for reagents, Albert Lai, David Pallas and David Thomas for helpful discussions, and Ken McDonald for technical assistance. This work was supported by grants to P.E.B. from the National Cancer Institute of Canada and the Canadian Institutes for Health Research.

Chapter 3: The Adenovirus E4orf4 Protein Induces Premature Activation of APC^{Cdc20} and Mitotic Arrest in Saccharomyces cerevisiae through a PP2A-Dependent Mechanism

3.1 Introduction

The interaction of E4orf4 with the B regulatory subunit of PP2A may promote cell death by several mechanisms. The E4orf4-PP2A complex may enhance the dephosphorylation of certain substrates, possibly by promoting substrate-PP2A interactions. Alternatively, the E4orf4-B interaction could prevent substrate access to the PP2A catalytic subunit, resulting in the inhibition of substrate dephosphorylation yielding hyper-phosphorylated proteins. Such inhibition could result from direct steric interference with substrate access or by an E4orf4-induced change in the conformation of the catalytic subunit such that the active site is no longer accessible to substrates. In addition to the possibility that E4orf4 modulates PP2A enzymatic activity, E4orf4 binding may also change PP2A localization resulting in a change is substrate specificity.

The majority of E4orf4 toxicity is relieved in *cdc55* cells (Kornitzer et al., 2001; Roopchand et al., 2001). While the specific mechanism by which expression of E4orf4 mediates cell death is unclear, PP2A/Cdc55 is likely to play a direct role. PP2A has been implicated in the control of mitotic events (Janssens and Goris, 2001). One possibility is that E4orf4 expression alters the ability of PP2A to regulate critical cell cycle transitions, resulting in inviability. This scenario is consistent with the observation that E4orf4 over-expression results in mitotic arrest and an increase in Clb2/cyclinB-associated Cdc28/Cdc2 kinase (Cdk1) activity in yeast and mammalian cells (Kornitzer et al., 2001; Roopchand et al., 2001; Brignole and Branton, unpublished data). Two anaphase-promoting complexes (APC) regulated by Cdk1 provide the temporal regulation required to coordinate sister chromatid separation with subsequent cytokinesis. Cdk1 activates APC^{Cdc20} promoting dissolution of securin and cohesins, and resulting in sister chromatid

separation (Cohen-Fix et al., 1996; Ciosk et al., 1998; Uhlmann et al., 1999; Uhlmann et al., 2000). Simultaneously, Cdk1 inhibits APC^{Hct1} activity thereby preventing anaphase exit (Zachariae et al., 1998). While the role of the APC in mitotic exit is clear, upstream regulators of Cdk1 and/or APC^{Cdc20} remain elusive.

E4orf4 provides a unique tool for identifying Cdc55-specific PP2A substrates. We have previously shown that the ability of E4orf4 to induce Cdk1 activity in yeast is dependent on the presence of *CDC55* (Roopchand et al., 2001). E4orf4, through its interaction with Cdc55, may modulate the activity of Cdc55-specific PP2A complexes towards selected substrates leading to mitotic arrest. To further characterize the mechanism of E4orf4-induced mitotic arrest we examined the activity of APC^{Cdc20} and APC^{Hct1} *in vivo* to precisely determine the transition point affected by E4orf4 expression. If APC^{Cdc20} substrates are stable this would suggest that the cells are arrested at metaphase while the stability of APC^{Hct1} substrates would indicate an anaphase arrest. Furthermore, to determine whether E4orf4 could promote Cdk1 and APC activities independently of mitosis we arrested cells in S phase with hydroxyurea (HU) and examined the activities of both complexes. We show that E4orf4 expression induces the premature activation of Cdk1 and APC^{Cdc20}, but not APC^{Hct1}. The E4orf4-induced premature activation of APC^{Cdc20} leads to unscheduled sister chromatid segregation, an event that may contribute to loss of cell viability. Furthermore, the effect of E4orf4 on the APC^{Cdc20} is dependent on a functional E4orf4-Cdc55 interaction, demonstrating that Cdc55/PP2A specifically regulates the APC^{Cdc20} complex. Based on our results we propose a mechanism whereby E4orf4 inhibits dephosphorylation of PP2A/Cdc55 substrates, and that Cdc55-PP2A plays an important regulatory role in anaphase exit.

3.2 Materials and methods

3.2.1 Strains, plasmids and media

The yeast strains used in this study (see Table 3-1) are all derivatives of W303, except where indicated. Wild type hemagglutinin-tagged E4orf4 (HA-E4orf4) and HA-E4orf4 point mutants were subcloned from mammalian expression vectors into the high copy pYES2 (Invitrogen) DNA vector under the control of the *GAL1* promoter. FLAG-tagged E4orf4 was cloned into the p424*GAL1* (ATCC) plasmid DNA vector. p424*GAL1*-FLAG-*CDC55* has previously been described (Roopchand et al., 2001). Yeast transformations were performed using the one-step method of Chen *et al.* (Chen et al., 1992). Transformants were selected on synthetic minimal media containing the appropriate auxotrophic supplements.

3.2.2 Cell synchronization-release experiments and FACS analysis

Yeast transformed with galactose-inducible control, FLAG-E4orf4 or HA-E4orf4 DNA plasmids were grown in 2% glucose-containing medium over night. Cells were diluted and transferred to medium containing 2% raffinose for 2 h after which E4orf4 expression was induced by the addition of galactose (2% final). Cells were harvested after 6 h of galactose induction. In other cases cells were treated with 0.2M hydroxyurea (Sigma) during galactose induction for 4.5 h to allow E4orf4 expression during S phase arrest. Cells were then released from the arrest into 2% raffinose- plus 2% galactose-containing medium and collected at 0, 1, 2 and 3 h post-release. For simplicity only the 0 and 3 hour time points are presented, as immunoblot analysis revealed similar results for all time points. Cells were processed for Western blotting (described below) as well as

fluorescent-activated cell sorting (FACS) analysis following the method outlined in Dien *et al.* (Dien et al., 1994). Propidium iodide stained cells were acquired on a FACScan instrument and analyzed with Modfit software.

3.2.3 Western blot analysis

Whole cell extracts were prepared by resuspending cells in yeast lysis buffer (25mM TrisCl pH 7.4, containing 125mM NaCl, 2.5mM EDTA, 1% Triton X-100 and protease inhibitors) followed by vortexing with acid-washed glass beads (Sigma). Protein was quantified by Bio-Rad protein assay reagent and 20 μg of total protein per sample was resolved by SDS-PAGE. Separated proteins were transferred to PVDF membranes (Millipore) and immunoblotted with the indicated antibodies. Anti-HA (HA.11, BAbCO), anti-(M2)FLAG (Kodak/Sigma-Aldrich), anti-tubulin (YOL1/34, Abcam) and anti-Myc (Covance) antibodies were all used at 1/1000 dilution. Membranes were incubated with secondary antibody linked to horse radish peroxidase (Jackson ImmunoResearch) at 1/10 000 dilution, followed by ECL detection (NEN Life Science Products).

3.2.4 Immunoprecipitation–protein kinase assay

Wild-type yeast expressing vector or E4orf4 were induced in medium containing 2% galactose for 5 h with or without 0.2M hydroxyurea. Cells were lysed as described above and 300 μg of whole cell extract was immunoprecipitated with 1 μg of rabbit polyclonal Clb2 antibody (Sigma) and 20 μl of protein A sepharose beads (50% slurry). Precipitates were washed four times in lysis buffer and one time in kinase assay buffer (50mM HEPES, pH 7.5, 1mM EGTA, 2 mM $MgCl_2$, 1 mM DTT). Immunoprecipitates were then incubated for 30 min at 30°C in 20μl of reaction mix containing kinase assay

buffer, 10 μg histone H1 (Sigma), and 10 μCi γ^{32}P-ATP (specific activity 6000 Ci/mmol). Kinase reactions were stopped by the addition of 10 μl of 4x sample buffer and heating samples at 100°C for 5 min. Samples were separated by SDS-PAGE using 10% polyacrylamide gels and the proteins transferred to PVDF. The level of H1 phosphorylation was determined by autoradiography.

3.2.5 Microscopy and sister chromatid separation experiments

The yeast strain carrying endogenous *TPD3* tagged with GFP at the N-terminus (MSG66) and *RTS1* tagged at the C-terminus with GFP (MSG167). has been previously described (Gentry and Hallberg, 2002). MSG66 and MSG167 was transformed with pYES2 or pYES2-HA-E4orf4 plasmids. Transformants were grown in glucose-containing medium, transferred to raffinose-containing medium and E4orf4 expression was induced with galactose. Visualization of GFP fluorescence was visualized on a BX60 microscope (Olympus) by using an enhanced green fluorescent protein (eGFP) filter set (Chroma Technology). Images were acquired using a charge-couple device camera (Olympus) with Magnafire software (Olympus) and analyzed using Photoshop (Adobe Systems)

The yeast strain (6803) carrying chromosome five marked with GFP at the centromere (CEN5-GFP) has been described (Michaelis et al., 1997). Briefly, 336 tandem Tet operators were integrated adjacent to the centromere of chromosome V in a strain expressing the Tet repressor fused to GFP. Control plasmid DNA or plasmid DNA expressing galactose-inducible HA-E4orf4 DNA was transformed into each of the above strains and cells were grown in 2% glucose-containing medium overnight then diluted

and resuspended into 2% raffinose-containing medium. E4orf4 expression was induced with the addition of galactose (2% final) for 4.5 hours in the presence of HU. Cells were fixed in 4% EM grade methanol-free formaldehyde (Polysciences Inc.) for 5 min, washed in phosphate buffered saline (PBS, 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4, pH 7.4) and mounted in a *Slowfade Light* anti-fade solution in 50% glycerol (Molecular Probes) according to the supplied protocol and used directly for fluorescence microscopy. Cells were examined using a Nikon TE200 inverted microscope equipped with a 100x 1.4 objective mounted on PE peizo z-drive/Improvision Orbit controller, and Hamamatsu ORCA-ERG camera. Image stacks (0.3μm optical sections with 17-21 stacks per image) were acquired using OpenLab software, and image de-convolution with Volocity 3.1 Restoration. Images shown are the extended focus of a z-series of 0.3μm optical sections. Imaging processing was performed with Adobe Photoshop CS. 367 cells from each sample were scored for separated sister chromatids.

Table 3-1 Yeast strains

Strain	Genotype	Source/Reference
MSG66	*MATα ade2-1 ura3-1 his3-11 trp1-1 leu2-3,112 GFP:TPD3*	Gentry and Hallberg, 2002
MSG167	*MATα ade2-1 ura3-1 his3-11 trp1-1 leu2-3,112 RTS1:GFP*	Gentry and Hallberg, 2002
6803	*MATα ade2-1 can1-100 leu2-3,112 his3-11,15 tetR-GFP:LEU2 TetOs:URA3PDS1-* myc_{18}*::LEU2 psi+*	Ciosk et al. 1998
425	*MATα* myc_{18}*-CDC20-TRP CDC16-*HA_3 *URA3 ade2-1 trp1-1 can1-100 leu2-3,112 his3-11,15 ura3*	Zachariae et al. 1998
491	*MATα* myc_9*–HCT1-LEU2 bar1::HIS3 ade2-1 trp1-1 can1-100 leu2-3,112 his3-11,15 ura3*	Zachariae et al. 1998
6142	*MATa ade2-1 can1-100 leu2-3,112 his3-11,15 ura3 GAL CDC5-*myc_{15} *::URA3 psi+*	Shirayama et al. 1998
7108	*MATα cdc20-3 ura3 leu2 his3 trp1 tetR-GFP LEU2 TetOs:URA3 PDS1-*myc_{18}*::LEU2*	Ciosk et al. 1998
6565	*MATa ade2-1 can1-100 leu2-3,112 his3-11,15 ura3 GAL, psi+ SCC1* myc_{18}*::TRP1*	Michaelis et al. 1997

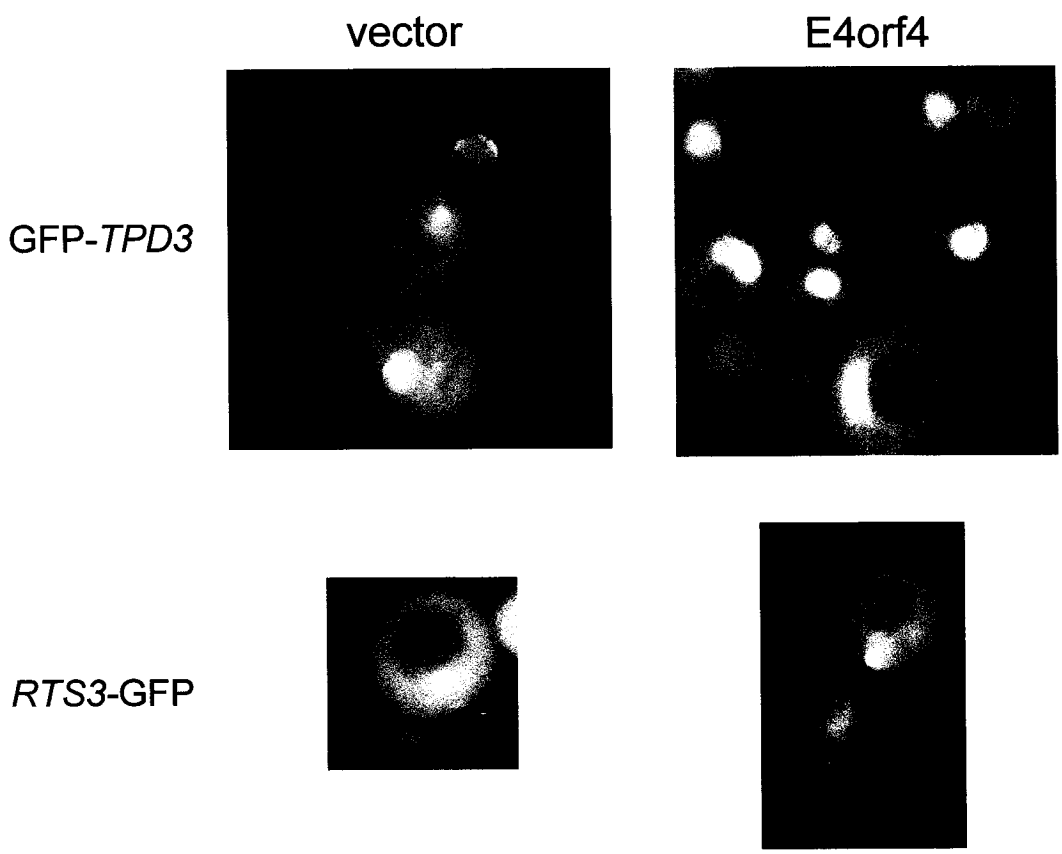
3.3 Results

3.3.1 E4orf4 prevents the proper localization of Cdc55-associated PP2A

In addition to a physical interaction that may alter PP2A activity, E4orf4 binding may also change PP2A/Cdc55 localization and thus alter the holoenzyme's access to critical substrates. PP2A subunits have over-lapping localizations; however, kinetochore localization is unique to Rts1-directed PP2A complexes and bud tip localization is specific to Cdc55-directed trimers (Gentry and Hallberg, 2002; Dobbelaere et al., 2003). During S- and M-phase, PP2A/Rts1 localizes to the kinetochore, and upon anaphase exit re-localizes to the bud neck, where it plays a role in septin ring dynamics and cytokinesis (Gentry and Hallberg, 2002; Dobbelaere et al., 2003). In contrast, PP2A/Cdc55 trimers localize specifically to bud tips in small to large budded cells and relocalize to the bud neck in post-telophase cells (Gentry and Hallberg, 2002). To determine if E4orf4 could affect the localization of PP2A/Cdc55 complexes, we examined the localization of the PP2A A subunit, Tpd3, which is common to all PP2A holoenzymes. Control or HA-E4orf4 plasmids were transformed into a yeast strain expressing a GFP-Tpd3 fusion protein. Cells were visualized by fluorescence microscopy to determine the localization of GFP-Tpd3p after 6 h of E4orf4 expression. While Rts1-directed localization of GFP-Tpd3 to kinetochores was undisturbed in cells expressing E4orf4, the Cdc55-dependant localization of Tpd3 at bud tips was abrogated (Figure 3-1). We examined the localization of a strain expressing an Rts1-GFP fusion as well to confirm that E4orf4 did not affect kinetochore localization of the PP2A-Rts1 complex. Rts1-GFP localization to the kinetochore was unaffected in E4orf4-expressing cells (Figure 3-1).

