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List of Abbreviations

Cdc: cell division cycle

Cdk: cyclin dependent kinase

DAG: diacylglycerol

EMM: Edinburgh Minimal Medium

GAP: guanine nucleotide activating protein

GEF: guanine nucleotide exchange factor

GS: β(1,3)-glucan synthase

GW: greatwall kinase

HU: hydroxyurea

iMTOC: interphase microtubule-organizing centers

MAPK: mitogen-activated-protein-kinase

MPF: mitotic-promoting factor

Pck (fission yeast), Pkc (budding yeast) PKC (mammals): protein kinase C

PDK: 3-phosphoinositide-dependent protein kinase

PRK/PKN: kinase C-related kinase/protein kinase N

SPB: spindle pole body

S.pombe: *Schizosaccharomyces pombe*

SRP: stress response pathway

TOR: target of rapamycin signaling

YES: yeast extract with supplements

The abbreviations that only appear once are not listed here.

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I would like thank to my supervisor Professor Iain Hagan, his passion for science and leadership has set up a goal that I hope to match some day. I want to specially thank Dr Agnes Grallert for her guidance and support in the lab.

Abstract

In fission yeast, mitotic commitment (irreversibly entering M-phase) is controlled by mitosis-promoting factor (MPF). The activity of MPF is regulated by the mitotic inhibitor kinase Wee1 and the mitotic inducer phosphatase Cdc25. Cells have evolved mechanisms that can check cell size, DNA integrity and the environment in order to regulate the activity of Wee1 and/or Cdc25 to ensure cell divide at appropriate time and conditions. Protein kinases are the key signaling factors that regulate cell cycle progression. In this report, I have used a chemical-genetics approach to demonstrate that protein kinase C (Pck1/2) in *Schizosaccharomyces pombe* (*S.pombe*) is involved in the control of mitotic commitment. Cells showed delay in entering M-phase after ATP analogue inhibition of Pck2.as (analog sensitive); and after releasing cells from Pck2.as inhibition, a burst of mitosis was observed at 25°C. At 36°C this burst of mitosis is delayed by 80 mins. Epstatic gene interaction tests showed that Pck2 regulates mitotic entry through Wee1, but not via Cdc25, nor the Pom1 regulated cell tip extension pathway or via the Plo1 regulated cell stress pathway. The Pmk1 regulated cell integrity pathway might not be directly involved in Pck2 regulated mitotic entry, but it did influence the timing of Pck2 regulated mitotic entry. I also observed that some of *pck2::kanMX6* cells have defects in interphase microtubule dynamics. Many *pck2::kanMX6* cells also have defects in polarized cell growth, suggesting Pck2 is involved new end take off (NETO).

Introduction

Cell reproduction arises from performing a sequence of ordered events in which the cell duplicates its contents and then divides into two daughter cells; this process is known as the cell cycle.

The most basic task of the cell cycle is to accurately duplicate the DNA in the chromosome and then equally segregate the copies into two genetically identical daughter cells. Thus, every cell cycle must have the two major phases, S-phase for chromosome duplication and M-phase for chromosome segregation. Most cells also require time to grow and double their contents, so most cell cycles also have gap phases G1 and G2 (figure1). The gap phase also provides time for the cell to monitor the internal and external conditions to ensure precise and accurate reproduction only occurs in the right context. Cells can also enter a specialized resting state known as G0. There are two commitment points, one near the end of G1 known as "START". After passing this point, the cell is committed to entry into S-phase and there is no way for reversing back to G1, another commitment point is near the end of G2 for mitotic entry.