Figure 3-1 E4orf4 prevents normal Cdc55-directed Tpd3 localization to bud tips, but does not affect Rts1 localization to the kinetochore

A strain expressing a GFP-Tpd3 fusion protein (MSG66) or an Rts1-GFP fusion protein (MSG167) was transformed with control or HA-E4orf4 plasmids. HA-E4orf4 expression was induced for 6 hours medium containing 2% galactose and the localization of GFP-Tpd3 and Rts1-GFP was examined by fluorescent microscopy.


vector
E4orf4
GFP-*TPD3*
RTS3-GFP

Therefore, E4orf4 appears to inhibit PP2A/Cdc55 trimers from localizing normally to bud tips but has no apparent effect on PP2A/Rts1 complexes.

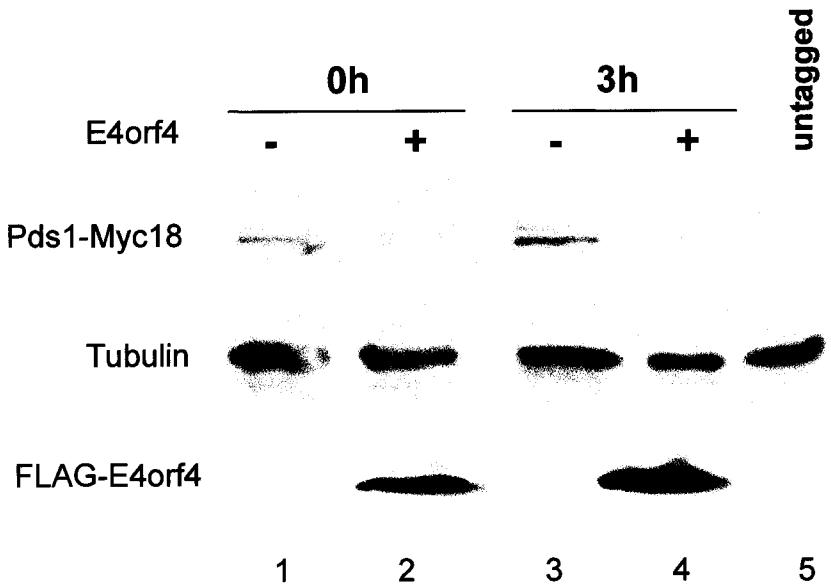
3.3.2 E4orf4 promotes premature Pds1 degradation and Cdc28-Clb2 activity

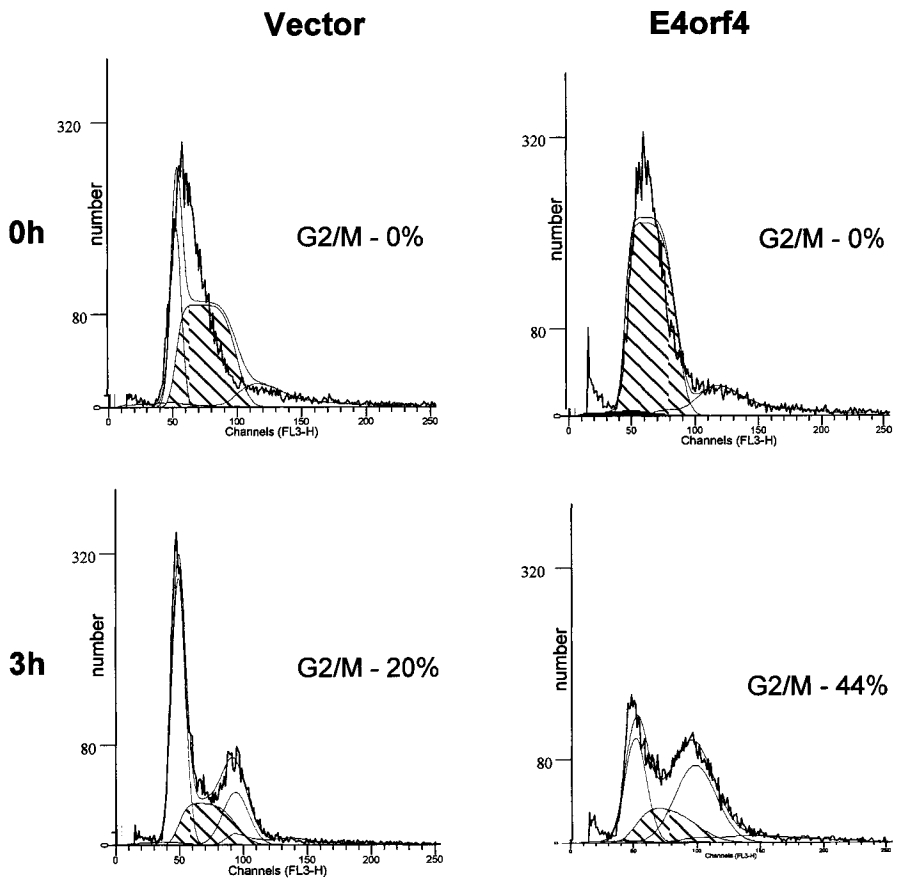
E4orf4 can induce high levels of Cdc28-Clb2 activity *in vitro* accompanied by the accumulation of cells with a 2N DNA content (Roopchand et al., 2001; Kornitzer et al., 2001). Phosphorylation of core subunits of the anaphase promoting complex (APC) by Cdc28-Clb2 is required for the activation of the Cdc20 form of the APC (APC^{Cdc20}), which promotes the metaphase to anaphase transition (Rudner and Murray, 2000). As E4orf4 induces high Cdc28-Clb2 activity, we investigated whether E4orf4 could indirectly promote the activity of APC^{Cdc20} through its effect on Cdc28-Clb2.

To test this possibility we examined the stability of securin/Pds1, an APC^{Cdc20} substrate. Ubiquitination and degradation of Pds1 by APC^{Cdc20} and the proteosome, respectively, is required for dissolution of cohesins and the metaphase to anaphase transition (Cohen-Fix et al., 1996; Ciosk et al., 1998). During S-phase, Pds1 is stable. To determine if E4orf4 expression results in unscheduled degradation of Pds1, a Pds1-18xMyc strain (6803) containing either control or FLAG-E4orf4 plasmid were grown under inducing conditions and simultaneously synchronized in S phase by incubation in 0.2M hydroxyurea (HU) for 4.5 h. Half of the HU-arrested cells were collected and the remainder were released into inducing medium without HU for an additional three hours. This synchronization protocol made it possible to determine if E4orf4 induced Pds1 degradation prematurely in S-phase-arrested cells. In addition, we could determine whether cells expressing E4orf4 3h after release from the HU arrest accumulated in

Figure 3-2A E4orf4 promotes premature degradation of Pds1

Yeast containing endogenous Myc-tagged *PDS1* (6803) were transformed with control or FLAG-E4orf4 plasmids. Cells were treated with 0.2M HU during galactose-induction as described in materials and methods. 20 μg of whole cell extract for each sample was subject to SDS-PAGE and immunoblotting with anti-Myc, anti-HA or anti-tubulin antibodies. On the right, cells were also processed for FACS analysis and the percentage of cells in G2/M phase is indicated.


0h
3h
untagged
E4orf4
-
+
-
+
Pds1-Myc18
Tubulin
FLAG-E4orf4
1
2
3
4
5


Vector
E4orf4
0h
3h
number
320
80
G2/M - 0%
G2/M - 0%
G2/M - 20%
G2/M - 44%
0
50
100
150
200
250
Channels (FL3-H)

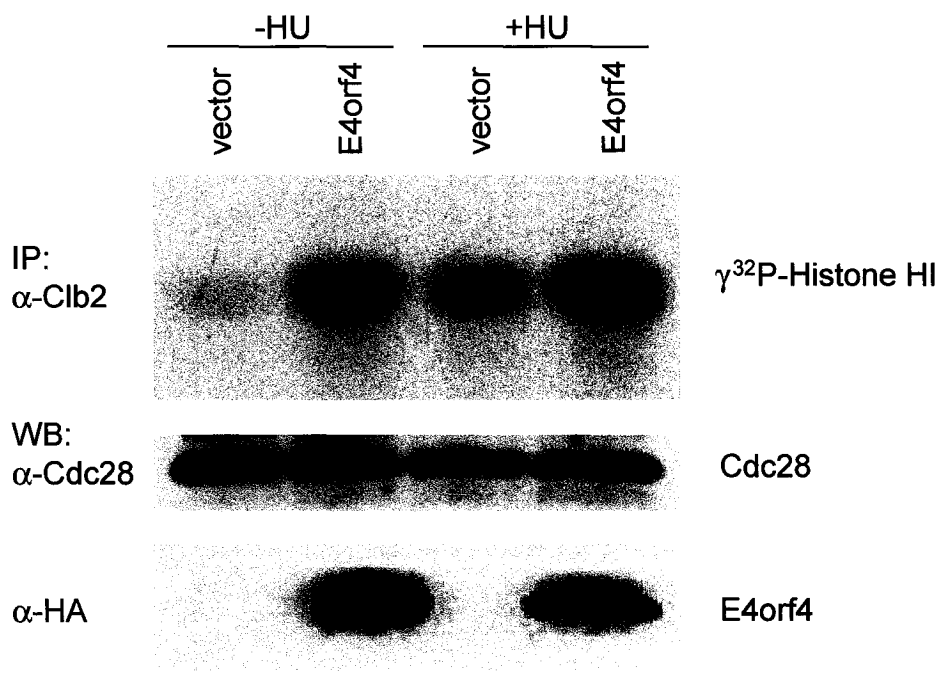
metaphase with stable Pds1 or after the metaphase to anaphase transition, where Pds1 is unstable.

Control or FLAG-E4orf4 plasmids were transformed into yeast expressing Myc-tagged Pds1 and cells were treated as described above. The HU arrest was confirmed by FACS analysis (Figure 3-2A, right panels). Pds1 stability was examined by immunoblot in HU-arrested cells (T=0h) and 3h after cells were released from the arrest (T=3h). In S phase Pds1 was stable in control cells, but found to be inappropriately unstable in cells expressing E4orf4 (Figure 3-2A, lanes 1 and 2). Three hours after release from the HU-arrest, cells re-entered the cell cycle. At this time 20% of control cells were in G2/M phase and with stable Pds1, whereas 44% of E4orf4-expressing cells were in G2/M and Pds1 levels remained greatly reduced compared to the control (Figure 3-2A, lanes 3 and 4, and right panels). Thus it appears that E4orf4 can actively promote the degradation of Pds1 both in mitosis and prematurely in cells blocked in S phase.

To confirm that E4orf4 induces Cdc28-Clb2 activity in HU-arrested cells, E4orf4 expression was induced for 4.5 hours in the absence or presence of HU. Cell extracts were subject to immunoprecipitation using anti-Clb2 antibodies and Clb2-Cdc28 kinase activity was assayed *in vitro* using γ-^{32}P-ATP and histone H1 as substrate. As we have previously reported, Figure 3-2B shows that E4orf4 expression causes a significant increase in Clb2-Cdc28 kinase activity in cycling cells. In addition, an increase in Clb2-Cdc28 was also clearly evident in HU-treated cells expressing E4orf4 (Figure 3-2B). Thus E4orf4 induces Clb2-Cdc28 activity prematurely in S phase.

Figure 3-2B E4orf4 inappropriately induces Cdc28-Clb2 activity in S phase

Strains transformed with vector or E4orf4 plasmid were grown in media containing 2% glucose, transferred media containing 2% raffinose. E4orf4 expression was induced in the presence of HU for 4.5 hours. Cells were lysed and equal amounts of protein were immunoprecipitated with anti-Clb2 antibody. The kinase activity of the immunoprecipitated Clb2-Cdc28 was assayed using histone H1 as substrate and γ^{32}P-ATP. 20ug of whole cell extract per sample was subject to SDS-PAGE and western blotting with anti-HA antibody or anti-Cdc28 antibody.


-HU
+HU
vector
E4orf4
vector
E4orf4
IP:
α-Clb2
γ32P-Histone HI
WB:
α-Cdc28
Cdc28
α-HA
E4orf4

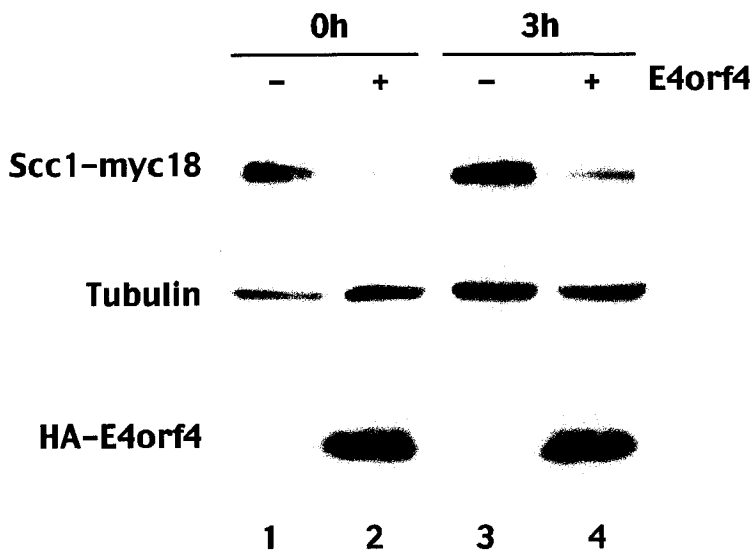
3.3.3 E4orf4 promotes the degradation of Scc1 accompanied by an increase in sister chromatid separation.

Pds1 binds to Esp1 and inhibits its cysteine protease activity against the cohesin subunit Scc1 (Cohen-Fix et al., 1996; Ciosk et al., 1998). After Pds1 is ubiquitinated by APC^{Cdc20} and degraded by the proteosome, Esp1 is free to degrade Scc1, promoting the dissolution of the cohesin complex and sister chromatid separation (Michaelis et al., 1997; Uhlmann et al., 1999; Uhlmann et al., 2000). To study the effect of E4orf4 on the stability of Scc1, experiments similar to those described above were conducted. FACS analysis confirmed the S phase arrest and showed that 3 hours after release from the HU arrest cells expressing E4orf4 accumulated with 62% of the population having a 2N DNA content compared to 41% for the control (Fig. 3-3A, right panels). Scc1 was present at high levels in control cells arrested with HU and 3 h after release from the drug (Figure 3-3A, lanes 1 and 3). In contrast, Scc1 was highly unstable in E4orf4-expressing cells in the presence of HU and Scc1 remained unstable for 3 h after release from the S phase-arrest (Fig. 3-3A, lanes 2 and 4). These results suggested that E4orf4 could promote the premature release of Esp1 from Pds1 resulting in inappropriate ubiquitination and degradation of Scc1.

Scc1 degradation promotes the collapse of the cohesin complex that holds sister chromatids together. Since E4orf4 was seen to promote the destruction of Scc1, we determined if this effect was also accompanied by an increase in sister chromatid separation. To visualize the separation of sister chromatids, a yeast strains carrying chromosome 5 marked with GFP at the centromere (CEN5-GFP) was transformed with control or FLAG-E4orf4 plasmid. Cells were incubated with HU during galactose

Figure 3-3A Expression of E4orf4 results in premature degradation of Scc1

Yeast containing endogenous Myc-tagged *SCC1* (6565) were transformed with control or HA-E4orf4 plasmids. Cells were treated with 0.2M HU during galactose-induction of E4orf4 as described in the materials and methods section. 20 μg of whole cell extract for each sample was subject to SDS-PAGE and immunoblot with the indicated antibodies. On the right cells from the same samples were also processed for FACS analysis and the percentage of cells in G2/M phase is indicated.


0h
3h
E4orf4
–
+
–
+
Scc1-myc18
Tubulin
HA-E4orf4
1
2
3
4

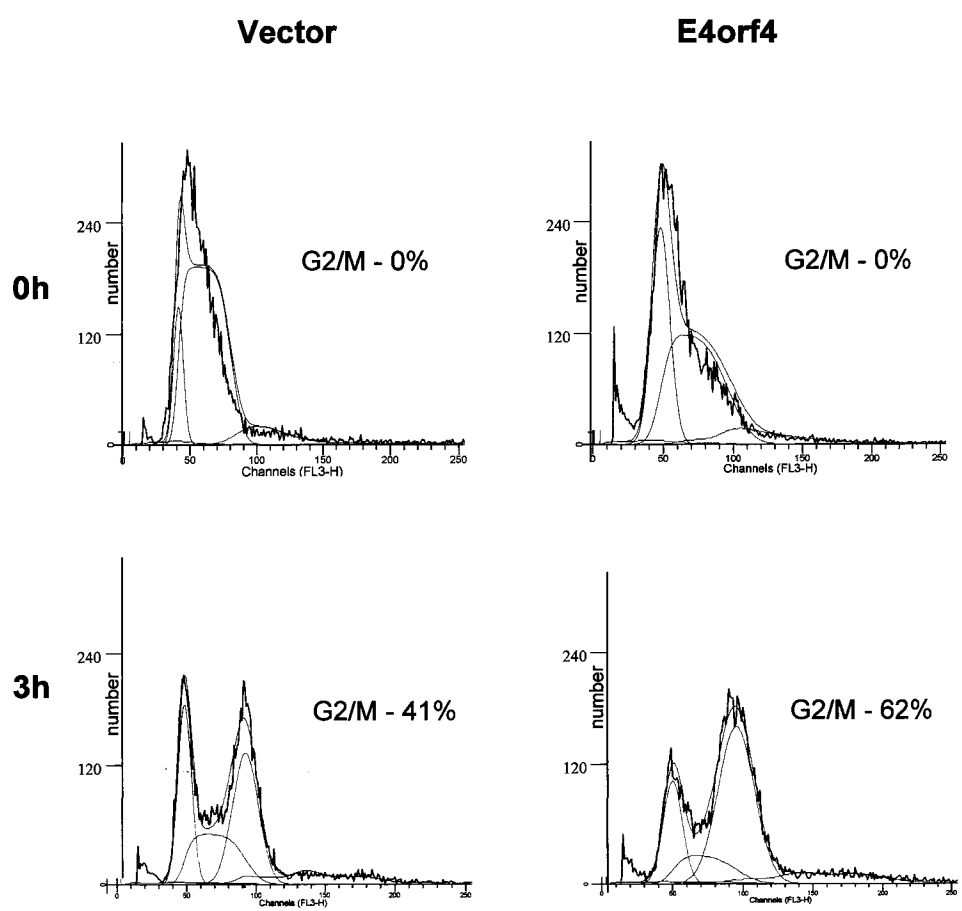
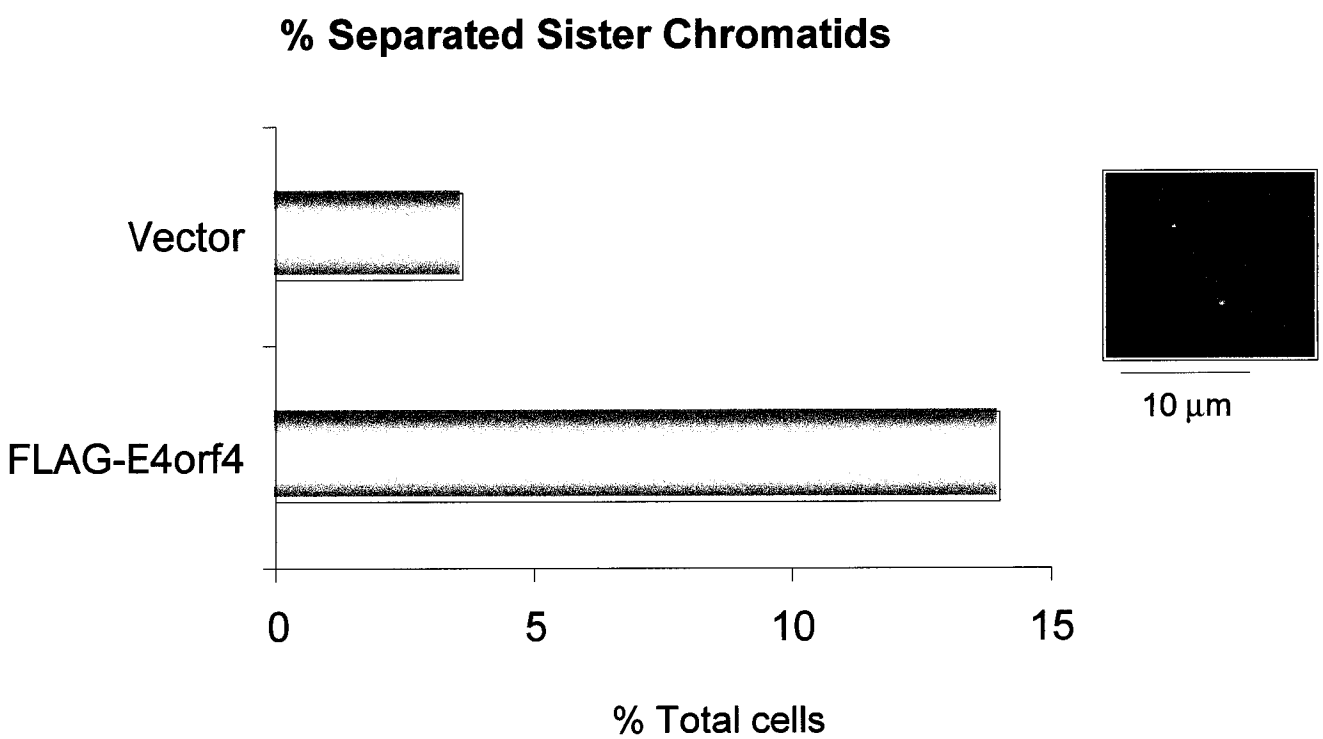

Vector
E4orf4
0h
3h
number
240
120
G2/M - 0%
G2/M - 0%
G2/M - 41%
G2/M - 62%
Channels (FL3-H)

Figure 3-3B Sister chromatid separation is increased in E4orf4-expressing cells.

Empty vector or FLAG-E4orf4 vector DNA was transformed into strains carrying chromosome 5 marked with GFP at the centromere (CEN5-GFP/ 6803) or RFP at the telomere (TEL5-RFP/ YJK2034). Cells were prepared and visualized by microscopy as described in the materials and method section. The number of cells with separated sister chromatids, visualized as two fluorescent dots, is graphed as a percentage of at least 300 cells scored for each sample.


% Separated Sister Chromatids
Vector
FLAG-E4orf4
0
5
10
15
% Total cells
10 μm

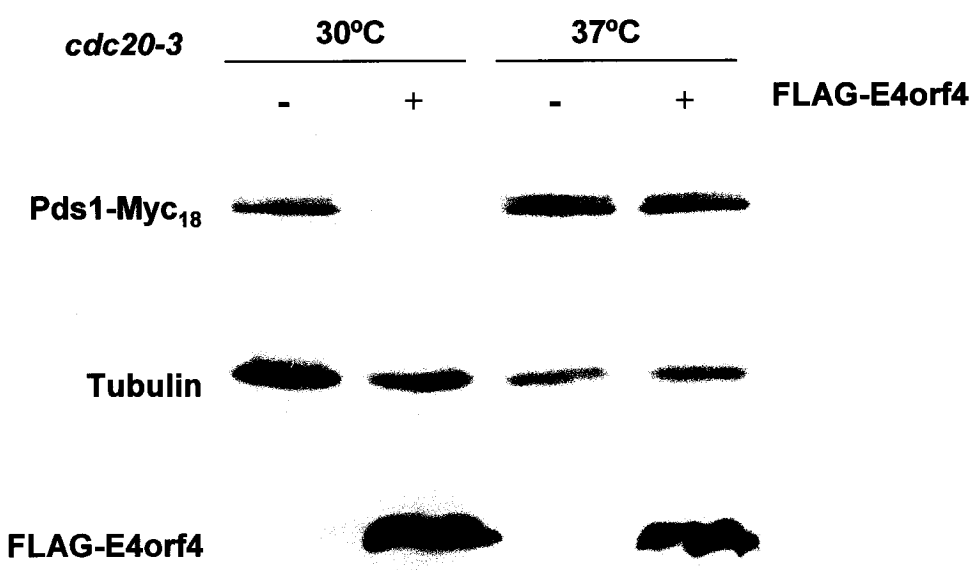
induction of E4orf4 expression, fixed and then visualized by fluorescence microscopy (see materials and methods). The right panel of Figure 3-3B shows a representative example of separated sister chromatids, characterized by the presence of two distinct spots. The percentage (%) of cells (n=386) containing separated sister chromatids was determined for control- and E4orf4-expressing cells. In cells expressing E4orf4 an approximately 4 fold increase in the number of cells with separated sister chromatids was observed (Figure 3-3B). Thus E4orf4 can induce premature sister chromatid separation.

3.3.4 E4orf4-induced Pds1 instability is APC-dependent

Previous studies have used strains harboring a temperature sensitive *cdc20-3* allele to show that Pds1 degradation is contingent on APC function (Shirayama et al., 1998). At 30°C, the *cdc20-3* allele is functional, but at 37°C APC^{Cdc20} is rendered inactive, resulting in stable Pds1 and a metaphase arrest (Shirayama et al., 1998). To determine if E4orf4-induced Pds1 destruction is dependent on APC^{Cdc20} the effect of E4orf4 expression on Pds1 stability was studied in the *cdc20-3* strain at 30°C and 37°C. Empty vector or FLAG-E4orf4 plasmids were transformed into the *cdc20-3* strain and cells were synchronized with 0.2M HU while in non-inducing raffinose medium. Cells were released from the cell cycle arrest into fresh media containing galactose to induce E4orf4 expression and cultured at 30°C or 37°C for 3 h. At 30°C, cells expressing E4orf4 induced Pds1 degradation as shown above in Figure 2A; however, at 37°C when APC^{Cdc20} is inactive, Pds1 was seen to be stable in both control and E4orf4-expressing cells (Figure 3-4). These data strongly suggest that E4orf4 can induce Pds1 degradation only through activation of APC^{Cdc20}.

Figure 3-4 E4orf4-induced Pds1 degradation is dependent on APC^{Cdc20}

The temperature sensitive *cdc20-3* strain (7108) transformed with empty vector or FLAG-E4orf4 plasmid DNA and grown in 2% glucose-containing medium. Cells were transferred to 2% raffinose-containing medium for 2 hours with 0.2 M hydroxyurea to synchronize cells. Cells were resuspended in fresh media containing 2% raffinose and 2% galactose to induce E4orf4 expression and cultured at either 30°C or 37°C for an additional 3 hours. Cells were collected and processed for western blotting with the indicated antibodies.


cdc20-3
30°C
37°C
-
+
-
+
FLAG-E4orf4
Pds1-Myc18
Tubulin
FLAG-E4orf4

3.3.5 Destruction of Pds1 and Scc1 are dependent on E4orf4 forming a functional interaction with the B subunit of PP2A

We have previously shown that E4orf4 requires a functional interaction with the Cdc55 subunit of PP2A to elicit the majority of its toxicity in yeast (Roopchand et al., 2001). Use was made of previously characterized E4orf4 point mutants to determine if the effect of E4orf4 on Pds1 and Scc1 was dependent on a functional interaction between E4orf4 and PP2A. R81AF84A and K88A represent Class I and Class II E4orf4 mutants, respectively, where the former does not interact with Cdc55 at all, but the latter interacts as efficiently as wild type E4orf4 (Marcellus et al., 2000; Roopchand et al., 2001). Despite these differences, both classes of mutants have been shown to be defective or significantly reduced in the induction of death in human cancer cells and yeast (Marcellus et al., 2000; Roopchand et al. 2001)

Wild type cells carrying Myc-tagged Pds1 or Scc1 were transformed with control plasmid or plasmids containing wild type E4orf4, R81AF84A or K88A. Cells were cultured in the presence of 0.2M HU and released from drug as described in materials and methods. Figure 3-5A shows that, as before, Pds1 was unstable in the presence of wild type E4orf4, in both HU-arrested and cycling cells (lanes 2 and 6); however, Pds1 was stable under both conditions with the class I mutant R81F84A (lanes 4 and 8) and largely stabilized with K88A (lanes 3 and 7). Comparable results were also obtained with Scc1 which remained stable in the presence of both the K88 and R81F84 E4orf4 mutants (Figure 3-5B). Thus the premature degradation of Pds1 and Scc1 induced by E4orf4 is

Figure 3-5A E4orf4-induced destruction of Pds1 is dependent on a functional interaction between E4orf4 and PP2A.

Empty vector, wild type FLAG-E4orf4, HA-K88 or HA-R81F84 were expressed in yeast strains carrying Myc-tagged *PDS1* (6803). Expression of wild type and mutant E4orf4 proteins was induced with galactose in the presence of 0.2M HU as described in the materials and methods. Cells were collected at 0 or 3 hours after release from the HU arrest and processed for SDS-PAGE followed by western blotting with the indicated antibodies.