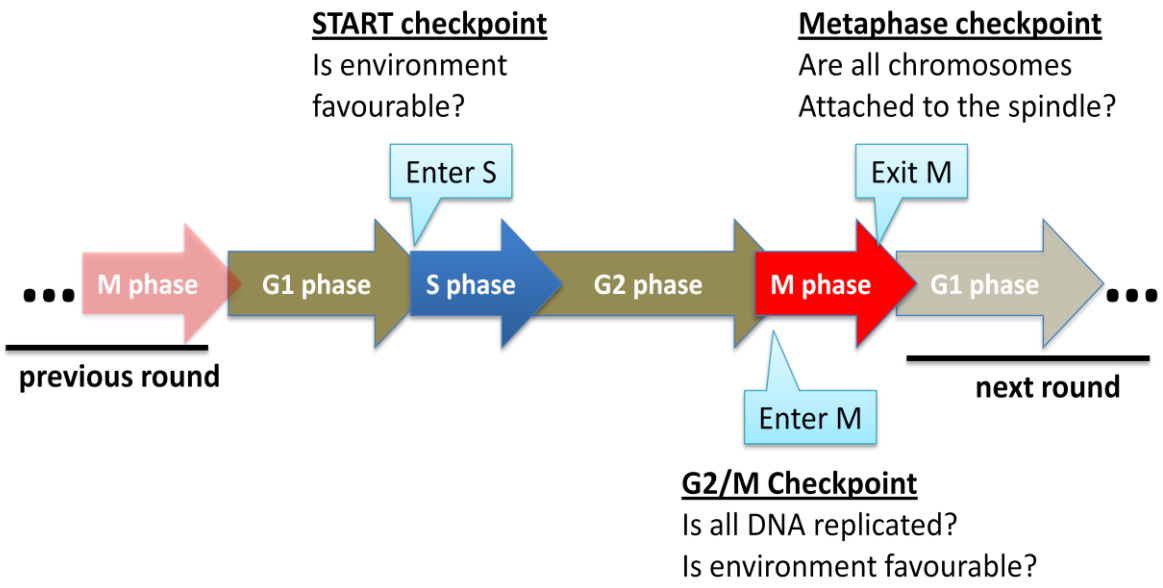


Figure 1 Cell cycle progression Each cycle generally consists of four phases to duplicate the genome and equally segregate the copies into two daughter cells. It also has three checkpoints to ensure that the cell divides faithfully.

Eukaryotic cells have evolved a complex network of regulatory proteins, known as the cell cycle control system, that control progression through the cell cycle. The cell cycle control system operates like an oscillator that triggers the progression through cell cycle events at three major regulatory transitions

called checkpoints. *Start* at the end of G1 before cells enter S-phase is for checking whether the conditions are appropriate for cell proliferation. *G2/M checkpoint* at the end of G2 is the decision of whether a cell should enter mitosis or not. The third one is the metaphase-to-anaphase transition in which the cell checks whether the sister-chromatids are properly attached to the mitotic spindle before triggering chromatid separation. These checkpoints provide molecular braking mechanisms that block cell cycle progression if a problem is encountered.

The central components of this system are a family of protein kinase called cyclin-dependent kinases or Cdks. Each complex phosphorylates various, but specific groups, of target proteins that initiate or regulate the major events of cell cycle. Cdks are activated by association with regulatory proteins called cyclins. Cdk activities rise and fall with the protein level of cyclin as the cell cycle progress. There are four classes of cyclins, G1-, G1/S-, S-, M-cyclin, they are defined by the stage of cell cycle in which they activate Cdks, and each generates a Cdk-cyclin complex that has a specific kinase activity.

The Cdk-cyclin complexes are considered to be the master regulators of the cell cycle; Cdk-cyclin complexes themselves are subjected to various regulatory mechanisms to fine tune their activity. One of the most important regulations is phosphorylation/ dephosphorylation of Cdk-cyclin complexes by a group of serine/ threonine and tyrosine protein kinases and phosphatases. Those enzymes provide a link that coordinates different cellular processes such as cell growth, stress sensing and DNA integrity with cell cycle progression.

This introduction will focus on cell division with an emphasis on protein kinase controls of mitotic commitment in the fission yeast *S. pombe*.