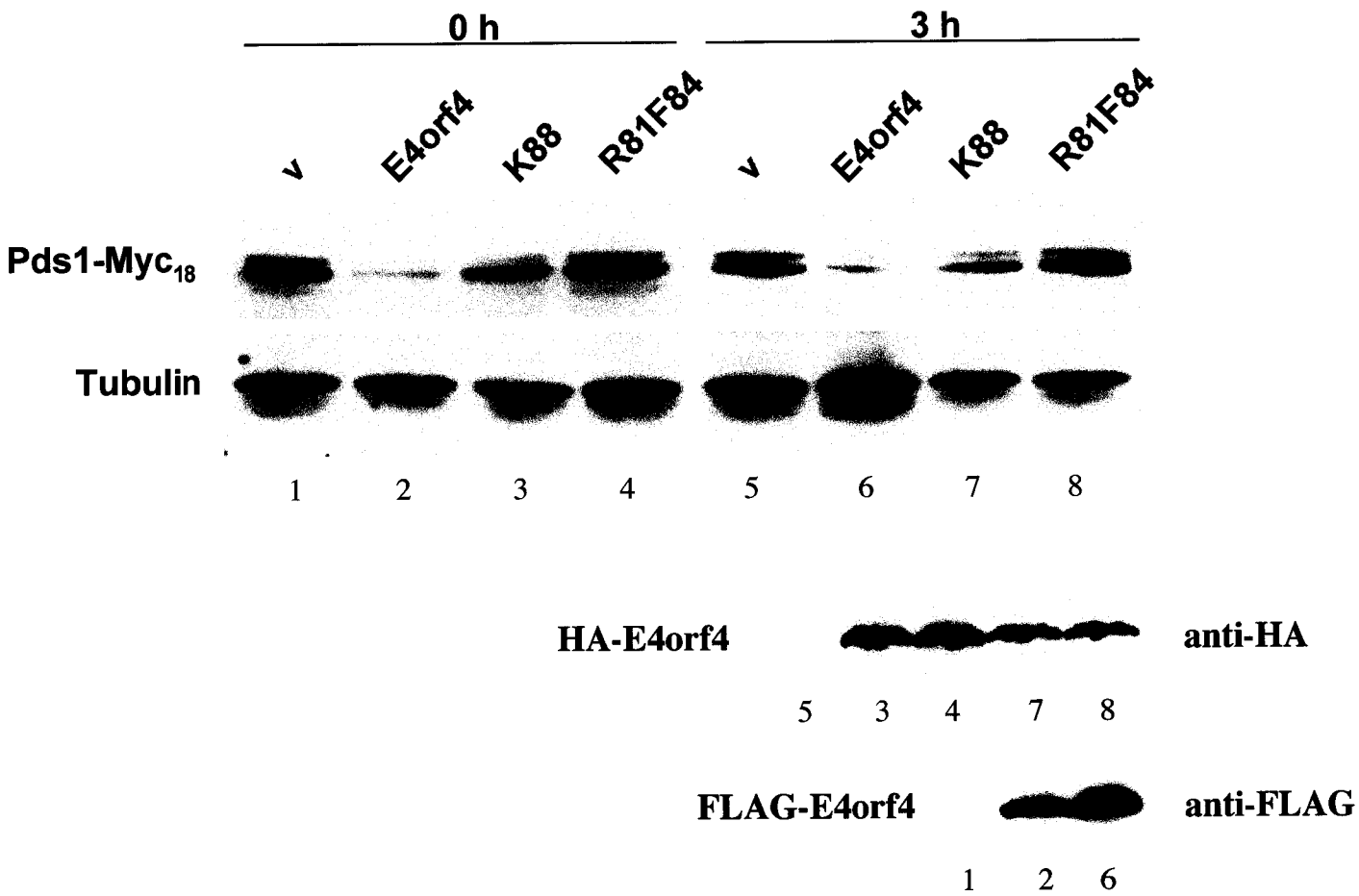
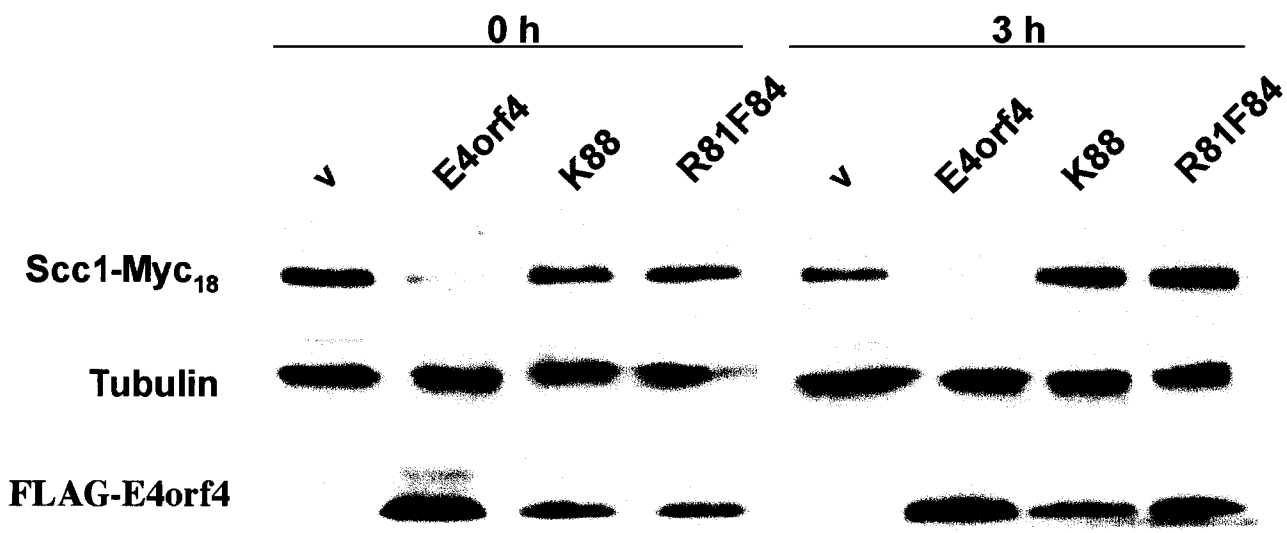

0 h
3 h
v
E4orf4
K88
R81F84
v
E4orf4
K88
R81F84
Pds1-Myc18
Tubulin
1 2 3 4 5 6 7 8
HA-E4orf4
anti-HA
5 3 4 7 8
FLAG-E4orf4
anti-FLAG
1 2 6

Figure 3-5B E4orf4-induced destruction of Scc1 is dependent on a functional interaction between E4orf4 and PP2A.

Empty vector, wild type FLAG-E4orf4, HA-K88 or HA-R81F84 were expressed in yeast strains carrying Myc-tagged *SCC1* (6565). Expression of wild type and mutant E4orf4 proteins was induced with galactose in the presence of 0.2M HU as described in the materials and methods. Cells were collected at 0 or 3 hours after release from the HU arrest and processed for SDS-PAGE followed by western blotting with the indicated antibodies.


0 h
3 h
v
E4orf4
K88
R81F84
v
E4orf4
K88
R81F84
Scc1-Myc18
Tubulin
FLAG-E4orf4

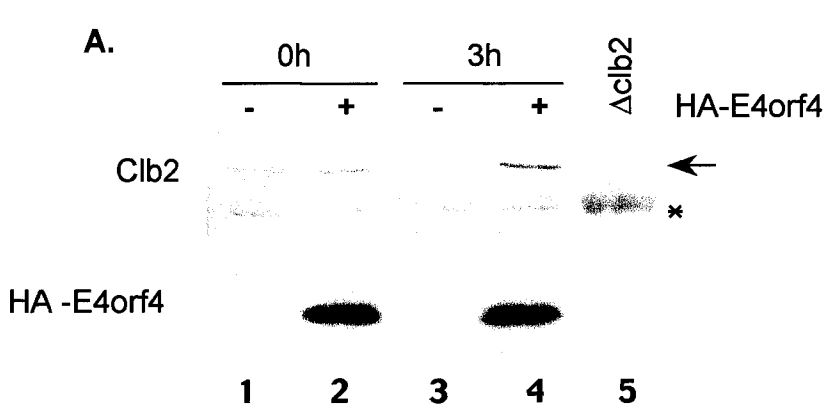
dependent on a functional interaction between E4orf4 and the Cdc55 regulatory subunit of PP2A.

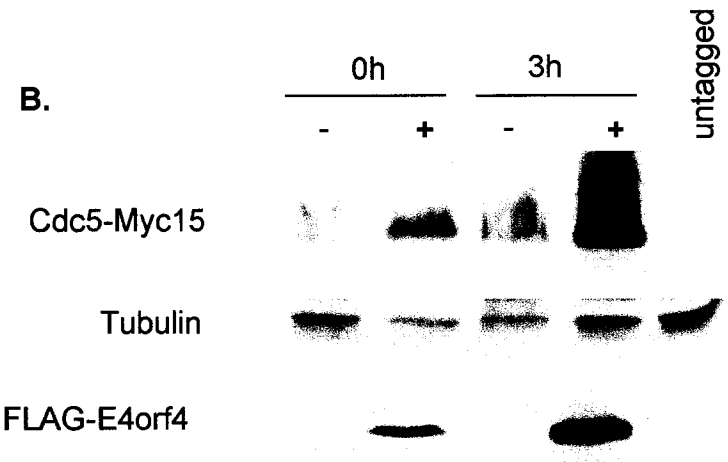
3.3.6 E4orf4 promotes Hct1 hyper-phosphorylation as well as the stabilization of Clb2, Cdc20 and Cdc5

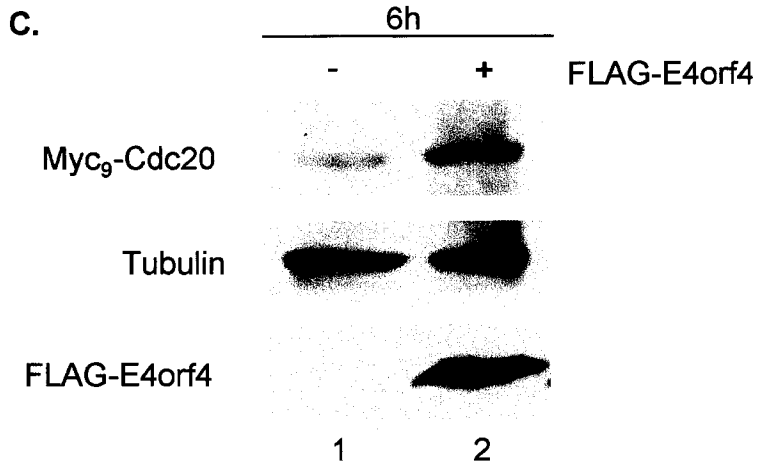
As E4orf4 expression promotes the activation of APC^{Cdc20}, studies were conducted to determine its effects on substrates of the second APC complex, APC^{Hct1}, that include Clb2, Cdc20 and Cdc5 (Noton and Diffley, 2000; Shirayama et al., 1998). Empty vector DNA or that expressing E4orf4 was transformed into yeast carrying Myc-tagged Cdc5 and an experiment utilizing HU as in Figure 3-2A (see materials and methods) was conducted on the stability of Cdc5. Figure 3-6A shows results obtained on the levels of endogenous Clb2 by western blotting using anti-Clb2 antibodies, and Figure 3-6B those of Cdc5-Myc using anti-Myc antibodies. With both Clb2 and Cdc5 the levels obtained in E4orf4-expressing cells were similar to or higher than control cells in the presence of HU, and they were considerably increased for at least 3 h upon removal of the drug. These results suggest that Clb2 and Cdc5 are stable regardless of the cell cycle phase in the presence of E4orf4. Similar studies were also conducted on Myc-tagged Cdc20. Figure 3-6C shows that E4orf4 expression also stabilizes Cdc20, again suggesting that APC^{Hct1} is inactive in E4orf4-expressing cells. APC^{Hct1} is activated by the dephosphorylation of Hct1 by the Cdc14 phosphatase; however, Hct1 is phosphorylated by Clb2-Cdc28, and Hct1 is not able to interact with the APC when in the hyper-phosphorylated form (Jaspersen et al., 1999; Zachariae et al., 1998). To address this possibility, the phosphorylation status of Myc-tagged Hct1 was examined in E4orf4-

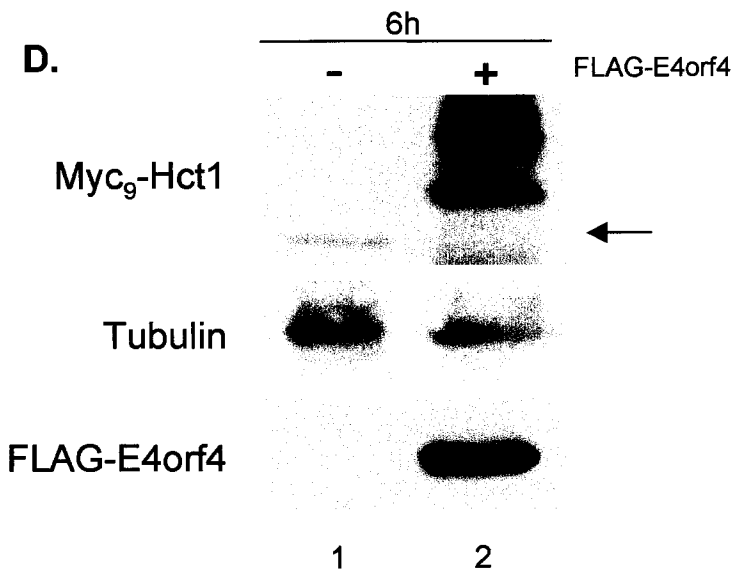
Figure 3-6 E4orf4 induces Hct1 hyper-phosphorylation and the stabilization of Clb2, Cdc5 and Cdc20.

A, B. E4orf4 promotes the stability of Clb2 and Cdc5. E4orf4 expression was induced in a strain carrying myc-tagged Cdc5 (6142) in the presence of 0.2M HU. Cells were released from the HU arrest into fresh galactose-containing medium and collected 0 or 3 hours after the release. Whole cell extract was prepared for SDS-PAGE followed by western blotting with the indicated antibodies. **C, D.** Empty vector or E4orf4 plasmid DNA was transformed into strains carrying myc-tagged *CDC20* or *HCT1* (425 and 491). Cells were collected 6 hours post-galactose induction of E4orf4 expression and prepared for SDS-PAGE and western blotting with the specified antibodies. The arrow in panel D indicates the hypo-phosphorylated form Hct1.


A.
0h
3h
Δclb2
HA-E4orf4
-
+
-
+
Clb2
*
HA -E4orf4
1
2
3
4
5


B.
0h
3h
untagged
-
+
-
+
Cdc5-Myc15
Tubulin
FLAG-E4orf4


C.
6h
-
+
FLAG-E4orf4
Myc9-Cdc20
Tubulin
FLAG-E4orf4
1
2


D.
6h
-
+
FLAG-E4orf4
Myc9-Hct1
Tubulin
FLAG-E4orf4
1
2

expressing cells by western blotting using anti-Myc antibodies. Figure 3-6D shows that in the presence of E4orf4 very high levels of the slower-migrating hyper-phosphorylated forms of Hct1 were evident.

These results suggest that the E4orf4-induced mitotic arrest could be due to failure to activate APC^{Hct1}. The absence of APC^{Hct1} activation may be a consequence of the constitutively high levels of Clb2-Cdc28 that result from effects of E4orf4 binding to Cdc55/PP2A holoenzymes.

3.4 Acknowledgements

We wish to thank the laboratories of Kim Nasmyth, Rodney Rothstein, Frank Uhlmann and Wolfgang Zachariae for kindly providing yeast strains as well as Lara Cushieri and James Knockleby for assistance with microscopy. This work was supported by grants to P.E.B. from the National Cancer Institute of Canada and the Canadian Institutes for Health Research and a studentship to D.E.R. from the McGill Faculty of Medicine.

Chapter 4: Use of the *Saccharomyces cerevisiae* deletion library for the identification of E4orf4 targets

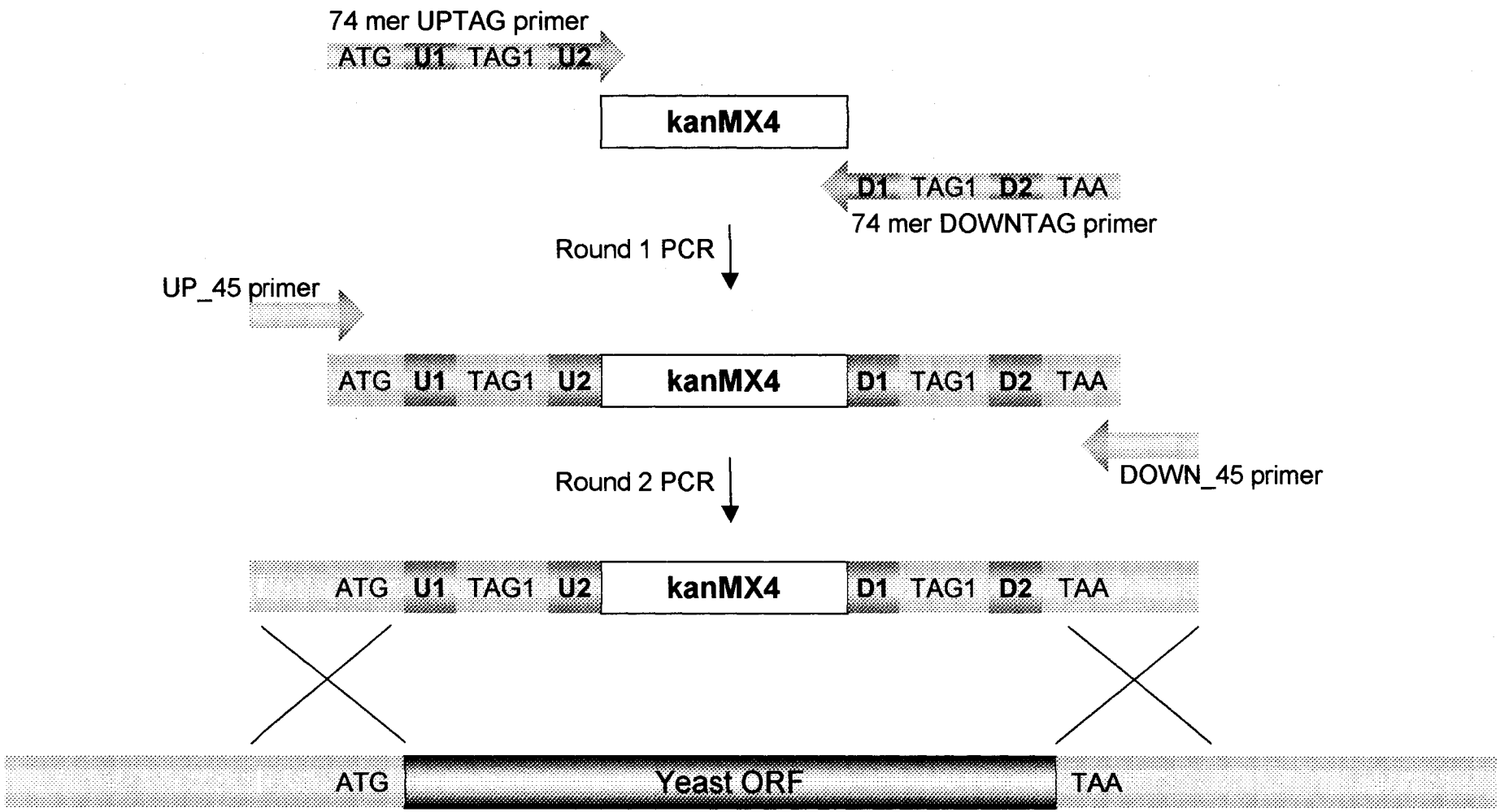
4.1 Introduction

The genome of *Saccharomyces cerevisiae* has been sequenced and consists of approximately 6200 known and predicted genes (Winzeler et al., 1999). Deletion mutants of each gene have been made through the combined efforts of several laboratories that form the yeast consortium. A PCR-based strategy was employed to introduce the kanMX4 cassette, encoding the KAN^R gene for G418 resistance, in place of each gene. This PCR strategy also incorporated a unique 20 base pair sequence tag at both the 5' and 3' ends of the kanMX4 cassette used to create each deletion strain, allowing relatively easy identification of strains (Shoemaker et al., 1996)(Figure 4-1). The deletion project has revealed the existence of ~1100 essential yeast genes with the remaining 5100 genes being non-essential in haploid cells (Winzeler et al., 1999). In addition, over 30% of the yeast genes remain functionally unclassified (Winzeler et al., 1999). The fact that over 80% of yeast genes are non-essential genes indicates that the genome is resilient to mutation and possesses a high degree of functional redundancy among proteins products.

In an effort to place all the non-essential yeast genes within genetic and functional networks, a system for global synthetic genetic analysis (SGA) was developed (Tong et al., 2001). The idea behind a synthetic genetic interaction is that when two genes are individually deleted in separate strains the strains may be viable or sick; however, when both of these genes are deleted within the same strain, the cells are no longer viable or are severely impaired for growth. An automated high throughput mechanism to analyze all the non-essential genes for synthetic genetic interactions has been established using a

Figure 4-1 Schematic of the PCR-based deletion strategy used for construction of deletion strains

The 74mer UPTAG primer (5'-3') contains 18 bases of homology to the region upstream of the yeast open reading frame (ORF), including the ATG, a common priming site U1 (GATGTCCACGAGGTCTCT), a unique 20 base barcode or tag sequence, followed by the common priming site U2 (CGTACGCTGCAGGTCGAC). The DOWNTAG 74mer primer (5'-3') contains 18 bases of homology to the region downstream of and including the stop codon of the ORF, the common priming site D1 (CGGTGTCGGTCTCGTAG), a unique 20 base barcode sequence, followed by the common priming site D2 (ATCGAT GAATTCGAGCTCG). U2 is homologous to a region 5' to the Kan gene and D2 is homologous to a region 3' to the Kan gene in the *kanMX*4 module. After the first round of PCR using the UPTAG and DOWNTAG primers the PCR product was used as template for a second round of PCR using the UP_45 and DOWN_45 primers. The upstream deletion primer (UP_45) is a 45mer containing the 45 bases directly upstream of the ORF (including the ATG). The downstream deletion primer (DOWN_45) is a 45 mer containing the 45 bases directly downstream of the ORF (including the stop codon, TAA). The final PCR product was then transformed into yeast to replace the target ORF with the *kanMX4* cassette by homologous recombination. Proper chromosomal integration of the *kanMX4* module was confirmed by PCR.



Chromosomal integration by homologous recombination

robotic system controlling a floating pin replicator (Tong et al., 2001). The 768 floating pins are not fixed in the base but float when they contact the agar plate, ensuring that contact is made evenly to all areas of the plate. The approximately 4700 viable yeast deletion strains have been arrayed in duplicate onto 16 solid medium plates in known locations. This collection of deletion strains can then be crossed to a query deletion strain of interest. The query strain or starter strain is generated from the strain in the deletion set simply by switching the mating type to *MAT*α and replacing the KAN^R gene with the NAT^R cassette, which encodes resistance to the drug nourseothricin. Through a series of automated pinning procedures, a query deletion strain of mating type *MAT*α can be mated to the entire set of deletions which are *MAT*a. These diploids are then pinned onto sporulation medium to generate tetrads of haploid cells, of which 25% should carry the double deletion mutant. After sporulation the cells are then pinned onto plates that allow the selection of either *MAT*a or *MAT*α haploids containing both deleted genes. The set of gene-deletion mutations are linked to the dominant selectable marker genes, KAN^R (for G418 resistance) and the query mutation is linked to the NAT^R gene (for nourseothricin resistance) allowing for the isolation of double mutants on selective growth medium containing these drugs. The final pinning step gives rise to an array of double-mutant haploid strains whose growth rate can be examined visually.

In an effort to identify genes which would be important for the mechanism of E4orf4-induced cell death we used the SGA system with some modifications. The logic was that if E4orf4 or the E4orf4-PP2A complex required a predominant target to elicit cell death, then deletion of this target would result in viable cells. With this in mind we

tailored the SGA system to allow introduction of E4orf4 into the array of deletion strains and the identification of potential E4orf4 targets.

4.2 Materials and Methods

4.2.1 Yeast strains

All studies were performed using the *S. cerevisiae* deletion strains created in the S288c background. The BY4741 (*MAT*a *his3Δ1 leu2Δ0 met15*Δ0 *ura3ΔO*) and BY4742 (*MATα his3Δ1 leu2*Δ0 *lys2*Δ0 *ura3*Δ0) are aliases for *MAT*a and *MAT*α S288C strains. Y3656 (*MAT*α, *can1*Δ*::MFA1pr-HIS3-MFα1pr-LEU2, his3Δ1, leu2Δ0, ura3Δ0, met15Δ0, lys2Δ0*) is the query or starter strain used for SGA. Y3656 has a dual reporter system integrated at the *CAN1* locus, which allows for isolation of either *MAT*a or *MAT*α haploids (see below). The Y3656 strain was transformed with pYES2-HA-E4orf4 (*URA* selection). Y3656 was also modified by replacing the *TRP1* gene with the NAT^R cassette to create Y3656 $trp1\Delta NAT^R$ to allow for the selection of the p424*GAL1*FLAG-*CDC55* (*TRP* selection) plasmid as well as pYES2-HA-E4orf4. The pYES2HA-E4orf4 and p424GAL1FLAG-*CDC55* were co-transformed into Y3656$trp\Delta NAT^R$ and the resulting strain was used as the query strain to be crossed against the SGA *MAT*a haploid deletion array (*MAT*a *XXXΔKAN* ^{R} *LYS2+ his3Δ1 leu2Δ0 met15*Δ0 *ura3ΔO,* where *XXX* represents a deleted gene).

4.2.2 Media and growth conditions for automated screening of deletions strains.

OmniTray plates (NUNC™) were used for all steps involving use of the Virtek robotic colony arrayer (Virtek Engineering Sciences, Inc.). For automated arraying, yeast cells were transferred using a 768 floating-pin replicator. The Y3656 starter strain

transformed with control pYES2 or pYES2HA-E4orf4 plasmid DNA. The resulting strains were grown overnight at 30°C in 5ml of selective liquid medium. The cultures were then plated onto solid -URA medium in OmniTray plates to obtain lawns of cells carrying the control or E4orf4 plasmid. Cells from the lawns were pinned onto fresh plates using the automated pin-replicator. The resulting query plates contained an array of 768 colonies of the starter strain carrying the empty vector control or E4orf4 plasmid DNA. Such query plates were used for mating to the array of deletion strains which were also arrayed in the 768 colony format.

The following media were prepared for each step of screening the array of deletion strains, which were kindly provided by the Bussey laboratory, with E4orf4.

1. YEPD (yeast extract, peptone and 2% dextrose) plates with supplemented adenine (120mg/L) were used to maintain the arrayed deletion strains. The deletion strains were mated to the Y3656 strain carrying the control or E4orf4 plasmids by pinning the arrayed colonies of strains onto plates containing yeast nitrogen base (YNB. Bioshop), 2% glucose and amino acid drop-out mix lacking lysine and uracil. Lack of lysine allowed for the selection of diploids and the absence of uracil selected for maintenance of the control or E4orf4 plasmid. Plates were incubated at 30°C for 2 days.

2. Diploids were sporulated on sporulation medium (containing potassium acetate, yeast extract, 0.05% glucose and complete amino acid mix) for 5 days at room temperature.

3. After spore germination, MATa hapoids carrying the E4orf4 plasmid were selected for on plates containing YNB, 2% glucose, amino acid drop out mix lacking arginine, histidine and uracil and containing 0.05 mg/ml of canavanine. Canavanine is a toxic

arginine analog. *CAN1* encodes the arginine permease which imports arginine into the cell as well canavanine. *CAN1* cells are canavanine sensitive and die in the presence of the drug, while *can1* cells are canavanine resistant and survive. A dual reporter system is integrated into the Y3656 strain at the *CAN1* locus and allows for selection of the *MAT*a or *MAT*α haploids depending on the amino acid composition of the selective media. In selecting for either type of haploid there are two levels of selection. First, the lack of histidine ensures that only *MAT*a hapoids will survive since the production of this essential amino acid is driven by the *MAT*a specific promoter, *MFA1pr*, in the Y3656 strain. Likewise, medium lacking leucine would allow only *MAT*α cells survived. Second, the presence of canavanine permits only haploids with the *MFA1pr-HIS-MFα1pr-LEU2* reporter replacing the *CAN1* gene at the *CAN1* locus to survive. The absence of arginine in the selection medium ensures that *CAN1* strains will import canavanine and die, while canavanine resistant Δ*can1* cells will survive. The absence of uracil allows for selection of the E4orf4 plasmid. Plates were incubated at 30˚C for 2 days and cells were pinned onto the plates described in this step twice to ensure proper selection.

3. To select for the MATa haploids with the KAN^R resistance marker, and thus the gene deletion, medium identical to the one used in step 2 was used except this time containing 200 mg/ml G418 (Sigma). Plates were incubated at 30˚C for 2 days

4. To finally induce expression of the galactose-inducible E4orf4 plasmid in the isolated *MAT*a haploid strains, cells were pinned onto selective medium containing YNB, 2% raffinose and 2% galactose, and amino acid drop out mix lacking uracil. Plates were incubated at 30˚C for 2 days

5. The final step after the identification of any deletion strain resistant to E4orf4 expression would involve going back and testing E4orf4 expression in the original deletion strain, either from the master plate or freezer stocks to verify the rescue of E4orf4-induced cell death.

In the case where both the E4orf4 and Cdc55 plasmids were co-transformed into Y3656 *trpΔNAT*^{R}, tryptophan was excluded from the selective medium to maintain the Cdc55 plasmid and nourseothricin was included in step 3 to ensure that surviving *MAT*a haploid cells were deleted for *TRP1* and therefore had need of the Cdc55 plasmid.

4.2.3 Screening of *MAT*a haploid deletion strain library

The *MAT*a haploid deletion strains were collected from the 16 plates of arrayed deletion strains and pooled to create a library of deletion strains. Cells were resuspended in 20% glycerol, aliquoted and frozen at -80°C. An aliquot of this mix was thawed and used to inoculate YPED liquid medium. Cells were grown to an optical density (OD) of ~0.6 and then co-transformed with pYES2-HAE4orf4 and p425*TEF*-FlagCDC55 plasmid DNA using a high efficiency yeast transformation protocol and plated onto selective 2% glucose-containing medium. Transformants were replica plated onto selective medium containing 2% galactose and 2% raffinose to allow for E4orf4 expression from the *GAL1* promoter. These galactose-containing plates were used for a second round of replica plating onto galactose-containing medium. Colonies that were able to survive the combined lethality of CDC55 and E4orf4 expression after two rounds of selection under E4orf4-inducing conditions were picked and analyzed further to determine the identity of the deletion strain.

4.2.5 Preparation of genomic DNA and identification of deletion strains

Genomic DNA was prepared according to the following protocol using 96 well PCR plates. 100 µl of Buffer A (0.6% Triton-X 100, 50 mM NaCl, 1 mM EDTA, 10 mM Tris pH 8) and glass beads were aliquoted into 96 well plates. Cells were resuspended into the buffer after which 25 µl of phenol (saturated with Tris, pH 8) and 25 µl of chloroform were added. The wells were capped and cells were vortexed for 4 minutes. Samples were centrifuged for 15 minutes at 3200 rpm and the supernatant was removed (~30 µl) to fresh tubes. Genomic DNA was precipitated with 3M ammonium acetate (pH 5.2) and 2.5 volumes of cold 100% ethanol, followed by washing with 100 µl of 70% ethanol and finally resuspended in 50 µl of 10 mM Tris (pH 7.4). Genomic DNA was then subject to PCR using the D2 reverse primer (CGGTGTCGGTCTCGAG) flanking the unique barcode tag and a forward primer (KAN D) corresponding to a sequence within the kanMX4 module (GATTCAGTCGTCACTCATG). The resulting PCR product was then sequenced using a third oligo, KAN Y (GATGCGAAGTTAAGT G), that anneals within the kanMX4 sequence, but upstream of the barcode sequence. After DNA sequencing the unique barcode could be read and then used to query a database containing all the unique barcodes and corresponding deleted genes.

Once the identity of the deleted gene was determined, the deletion strain was obtained from the strains arrayed on the master SGA plates or from the freezer stocks. The deletion strains were then transformed with E4orf4 and CDC55 plasmids to verify whether the cell death rescue was reproducible.

4.3 Results

4.3.1 Use of automated SGA to screen for E4orf4 targets

In the initial trial with the SGA high throughput system, the galactose-inducible control or E4orf4 plasmid was transformed into the starter strain and mated to the deletion array. After sporulation of the diploids, selection for *MAT*a hapoids and selection of the deletion with G418 resistance, E4orf4 expression was induced by pinning onto medium containing 2%galactose and 2% raffinose, as described in section 4.2.2. There was barely any difference seen in the growth of colonies between the E4orf4 plates and the vector control plates after the induction of E4orf4. The reason for this may have been due to the existence of sufficient cell growth before enough E4orf4 could be expressed to allow differences in growth to be apparent.

As visual difference in cell growth between the plates expressing vector control and E4orf4 plasmids was needed in order for the screen to be feasible, we made use of an earlier observation that over-expression of Cdc55 and E4orf4 together in wild type strains was more toxic than just expression of E4orf4 alone. To allow for the selection of the Cdc55 galactose-inducible plasmid (p424*GAL1*FLAG-*CDC55*) in the Y3656 strain the *TRP* gene was deleted using the NAT^R cassette to create Y3656$trp\Delta NAT^R$ and the E4orf4 and Cdc55 plasmids were co-transformed into this strain. As a control for our pilot study we co-transformed the two empty vectors (pYES2 and p424*GAL1*) into Y3656$trp\Delta NAT^R$ as well. These query strains were mated to the deletion array and the same pinning procedures were followed as described in section 4.2.2, except this time the selection

medium was modified to allow for the selection of the additional Cdc55-containing plasmid.