Controls for Mitotic Entry

1.1 Introduction

M-phase consists of mitosis for nuclear division and cytokinesis for cytoplasmic division. According to early observations of gross changes in the structure and behavior of chromosomes and spindles by light microscopy, mitosis is further divided into five stages prophase, prometaphase, metaphase, anaphase, and telophase. Mitosis is followed by cytokinesis to complete the cell division, a process of cleavage by which the cytoplasm is divided in to two by an actin and myosin based contractile ring to create two daughter cells, each of which contains one nucleus. Although, the staging system has been questioned recently as mitosis in many different organisms appear not to follow five steps exactly for example the nuclear envelope never breaks down in many organisms such as *S.pombe* (Pines and Rieder 2001), The underlying mitotic control system seems to be universal in all eukaryotic cells.

The entry of mitosis is marked by activation of a ubiquitous protein kinase complex known as MPF, which contains a catalytic subunit Cdc2 (Cdk1 in fission yeast) and a regulatory subunit called cyclin B. In fission yeast, MPF is constitutively nuclear(Pines and Rieder 2001); its activation is control by a number of protein kinases and phosphatases. The balance of activity between those enzymes determines the timing of mitosis.

1.2 Mitotic commitment is controlled by mitosis-promoting factor (MPF)

Genetic and biochemical approaches were used throughout the 70s and early 80s to identify the key factors that control the timing of mitotic commitment. The rationale behind these experiments was simple: deleting /overexpressing candidate genes or enhancing candidate proteins advances mitosis in cells proceeding through the cell cycle, or enables mitotic entry in cells naturally arrested in the cell cycle. The network of mitotic control was established mainly from studies of two different types of yeast mutants: cell cycle division (*cdc*) mutants were initially isolated in a genetic screen in budding yeast (Hartwell, Culotti et al. 1970), Some cdc mutants are temperature sensitive, unable to complete cell division at the restrictive temperature. These cells either become blocked at one of the cell cycle events such as DNA synthesis and cell division (Nurse, Thuriaux et al. 1976), or divide at an increased cell size at the restrictive temperature (Nurse and Thuriaux 1980). In contrast, *wee* mutations advance cells into

mitosis, and cells divide at a reduced cell size. *cdc2*$^{+}$, *cdc25*$^{+}$ and *wee1*$^{+}$ products were then identified as three main factors that determine the overall timing of mitotic commitment (Nurse 1990). Deletion of the dosage-dependent mitotic inhibitor *wee1*$^{+}$ or overexpression of the dosage-dependent inducer *cdc25*$^{+}$, advances cells into mitosis. The *cdc2* mutants that have a *wee* phenotype, *cdc2.w* mutations were dominant and allele specific for genetic interaction with *cdc25* and *wee1* (Fantes 1981). For example, *cdc2.1w* was first isolated as *wee2*$^{+}$ mutant (Nurse and Thuriaux, 1980), and it is largely insensitive to Wee1. In contrast, *cdc2.3w* can bypass the requirement of *cdc25*$^{+}$ to induce mitosis. Furthermore, dosage of *cdc2*$^{+}$ has virtually no impact on the timing of mitotic commitment (Nurse and Thuriaux 1980). Thus, Cdc2 activity is rate limiting for mitotic commitment, and is inhibited by Wee1, after which this inhibition can be relieved by Cdc25.

1.2.1 Cdc13 and Cdc2 are the key components of MPF

Unlike any other model organisms that are used in cell-division studies, fission yeast is able to use a single cyclin-cdk complex for mitotic control. Although three cyclin genes *cig1*$^{+}$, *cig*2^{+} and *cdc13*$^{+}$ have been discovered in this organism to date, Cdc13 alone is sufficient to enable Cdc2 kinase activity to control both transit through START and mitotic entry (Fisher and Nurse 1995). Deletion of *cdc13*$^{+}$ blocks mitotic commitment, and the temperature sensitive mutant *cdc13-117* has partial function that allows activation of Cdc2 but only after certain mitotic events take place (Moreno, Hayles et al. 1989). Phosphorylation of thr167 of Cdc2 by protein kinase CAK is required for the formation of Cdc2-Cdc13 complex (Draetta 1993) (figure 2).