The Y3656*trpΔNAT*^{R} strain expressing both empty vectors or E4orf4 and Cdc55 were successfully mated to the deletion strains. The diploid cells containing the control vectors were successfully sporulated and the *MAT*a haploids were obtained on selective media. In contrast, we could not obtain viable MATa haploids cells after sporulation of the diploid cells carrying the E4orf4 and Cdc55 plasmids. Most of the cells (~70%) pinned onto the *MAT*a selection medium never grew to form colonies and appeared to be dead even before E4orf4 and Cdc55 expression was induced on galactose-containing medium. This lack of viability could have been due the combination of leakiness from the *GAL1* promoter, allowing low amounts of E4orf4 and Cdc55 to be expressed, in combination with the selective pressure on the cells from treatment with canavanine. Alternatively, the low amounts of E4orf4 and Cdc55 expression may have interfered with sporulation of the diploids; however this seems unlikely because all the diploid strains would have one copy of the *CAN1* gene intact, rendering them all sensitive to the presence of canavanine in the medium. To overcome this unforeseen technical difficulty, the approach to screening the yeast deletion strains with E4orf4 was again modified.

4.3.2 Screening of MATa haploid deletion library

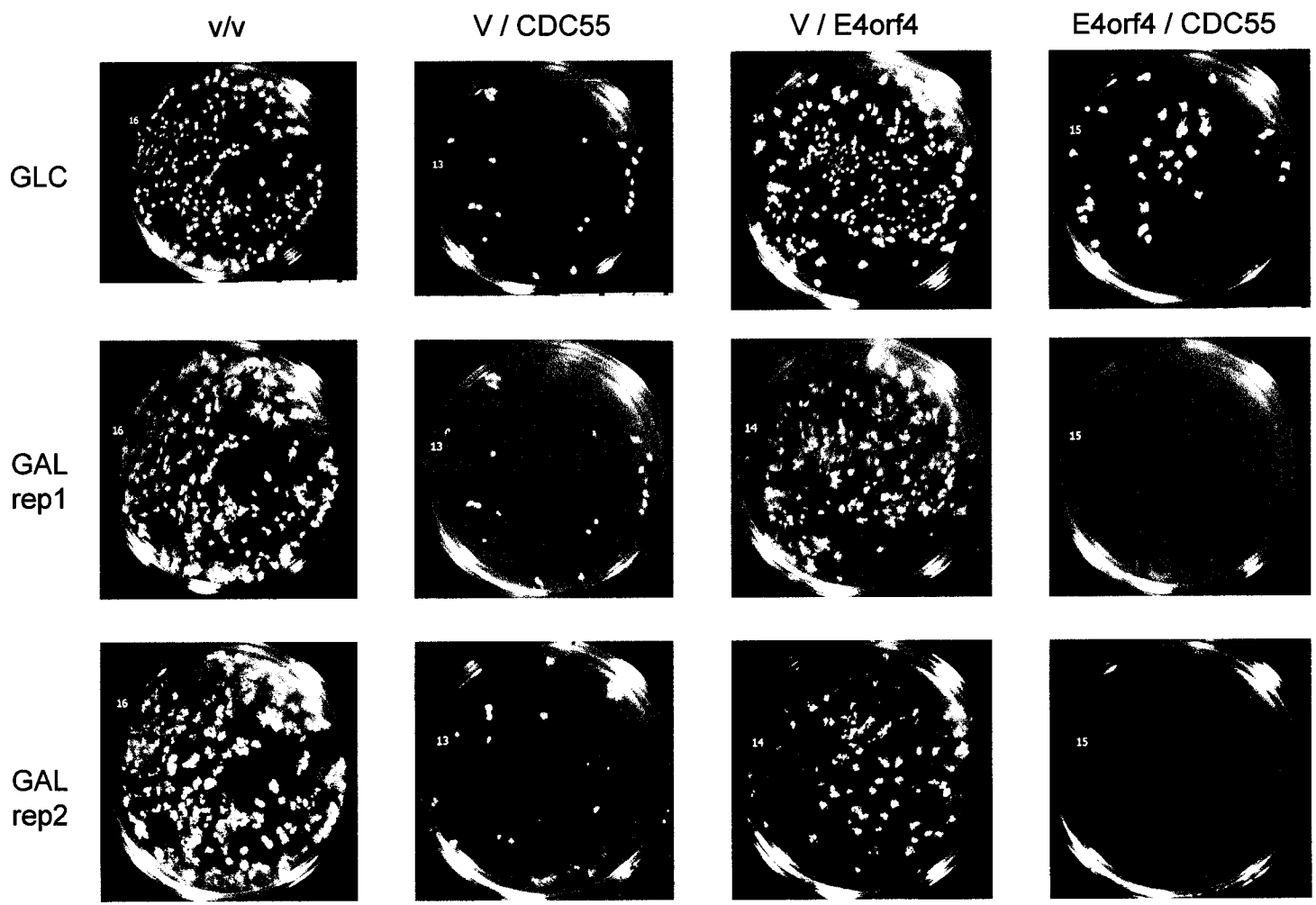
All the cells were collected from the 16 plates of arrayed deletion strains and used to create a library of deletion strains into which the E4orf4 and Cdc55 plasmid DNAs could be co-transformed using conventional yeast protocols. As mentioned previously, expression of E4orf4 alone in this type of screen did not yield clear differences in colony

growth when compared with the vector control therefore both E4orf4 and Cdc55 had to be expressed. In an initial test experiment using the pYES2-HAE4orf4 and p424*GAL1*-FLAG-*CDC55* plasmids we observed that the difference between the colony growth on plates expressing those plasmids and control plasmids was not as dramatic as needed to perform the screen. To overcome this we tried expressing the pYES2-HA-E4orf4 construct in combination with *CDC55* under different constitutive promoters in either centromeric or high copy 2μ plasmids. The E4orf4 and Cdc55 plasmids were co-transformed into the wild type BY4741 strain and cells were plated onto selective glucose containing medium. The Transformants were then replica plated onto plates containing 2% raffinose and 2% galactose to induce E4orf4 expression. Pilot studies indicated that the combination of the galactose-inducible E4orf4 with Cdc55 under the control of the consitutive *TEF* promoter in a high copy plasmid gave the most toxic phenotype compared to controls after replica plating onto galactose-containing medium (Figure 4-2). This combination of plasmid was therefore used to screen the yeast deletion strain library as described in section 4.2.3.

Approximately 20, 000 colonies were screened, representing about a four fold coverage of the deletion set. Of these, 206 colonies grew after two rounds of replica plating onto galactose-containing medium. Genomic DNA was obtained from single colonies and primers were used to amplify by PCR the region containing the unique barcode sequence identifier for each colony as described in sections 4.2.5. The resultant PCR products were subject to DNA sequencing using a third internal primer to identify the barcode sequence. The barcode sequence was used to query a database from which the corresponding deletion strain could be identified. A total of 104 deletions were

Figure 4-2 Efficiency of cell death mediated by expression of E4orf4 and Cdc55

Control, pYES2-HA-E4orf4 or p424*TEF*-FLAG-*CDC55* plasmids were co-transformed into BY4741 wild type yeast by the one-step method {Chen, 1992 #423}. Cells were plated on solid medium containing 2% glucose and incubated for 2 days at 30°C to select for Transformants. Cells were then replica plated onto medium containing 2% galactose and 2% raffinose to induce E4orf4 expression (Rep1). These plates were used to do a second round of replica plating (Rep2) onto fresh plates.


v/v
V / CDC55
V / E4orf4
E4orf4 / CDC55
GLC
GAL rep1
GAL rep2
16
13
14
15

successfully identified by this method. In 50 cases deletions could not be identified due to unsuccessful PCR from the genomic DNA even after multiple attempts. The other 52 cases gave PCR products to be sequenced; however, no identification was made because of incomplete barcodes, unreadable DNA sequences, or the barcode did not match any deletion in the data base. Mistakes may have occurred during the sequencing stage; however, repeating the DNA sequencing for these 52 clones gave the same negative results.

For each of the 104 deletion strains that were identified, the corresponding *MAT*a haploid strains from the master SGA plates were transformed with the E4orf4 and Cdc55 plasmids to verify the rescue. Spotting assays were performed with each of the 104 deletions that were identified and of these 22 deletions appeared to be either partially or completely resistant to E4orf4 and Cdc55 co-expression (Table 4-1). To further confirm that these deleted genes were targets of E4orf4, strains were thawed from the original glycerol stocks of all the arrayed deletion strains. E4orf4 and Cdc55 plasmids were transformed into these original strains and spotting assays were performed to determine whether these deletions would be resistant to E4orf4 and Cdc55 expression. In all cases, the rescue of cell death promoted by co-expression of E4orf4 and Cdc55 observed previously in the strains from the plate stocks was not reproducible in the deletion strains taken from the original glycerol freezer stocks (Table 4-1). The reason for this non-reproducibility is unclear but may involve the ability of the yeast strains obtained from the plates to down-regulate the expression of E4orf4 and/or Cdc55. Over time, cells subject to repeated sub-culturing, as is the case for the deletion strains arrayed on the plates, can develop mutations. Since the deletion strains obtained from the original

freezer stocks were subject to significantly less sub-culturing, it is likely that the results obtained with these strains are more reliable.

Table 4-1 Deletion strains identified from screen with E4orf4 and Cdc55

Gene	ORF name	Tested with deletion from plate	Tested with deletion from freezer stocks
ybr067c	*TIP1*	+	-
ybr150c	*TBS1*	-	
ybr208c	*DUR1*	-	
ybr275c	*RIF1*	-	
ybr281c	Hypothetical ORF	+	-
ybr284w	Hypothetical ORF	+	-
ycl022c	Hypothetical ORF	+/-	-
ycl023c	Hypothetical ORF	+	-
ycl027w	*FUS1*	-	
ycl047c	Hypothetical ORF	-	
ycl050c	*APA1/DTP1*	-	
ycl050c	*APA1*	-	
ycr030c	*SYP1*	-	
ycr068w	*AUT5*	-	
ycr075c	*ERS1*	-	
ydl179w	*PCL9*	+/-	-
ydr007w	*TRP1*	-	
ydr102c	Hypothetical ORF	-	
ydr163w	*CWC15*	-	
ydr198c	Hypothetical ORF	-	
ydr256c	*CTA1*	-	
ydr316w	*OMS1*	-	
ydr395w	*SXM1*	-	
ydr409w	*SIZ1*	-	
ydr492w	*IZH1*		
ydr533c	*FIT1*	-	
ydr539w	Hypothetical ORF	-	
yel007w	Hypothetical ORF	-	
yel008w	Hypothetical ORF	-/+	-
yel041w	Hypothetical ORF	+	-
yel062w	*NPR2*	-	
yer037w	*PHM8*	-	
yer142c	*MAG1*	+	-
yer179w	*DMC1/ISC2*	-	
yer187w	Hypothetical ORF	-	
yfr033c	*QCR6*	+/-	-
ygl032c	*AGA2*	-	
ygl081w	Hypothetical ORF	-	
ygl221c	*NIF3*	-	
ygl250w	Hypothetical ORF	-	
ygr227w	*DIE2/ALG10*	-	
ygr244c	*LSC3*	-	
yhl028w	*WSC4*	+/-	-

yhr125w	Hypothetical ORF	-	
yil030c	*SSM4*	-	
yil094c	*LYS12*	-	
yir014w	Hypothetical ORF	-	
yjl071w	*ARG2*	-	
yjl146w	*IDS2*	-	
yjl164c	*SRA3/TPK1*	-	
yjl210w	*PAS5/PEX2/CRT1*	-	
yjl213w	Hypothetical ORF	+	-
yjl216c	Hypothetical ORF	-	
yjr058c	*APS2*	-	
yjr100c	Hypothetical ORF	-	
yjr128w	Hypothetical ORF	-	
yjr130c	*STR2*	-	
ykl023w	Hypothetical ORF	-	
ykl067w	*YNK1/NDK1*	-	
ykl211c	*TRP3*	-	
ykr011c	*TOS5*	-	
ykr023w	Hypothetical ORF	-	
ykr052c	*MRS4*	-	
yll052c	*AQY2*	-	
ylr031w	*GAT3*	-	
ylr096w	*KIN2*	-	
ylr099c	*ICT1*	-	
ylr343w	*GAS2*	-	
ylr427w	*MAG2*	-	
yml102w	*CAC2*	-	
ymr144w	Hypothetical ORF	-	
ymr164c	*MSS11*	-	
ymr214w	*SCJ1*	-	
ymr262w	Hypothetical ORF	+ / -	-
ymr304c-A	Hypothetical ORF	+	-
ymr322c	*HSP34*	-	
ynl041c	*COD2*	-	
ynl093w	*YPT53*	-	
ynl093w	*YPT53*	-	
ynl095c	Hypothetical ORF	-	
ynl098c	*RAS2*	+/-	-
ynl122c	Hypothetical ORF	+	-
ynl159c	*ASI2*	-	
ynl223w	*AUT2*	-	
ynl257c	*SIP3/SNF1*	-	
ynl264c	*PDR17*	+/-	-
ynl266w	Hypothetical ORF	-	
ynl291c	*MID1*	-	
ynl299w	*TRF5*	-	
ynr058w	*BIO3*	-	
ynr069c	*BSC5*		
yol155c	Hypothetical ORF	+/-	-
yor275c	*RIM20*	+/-	-
yor276w	*CAF20/CAP20*	-	
yor298c-A	*MBF1*	-	
yor301w	*RAX1*	-	
yor338w	Hypothetical ORF	+	-

ypl019c	*VTC*	-	
ypl034w	Hypothetical ORF	-	
ypr013c	Hypothetical ORF	+/-	-
ypr026w	*ATH1*	+	-
ypr079w	*MRL1*	-	
ypr154w	*PIN3*	-	
ypr192w	*AQY1*	-	

+ : almost complete rescue of cell death
+/- : intermediate or partial rescue of cell death

4.4 Acknowledgements

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Chapter 5: General Discussion

Previous studies correlated the binding of E4orf4 to the Bα subunit of PP2A with the induction of p53-independent cell death in mammalian cancer cell lines (Shtrichman et al., 1999; Marcellus et al., 2000). Little is known about the effects of E4orf4 on PP2A activity or what the critical PP2A substrates might be that effect the cell death response. As the PP2A enzyme is well conserved across eukaryotic species, we chose to address the mechanism of E4orf4-induced cell death using the yeast *Saccharomyces cerevisiae*, an organism that is much more amenable to genetic manipulation than mammalian cells. In these studies we have demonstrated the feasibility of using yeast as a model system for the investigation of the effects of E4orf4.

E4orf4 expression in yeast is lethal and confers a readily identifiable elongated morphology somewhat similar to that observed in *cdc55* strains cultured at low temperature. By expressing E4orf4 in *cdc55* and *rts1* strains, we found that the homolog of the B class regulatory subunit in yeast, *CDC55*, was important for mediating E4orf4-induced cell death, whereas *RTS1*, which encodes the homolog of the B' PP2A regulatory subunit, was not. The presence of E4orf4 did not affect the interaction between Cdc55 and the Tpd3 A subunit; however, Tpd3 could not associate with E4orf4 in a *cdc55* strain. Therefore, E4orf4 requires an interaction with the Cdc55 subunit in order to associate with PP2A. In contrast, E4orf4 does not interact with the Rts1 subunit as demonstrated in co-immunoprecipitation experiments. E4orf4 was also found to interact with PP2A complexes containing either of the yeast C subunits, Pph21 or Pph22. As E4orf4 interacts exclusively with the Cdc55-PP2A complex and not with the Rts1-PP2A

complex, E4orf4 appears to be a useful tool for differentiating between substrates acted upon by these two classes of PP2A trimer.

Both Class I and Class II E4orf4 point mutants are highly defective for killing mammalian cancer cell lines; however Class II mutants can bind the mammalian Bα subunit, suggesting that E4orf4 binding to Bα is required, but not sufficient to elicit cell death (Marcellus et al., 2000; Champagne et al., 2004). As seen in experiments using mammalian cells, the Class I R81F84 E4orf4 mutant showed no interaction with Cdc55; however, the Class II K88 mutant interacted with Cdc55 as well as wild type E4orf4. In yeast cell growth assays both the R81/F84 and K88 mutant induced a very similar, modest inhibition of cell growth. Therefore, while K88 can interact with Cdc55, it only yields a partial toxicity similar to R81F84 suggesting that while E4orf4 requires binding to Cdc55 to elicit its effect, this binding is insufficient for E4orf4 to mediate its full phenotype. Alternatively, the Cdc55-K88 interaction may not be biologically functional. Cdc55 is clearly important for E4orf4-mediated cell death, as most of the inhibitory activity is relieved in *cdc55* strains; however, the partial toxicity seen with E4orf4 in *cdc55* cells, and with the class I and class II mutants in wild type cells clearly indicates that a Cdc55-independent function may also exist.