In *S. pombe*, Cdc2 is the only Cdk required for both S-phase and mitosis. The functional specificity of S- and M- cyclins are considerably overlapped in yeast. Analysis of the *cdc13-117* mutants suggests that the G1 cyclin activity of Cig2 can be effectively substituted by Cdc13 (Fisher and Nurse 1996). Therefore, a single B type cyclin, Cdc13, drives the periodic formation of cdc2-cyclin complex to give oscillation of Cdc2 kinase activity that can promote the ordered progression into both DNA replication and mitosis (Fisher and Nurse 1996).

1.2.2 MPF is universal

Microinjection experiments in sea-urchin and frog eggs also demonstrated that Cdc2 and cyclinB are key components of MPF, which form a protein complex required for entry of mitotic and meiotic M-phase (Lohka, Hayes, and Maller 1988). Yeast genetics was used to clone the human homologue of *cdc2*. Human *cdc2* is 63% identical in primary amino acid sequence to the yeast gene (Lee and Nurse 1987);

and the human *cdc2* gene can substitute for the yeast *cdc2*$^{+}$ gene. Furthermore, human cyclin homologues were cloned, which coimmunoprecipitates to the human Cdc2, and are also destroyed at the end of mitosis (Pines and Hunter 1989), suggesting that this mitotic control system is both functionally and structurally conserved from yeast to human, and that this control mechanism might be universal in all eukaryotes.

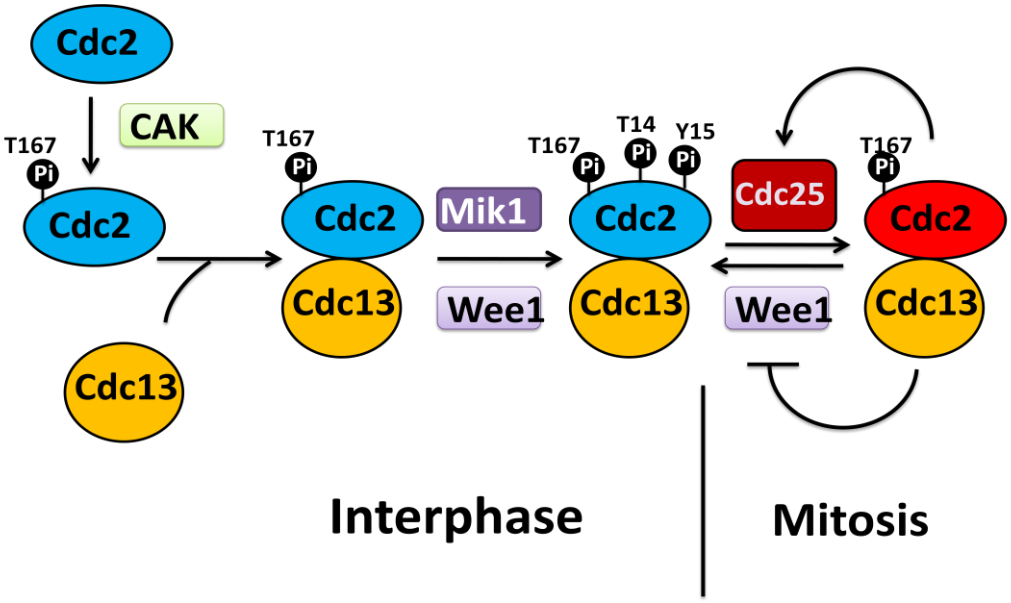


Figure 2 Regulation of mitosis-promoting factor (MPF) the drives mitotic entry Phosphorylation of Cdc2 at T167 CAK is required for formation of MPF complex. Phosphorylation at T14 and Y15 by Wee1 and Mik1 inhibit MPF activity, Cdc25 removes Phosphorylation at T14 and Y15 and activates MPF activity.

1.3 MPF activity is regulated by Wee1 and Cdc25

The molecular basis of the "all-or-nothing" mechanism of mitotic commitment is not well understood. One possible model is that in late G2 phase the level of active Wee1 and active Cdc25 is in a balance that forms a futile cycle over Cdc2, and keeps this master regulator in a restrained state. Once the conditions for cell division have been satisfied, e.g. the cell growth reaches an appropriate cell length; the overall activity of Cdc25 overcomes that of Wee1. Activated Cdc2 then reaches an efficient level to activate a positive feedback loop that further boosts activity of Cdc2 to the critical level that is required for mitotic commitment. It is thought that during the cell cycle, the periodically fluctuating levels of cyclin are the primary determinant of Cdk activity and specificity; several additional mechanisms such as protein phosphorylation fine-tune Cdk activity.

More recently developed biochemical, molecular and structural biology approaches not only furthered our understanding of those known controls, but also identified more upstream molecules that regulate mitotic commitment through MPF. Immunoprecipitation showed that Cdc2 is multiply phosphorylated on serine, threonine and tyrosine residues in exponentially growing fission yeast. The level of Cdc2 phosphorylation reaches a maximum level in late G2, and as cells enter mitosis, it decreases (Gould and Nurse 1989). The protein kinase activity of Cdc2 toward histone H1 however, shows an inverse pattern: it increases from a low level in the late G2 to increase to its maximum level in mitosis (Moreno, Hayles et al. 1989). This suggests that phosphorylations of Cdc2 in late G2 and M phase have a negative impact on kinase activity of Cdc2. One of the phosphorylation sites is located at residue tyrosine 15 (Y15), within a part of the postulated nucleotide binding domain of Cdc2. Substitution of Y15 by non-phosphorylatable phenylalanine (*cdc2.F15*) advances cells prematurely into mitosis (Gould and Nurse 1989). Therefore, the kinase activity of Cdc2 is required for the G2/M-phase transition, and phosphorylation of Y15 directly regulates the kinase activity of Cdc2. Thus, there must be at least one protein kinase and phosphatase that regulates mitotic commitment on upstream of Cdc2.

Wee1 contains a protein kinase domain and its activity is an element in mitotic control (Russell and Nurse 1987), Although in the 80s the researchers could not identify any protein phosphatase motif on Cdc25, experiments showed Cdc25 is a dosage-dependent mitotic inducer that functions in opposition to Wee1 (Russell and Nurse 1987), and Cdc25 was later shown to be the tyrosine phosphatase (Gautier and Kirschner 1991). Furthermore, inactivation of Cdc25 results in a late-G2 arrest in which Cdc2 becomes maximally phosphorylated, and the *cdc2.F15* mutant can rescue *cdc25-22* mutant at restrictive temperature by bypassing the requirement of $cdc25^{+}$ gene function for mitotic entry (Gould and Nurse 1989). Thus, Wee1 and Cdc25 regulate the kinase activity of MPF via protein phosphorylation and dephosphorylation respectively on specific residue(s) of Cdc2, and Y15 might be one of the key regulatory sites.

In *S.pombe*, Wee1 was first thought to be the only kinase that directly phosphorylates Y15 of Cdc2 (Gould and Nurse 1989). However, this was soon questioned after the observation of tyrosine phosphorylation of Cdc2 in *wee1* mutants (Gould, Moreno et al. 1990; Lundgren, Walworth et al. 1991). Mik1 was then identified as a protein kinase that functions redundantly with Wee1 in the negative control of Cdc2 (Lundgren, Walworth et al. 1991). $mik1^{+}$ was able to complement mutation in the *wee1*, and *wee mik1* double mutation is far more severe than either mutant alone. However, $mik1^{+}$ deletion has no *wee* phenotype and cannot rescue *cdc25* mutants, suggesting it is regulated differently

(Christensen, Bentley et al. 2000). The role of *wee1*$^{+}$ in the inhibitory phosphorylation of Y15 was then re-evaluated: allelic specific mutant *cdc2-r4* is competent to be a substrate of tyrosine kinase, and it is able to rescue *mik1*$^{+}$ *wee1*$^{+}$ double mutant without being phosphorylated at tyrosine residue. Furthermore, Cdc25 is not essential in the *mik1*$^{+}$ *wee1*$^{+}$ double mutant (Lundgren, Walworth et al. 1991). Thus, *wee1*$^{+}$ and *mik1*$^{+}$ both encode *cdc2*$^{+}$ tyrosine kinase activity. Wee1 is involved in linking cell size sensing with mitotic commitment, whereas, Mik1 is involved in linking DNA damage and DNA replication checkpoint with mitotic commitment (details are described in later sessions). Therefore, Wee1 and Mik1 as well as Cdc25 can be considered as transducers that receive signals from different cellular process and pass their counteracting activities onto MPF contro; in the form of phosphorylation/dephosphorylation; and the balance between them determines the timing of mitosis.