While the Class I and Class II E4orf4 mutants induced a partial growth delay, they were not sufficient to generate the aberrant elongated morphology associated with a significant proportion of E4orf4-expressing yeast cells. In the case of R81F84 this effect can be explained by the absence of E4orf4 and Cdc55 interactions, suggesting that a stable E4orf4-PP2A complex is required for this phenotype; however, the K88 mutant, which does interact with Cdc55, did not show any significant morphological

abnormalities. The results obtained with the K88 mutant indicate either that the interaction between E4orf4 and Cdc55 is insufficient to promote the morphological phenotype or that the complex of PP2A with this E4orf4 mutant is non-functional in this respect. In addition, expression of E4orf4 in the *cdc55* strain does not increase the number of elongated cells in the culture (unpublished data). E4orf4 therefore depends on a functional interaction with Cdc55 to promote the abnormal cell morphology. These findings are in agreement with results of earlier studies that have demonstrated that PP2A is involved in yeast cell morphogenesis (Healy et al., 1991; Lin and Arndt, 1995; Evans and Stark, 1997; Ronne et al., 1991; van Zyl et al., 1992). Elongated buds are often the result of failure of buds to undergo the switch from apical growth (elongation of the bud) to isotropic growth (growth of the bud in all directions). Apical growth occurs during S and G2 phases that represent periods of low Cdk1 activity due to inhibitory Swe1 phosphorylation of Y19 on Cdc28 (Lew and Reed, 1995b). Isotropic growth occurs during periods of high Cdk1 activity when Swe1 becomes inactivated in early mitosis (Lew and Reed, 1995b). Swe1 stability is regulated by Cdc55-PP2A (Yang et al., 2000), leading to the possibility that E4orf4 may induce the morphogenesis checkpoint, resulting in stable Swe1, a G2 arrest and the presence of elongated buds. This possibility appears not to be the case as in our studies as we have consistently observed that E4orf4 induces mitotic arrest with high levels of Cdk1 activity, suggesting that Swe1, which is inhibitory to Cdk1, is inactivated in these cells. Why this morphology is consistently observed in only a fraction (about one third) of the population of yeast expressing the E4orf4 gene remains unclear, but could be due to differential levels of E4orf4 protein expression

within individual cells or timing of optimal E4orf4 expression relative to the stage of the cell cycle.

It has previously been reported that strains harboring *cdc55rts1* double deletions show extremely slow growth compared to wild type cells or those lacking just one of the genes encoding these PP2A subunits (Shu et al., 1997). We demonstrated that E4orf4 does not require *RTS1,* as E4orf4 expression in the *rts1* strain induced a slow growth phenotype similar to that which would be expected in a *cdc55/rts1* strain, thus raising the possibility that E4orf4 could simply be binding and inactivating the Cdc55 subunit. As the E4orf4-induced phenotype does not completely mimic the *cdc55* strain, this possibility seems unlikely. While *cdc55* strains grow somewhat slower than wild type strains at 30°C, the effect of E4orf4 expression in wild type cells is significantly more severe. Furthermore, over-expression of *CDC55* in E4orf4-expressing cells does not reverse E4orf4-induced toxicity, but rather exacerbates it.

E4orf4-Cdc55/PP2A complexes possess high levels of phosphatase activity as determined by *in vitro* phosphatase assays using a phosphorylated peptide as substrate. Similar results were obtained in mammalian cell lines (Marcellus et al., 2000; Brignole et al., unpublished results). While E4orf4 does not inhibit the catalytic activity of the phosphatase, we believe that E4orf4 may affect PP2A activity against selected substrates. We show that E4orf4 expression prevents Cdc55-directed PP2A trimers from localizing normally to bud tips. This pool of Cdc55-PP2A that is lost at the bud tips may be re-localized elsewhere in the cell. This loss of localization to bud tips may result in a change in PP2A substrate specificity, with some substrates being left in a hyper-phosphorylated state and others being preferentially dephosphoryated. E4orf4 could thus be used as a tool

to identify downstream targets and/or functions of PP2A that are modulated by its expression. In light of the E4orf4-induced mitotic delay, possible PP2A targets include regulators of the cell cycle.

Previous genetic studies in *Saccharomyces cerevisiae* have implicated Cdc55 in mitotic control (Wang and Burke, 1997; Minshull et al., 1996). We have demonstrated that E4orf4 modulates only the Cdc55 form of PP2A to promote a sustained as well as premature activation of the Cdk1/Clb2-Cdc28 complex. E4orf4 appears to require the activity of Cdk1 to mediate cell death as in *cdc55* cells where E4orf4-induced cell death is almost completely reversed, no increase in Cdk1 activity is observed. The phosphorylation of APC subunits by Cdc28-Clb2 is necessary for the activation of APC^{Cdc20} (Rudner and Murray, 2000). An earlier study of E4orf4 in yeast proposed that E4orf4 could enhance PP2A activity against APC core subunits, inducing the inactivation of the APC and the stabilization of both APC^{Cdc20} and APC^{Hct1} substrates (Kornitzer et al., 2001). The authors proposed that the E4orf4-induced inhibition of the APC complexes in combination with the increased Cdk1 activity created conflicting signals that resulted in death. In the present study we re-visited this idea by examining the stability of various APC substrates as well as the downstream Esp1 substrate, Scc1. We have found in disagreement with Kornitzer *et al.* that APC^{Cdc20} is indeed active in E4orf4-expressing yeast cells. E4orf4 was able to induce Cdc28-Clb2 activity even in S phase arrested cells when this complex is normally not active, indicating that E4orf4 can induce a premature mitotic state. Furthermore, E4orf4 is able to activate APC^{Cdc20} prematurely, resulting in the destruction of Pds1, likely by promoting Pds1 ubiquitination and degradation by the proteosome. The Pds1 instability induced by E4orf4 was dependent

on the function of APC^{Cdc20} as well as the formation of functional E4orf4-Cdc55 complexes. As the Clb2-Cdc28 has been shown to phosphorylate APC subunits and contribute to the activity of APC^{Cdc20} (Rudner and Murray, 2000), the premature activation of APC^{Cdc20} in E4orf4-expressing cells presumably resulted from the E4orf4-induced increase in Clb2-Cdc28 activity, an effect that is dependent on Cdc55-PP2A. Esp1 is known to be released upon Pds1 destruction and we observed that the Esp1 target, Scc1, was unstable in E4orf4-expressing cells and that this effect was dependent on a functional E4orf4-Cdc55 interaction. Hct1 is phosphorylated by Clb2-Cdc28 and can not interact with the core APC complex in its hyper-phosphorylated state (Zachariae et al., 1998; Huang et al., 2001; Yeong et al., 2001). Due to the high Cdk1 activity induced by E4orf4-Cdc55/PP2A complexes, Hct1 remained in a highly phosphorylated state preventing the formation of an APC^{Hct1} complex. The absence of APC^{Hct1} activity in the presence of E4orf4 was confirmed by the stability of its substrates, Cdc20, Cdc5 and Clb2.

Degradation of Scc1 causes dissolution of the cohesin complex resulting in sister chromatid separation. In cells expressing E4orf4 we observed a 4-fold increase in sister chromatid separation relative to control cells expressing the control plasmid. There are three possible explanations for why the sister chromatid separation was not more extensive, given the high degree of Scc1 degradation induced by E4orf4. First, there are two pools of Scc1, chromatin-bound and free soluble. Chromatin bound Scc1 phosphorylated by Cdc5 is the form of Scc1 that is preferentially cleaved by Esp1 (Hornig and Uhlmann, 2004). E4orf4 may indiscriminately promote the degradation of both of these pools of Scc1, resulting in a relatively low percentage of separated sister

chromatids. Second, because only one of the sixteen yeast chromosomes was marked with GFP in the yeast strain used, the fraction of separated sister chromatids scored may be an underestimate of the actual percentage. Finally, cells exhibiting sister chromatid separation may be preferentially lost due to cell death. We believe that the premature separation of sister chromatids and consequent mis-segregation of chromosomes or the retention of cells for long periods in mitotic arrest may be plausible explanations for E4orf4-induced cell death.

By what mechanism is E4orf4 able to promote a mitotic state in cells? Earlier studies have correlated the expression of adenovirus E4orf4 with the dephosphorylation of proteins involved in viral gene expression, such as the AP1 transcription factor subunit c-Fos, and E1A (Muller et al., 1992; Whalen et al., 1997), with phosphorylation changes in SR splicing factors (Kanopka et al., 1996; Kanopka et al., 1998) and with the transcription factor E4F, which regulates expression of the early region 4 of human adenoviruses (Bondesson et al., 1992). It cannot be entirely ruled out that the E4orf4-PP2A complex directly promotes these dephosphorylation events; however, the kinetics with which c-Fos and E1A become hypo-phosphorylated in the presence of E4orf4 suggest that the effect may be induced by an indirect mechanism, such as the inhibition of an appropriate protein kinase (Muller et al., 1992). E4orf4 may, alternatively, inhibit the dephosphorylation of selected substrates. The binding of E4orf4 to the B/Cdc55 subunit may promote the relocalization of the PP2A away from its usual substrates. Indeed, we observed that E4orf4 impairs normal Cdc55-directed PP2A localization to bud tips, but not Rts1-directed PP2A localization to the kinetochore. This loss of localization at bud

tips could result in the failure of PP2A to act on targets at this region of the cell, and possibly the redirecting of the enzyme to other substrates.

There is increasing evidence to suggest that the interaction of E4orf4 with B/Cdc55-containing PP2A holoenzymes results in the inhibition of activity of this pool of PP2A enzymes, at least against some substrates. The B55/Cdc55 regulatory subunit defines substrate specificity and is composed of seven WD40 repeats, believed to be responsible for mediating protein-protein interactions. The interaction of E4orf4 with Cdc55 could physically prevent certain substrates from binding to the PP2A holoenzyme and possibly favour the interaction of others. This possibility predicts that many of the usual substrates of B55/Cdc55-containing PP2A complexes will be hyperphosphorylated in response to E4orf4, and certain others might be hypophosphorylated. In agreement with this idea we have observed that E4orf4 expression can promote the hyper-phosphorylation of PP2A substrates both *in vivo* and *in vitro* (Brignole and Branton, unpublished results). Binding of E4orf4 appears to inhibit the ability of purified Bα–containing PP2A complexes to dephosphorylate phosphorylase *a* and histone H1 *in vitro* (Brignole and Branton, unpublished data) In addition, two substrates of PP2A, 4EBP-1 and p70-S6K were hyper-phosphorylated in E4orf4-expressing cells (Brignole and Branton, unpublished data). The phosphorylation of another PP2A substrate, vimentin, was not affected by E4orf4, indicating that E4orf4 does not modulate the dephosphorylation of all PP2A substrates (Brignole and Branton, unpublished data).

Okadaic acid (OA), a fairly specific PP2A inhibitor, especially at lower concentrations, can promote a mitotic state (Zheng et al., 1991; Sakurada et al., 1992 #1020; Lerga et al., 1999 #1021}. It has been observed that E4orf4-induces mitosis and

an increase in Cdk1 activity *in vitro* in mammalian cancer cell lines and this effect is enhanced upon treatment with OA (Brignole and Branton, unpublished results). Furthermore, OA has been shown to inhibit cyclin B ubiquitination and degradation in *Xenopus* egg extracts, suggesting that a PP2A-like activity is required for mitotic exit (Vorlaufer and Peters, 1998). As we have found that E4orf4 also induces a state of high Cdk1 activity and prevents mitotic exit in yeast, it is reasonable to propose that, when bound to Cdc55, E4orf4 may prevent certain substrates involved in mitosis from being dephosphorylated by PP2A. Furthermore, as E4orf4 only interacts with the Cdc55 subunit of PP2A, any effect would be specific to Cdc55-associated PP2A trimers, making E4orf4 a more specific inhibitor than okadaic acid.

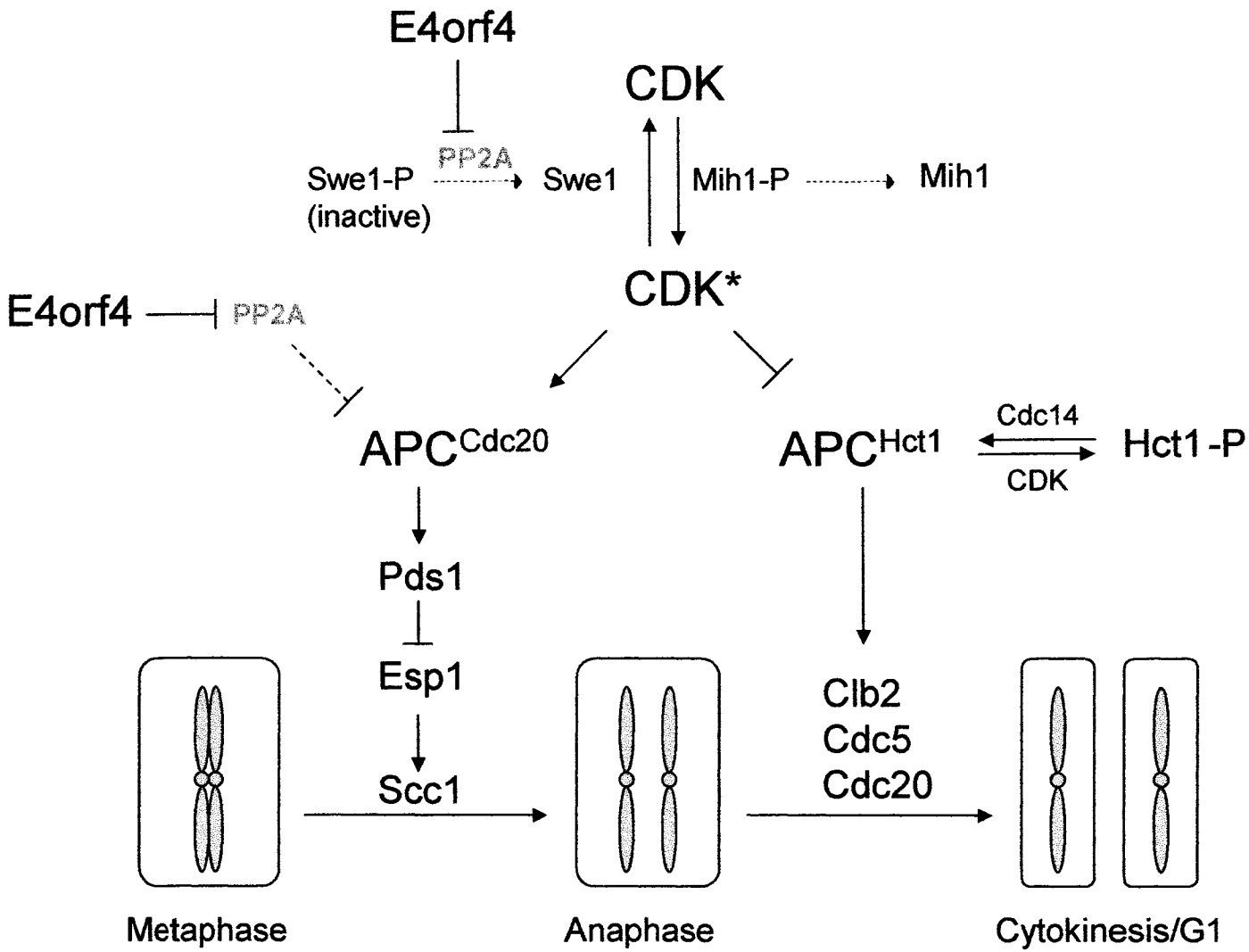
The identities of the PP2A substrates modulated by E4orf4 have yet to be fully elucidated. In yeast, Cdc28 activity is controlled by inhibitory phosphorylation on tyrosine 19 (Y19). The kinase (Swe1), but not the phosphatase (Mih1), that controls Cdc28-Y19 phosphorylation, is regulated either directly or indirectly by Cdc55-PP2A (Yang et al., 2000). Swe1 is inactivated by phosphorylation and is subsequently ubiquitinated and degraded by the proteosome {Sia et al., 1998; McMillan et al., 2002). In *cdc55* strains arrested in mitosis, either genetically or with nocodazole, Swe1 remains stable compared to wild type cells (Yang et al., 2000). In a *cdc55pph21pph22* strain arrested in mitosis the Swe1 turnover is identical to that seen in wild type cells, indicating that in the absence of PP2A activity Swe1 cannot be dephosphorylated and stabilized. These data suggest that the Cdc55 regulatory subunit may prevent PP2A from dephosphorylating Swe1 and it has been proposed that the loss of *CDC55* results in an unrestrained or misdirected PP2A activity, which promotes the dephosphorylation and

stabilization of Swe1 (Yang et al., 2000 #452). In immunoprecipitation-western blot experiments we have observed increased amounts of Tpd3 associated with Cdc55 in the presence of E4orf4 compared to the amount of Tpd3 complexed with Cdc55 in the absence of E4orf4 (M. Mui and P.E. Branton, unpublished results), suggesting that E4orf4 may promote the association of Cdc55 with the holoenzyme. The increase in Cdc55 containing trimers may restrain PP2A activity against Swe1, promoting its hyper-phosphorylation and degradation and resulting in active Clb2-Cdc28. Alternatively, E4orf4 may physically block PP2A from promoting the dephosphorylation of Swe1 and/or other substrates involved in allowing the activation of Cdc28. PP2A may be involved in dephosphorylating APC core subunits resulting in the inactivation of the APC complex (Lahav-Baratz et al., 1995). Given this possibility, E4orf4 could prevent PP2A from dephosphorylating APC subunits resulting in an active APC complex. (Lahav-Baratz et al., 1995). Figure 5-1 summarizes a model for the E4orf4-mediated activation of Cdk1 and APC^{Cdc20}.

Genetic studies in yeast have shown that depleting the cell of Pph21 and Pph22 arrests cells in G2, preventing entrance to mitosis. It would appear that PP2A activity is required for mitotic entry in *S. cerevisiae*. This scheme is different from the situation seen in Xenopus and fission yeast systems where PP2A activity negatively regulates mitotic entry, and cells depleted for PP2A enter mitosis prematurely. If E4orf4 inhibits PP2A activity against specific substrates why do yeast cells not arrest on G2? We have demonstrated that the E4orf4 specifically modulates the Cdc55-PP2A complex and not the entire pool of PP2A enzymes, which is the case with the above mentioned genetic study. Furthermore, E4orf4 may not inhibit the activity of Cdc55-PP2A against all

Figure 5-1 Model for activation of Cdk1 and APC^{Cdc20} by E4orf4

Cdk1 phosphorylation is controlled by Swe1 and Mih1. E4orf4 induces Cdk1 activity and Cdc55-PP2A is known to control the stability of Swe1. We hypothesize that the E4orf4-PP2A interaction may promote the hyper-phosphorylation and instability of Swe1 and this may be a contributing factor leading to the increase in Cdk1 activity. E4orf4-induced Cdk1 activates APC^{Cdc20} (yielding unstable Pds1 and Scc1) and inhibits APC^{Hct1} (resulting in stable Clb2, Cdc5, Cdc20 and hyper-phosphorylated Hct1). Cdc14 dephosphorylates Hct1 allowing it to interact with the APC complex and promote anaphase exit. Cdk1 phosphorylates Hct1, preventing it from associating with the core APC complex, thus preventing anaphase exit. In addition, the phosphorylation of APC core subunits is required for APC^{Cdc20} activity. If PP2A is involved in the dephoshorylation of APC subunits, inhibition of PP2A activity towards APC subunits may also contribute to APC^{Cdc20} activity.


E4orf4
CDK
PP2A
Swe1-P
(inactive)
Swe1
Mih1-P
Mih1
CDK*
E4orf4
PP2A
APC Cdc20
APC Hct1
Cdc14
CDK
Hct1-P
Pds1
Esp1
Scc1
Clb2
Cdc5
Cdc20
Metaphase
Anaphase
Cytokinesis/G1

substrates. This possibility allows use of E4orf4 to delineate specific functions mediated by the Cdc55-PP2A complex. Based on the present studies we propose a model that involves E4orf4 acting as a Cdc55-specific PP2A inhibitor of certain substrates whose hyper-phosphorylation could explain the observed defects in mitosis. The possibility that expression of E4orf4 may also lead to the hypo-phosphorylation of selected substrates to promote the mitotic arrest is not excluded.

In mammalian cells, there appears to be two separate pathways through which E4orf4 can mediate cell death, a cytoplasmic Src-dependent pathway involving Src-mediated phosphorylation of E4orf4 and a nuclear Src-independent pathway (Robert et al., 2002). The contribution of either pathway is possibly determined by the availability of Src-specific targets that are modulated by the E4orf4-Src complex as well as the activity of the signaling pathways involved in a given transformed cell type (Robert et al., 2002). The nuclear pathway of E4orf4-mediated cell death may be entirely dependent on PP2A. There is no yeast homolog of Src; however, it would be of interest to determine whether E4orf4 can be phosphorylated by some other kinase in yeast. It is more likely that the E4orf4-induced lethality in yeast is due to the nuclear pathway given that the mitotic events observed to be induced by E4orf4 occurs in the nucleus. It remains to be determined if the effects of E4orf4 on the APC substrates are similar in mammalian cancer cell lines and whether these effects can be seen with an E4orf4 mutant defective for mediating the cytoplamic pathway of cell death (E4orf4-Y3F).

From studies on the localization of PP2A subunits in yeast we know that Cdc55 can localize normally, although at reduced frequency in the absence of holoenzyme formation. These data suggest that the Cdc55 sequence contains encoded localization

information and that it may have functions outside the holoenzyme. It will be interesting the see the effects of E4orf4 in cells carrying a Cdc55 mutant that cannot interact with core enzyme, but still retain binding to E4orf4. Such a mutant would help define which effects of E4orf4 are dependent on the holoenzyme and which are dependent on Cdc55 alone. It would also be of interest to study the effects of E4orf4 in a strain carrying a Cdc55 mutant defective for E4orf4 binding. Such a Cdc55 mutant would be useful to study the Cdc55-independent effects of E4orf4. Such mutants are currently being generated.

Our screening of the collection of yeast deletion strains for E4orf4 did not yield any potential E4orf4 targets. If the primary target of the E4orf4-Cdc55/PP2A complex is an essential gene this approach would not have identified such a protein. It is also possible that Cdc55 is the only primary target of E4orf4. By modulating the activity of Cdc55/PP2A trimers, E4orf4 may affect several downstream target proteins, none of which when deleted alone would rescue E4orf4-mediated cell death. In that case a proteomics approach may be useful in determining which proteins are more or less abundant or hyper- or hypo-phosphorylated in the presence of E4orf4.

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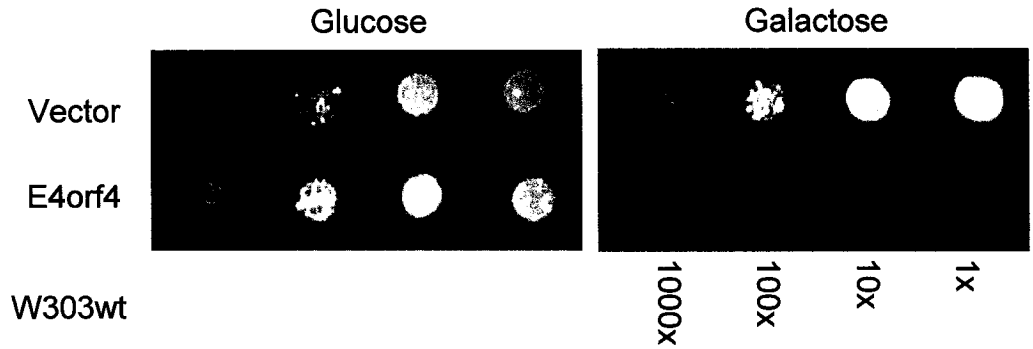
Appendix

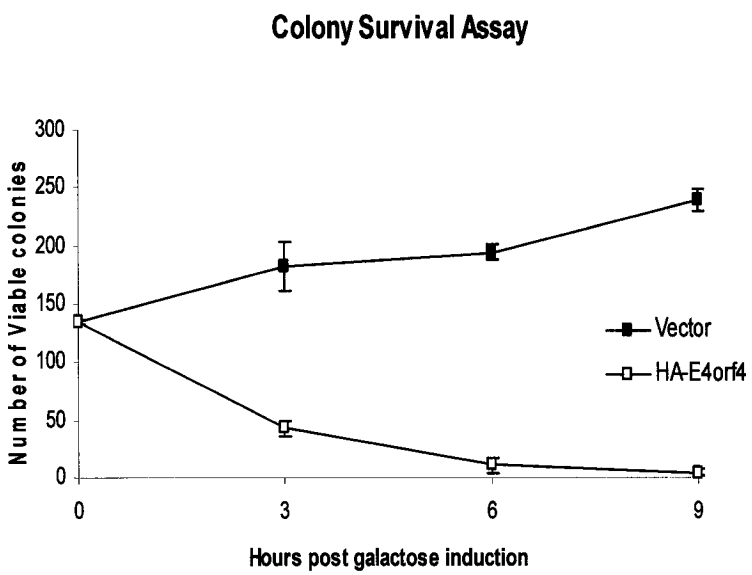
Figure A-1 Effect of E4orf4 in the W303 strain background

pYES2 or pYES-HA-E4orf4 were transformed into W303wt cells. **A**. Spotting assays and **B**. colony survival assays were performed as previously described (Roopchand et al., 2001). **C**. Cells were also collected at each time point for the colony survival assay. Whole cell extracts were prepared and subject to SDS-PAGE, followed by immunoblot with anti-HA antibody.

A


Glucose
Galactose
Vector
E4orf4
W303wt
1000x
100x
10x
1x

B


Colony Survival Assay
Number of Viable colonies
300
250
200
150
100
50
0
0
3
6
9
Hours post galactose induction
Vector
HA-E4orf4

C

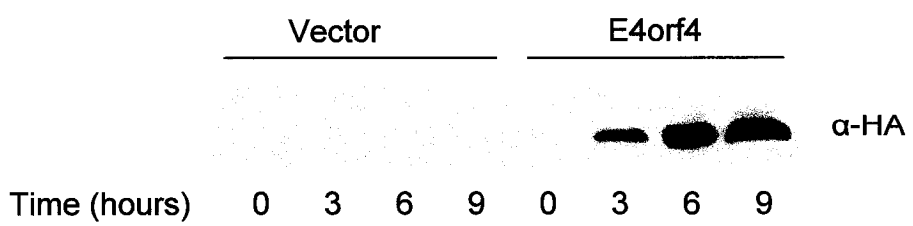
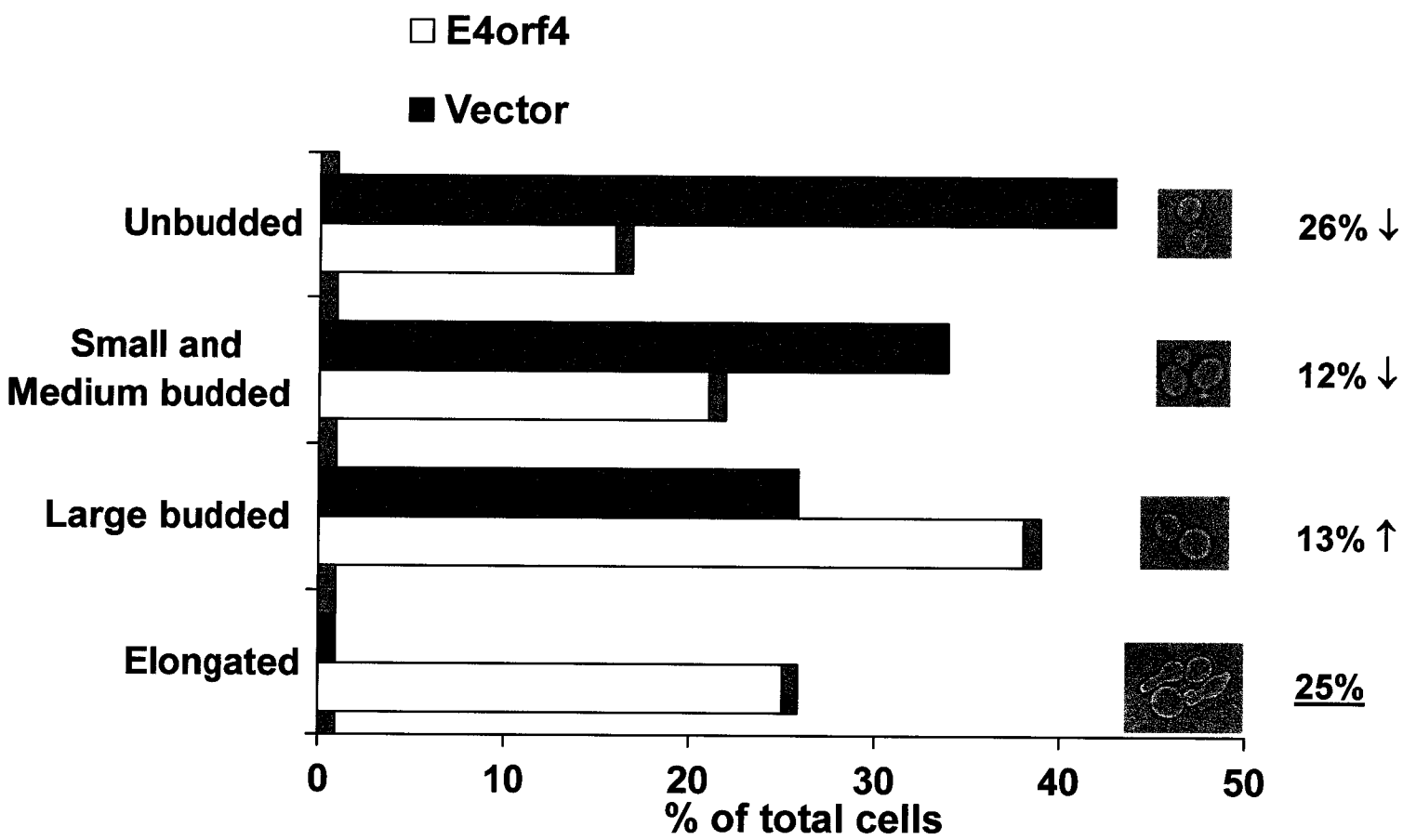

Vector
E4orf4
α-HA
Time (hours)
0 3 6 9 0 3 6 9

Figure A-2 Budding morphologies of yeast expressing E4orf4

W303 yeast cells (strain 6803) were transformed with control or FLAG-E4orf4 plasmid. Cells were grown under inducing conditions for 6 hours to allow E4orf4 expression. Cells were then collected and fixed for microscopy as described in the materials and methods. Photographs were taken and at least 300 cells were scored according to whether they were unbudded, had small to medium sized buds, were large budded or had an elongated bud morphology. The graph represents the percentage of the population having each of these morphologies for both control and E4orf4-expressing cells. The photographs on the right are representative of each morphology observed. The percentages on the right indicate whether the E4orf4-expressing cells showed and increase (↑) or decrease (↓) in a particular bud morphology.


E4orf4
Vector
Unbudded
Small and
Medium budded
Large budded
Elongated
26% ↓
12% ↓
13% ↑
25%
0
10
20
30
40
50
% of total cells

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Diana Roopchand

HAS SUCCESSFULLY COMPLETED A BASIC

LABORATORY COURSE IN

RADIATION SAFETY

April 2001

Radiation Safety Officer
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Manager, Environmental Safety
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