In *Xenopus* eggs, MPF activation promotes a positive feedback loop that increased the activity of Cdc25 and inhibits Wee1 by protein phosphorylation to achieve rapid, complete and irreversible onset of mitosis (Hoffmann, Clarke et al. 1993). Consistently, the prior activation of Cdc2 is required for the activation of Cdc25 bulk activity in fission yeast (Kovelman and Russell 1996).

1.4 Positive feedback loop

The phosphorylation and activation of Cdc25 by the Cdc2 mediated positive feedback loop can be recognized by a phospho-specific antibody called mitotic protein monoclonal 2 (MPM2). The persistence re-activity of Cdc25 to MPM2 antibodies after removal of MPF activity from *Xenopus* egg extracts led to a searching for other protein kinases that function upstream of Cdc25 in the positive feedback loop (Izumi and Maller 1995).

1.4.1 SPB recruitment of Polo kinase regulate mitotic commitment

Xenopus Polo kinase directly binds and activates Cdc25, and this activation can be recognized by MPM2 (Kumagai and Dunphy 1996; Abrieu, Brassac et al. 1998). Moreover, M-phase is blocked after microinjection of anti-Plk1 (Polo kinase homologue in human) antibodies in non-transformed human cells (Lane and Nigg 1996). Therefore, Polo kinase is an integral part of the positive feedback loop. Polo kinase plays several different roles in a number of distinct cell cycle events ranging from the formation of the mitotic spindle to cytokinesis (Glover, Hagan et al. 1998). The detailed molecular basis of Polo kinase in mitotic control is not fully understood, but genetic and biochemical data in fission yeast

showed that the spindle pole body (SPB) association of Plo1 (Polo kinase in *S.pombe*) controls mitotic commitment. Plo1 might activate Cdc25 at the pole. Alternatively, Plo1 might inactivate Wee1, because Wee1 is heavily phosphorylated during the feedback activation (Tang, Coleman et al. 1993), and Polo kinase contains polo box that help it to bind to target protein already phosphorylated (Glover, Hagan et al. 1998). The affinity of Plo1 for the SPB is regulated through at least three factors.

1). Plo1 associate with SPB through Cut12

The functions of Plo1 in mitotic commitment were initially identified through studies of another gene *cut12*$^+$. *cut12*$^+$ was initially identified and characterized as the *stf1*$^+$ gene; *stf1*$^+$ mutations suppress the temperature sensitive growth defect of *cdc25.22* cells, and *stf1.1* (*cut12.s11*) mutation is able to support ten cell divisions of cells with no *cdc25*$^+$ (Hudson, Feilotter et al. 1990). *cut12*$^+$ encodes an essential component of SPB, and it is required for bipolar spindle formation in *S.pombe*, loss function mutation of *cut12*$^+$ leads to one SPB work while one failed during mitosis (Bridge, Morphew et al. 1998), suggesting that *cut12*$^+$ plays a role in mitotic control, possibly through a SPB associated mechanism.

Co-immunoprecipitation and yeast two hybrid experiments showed that Plo1 associates with Cut12 (MacIver, Tanaka et al. 2003). The molecular mechanism by which Cut12 mediated Plo1 polar localization though Cut12 regulate mitotic commitment is not clear. However, the exclusive study of two point mutations on *cut12*$^+$ has helped to clarify the function of Cut12 in SPB recruitment of Plo1 and mitotic control. In wild type cells, Plo1 associates with mitotic and late G2 SPB. This SPB association of Plo1 is the earliest marker for mitotic entry recorded to date, and the kinase activity of Plo1 is important in mitotic entry (Mulvihill, Petersen et al. 1999). In *cut12.1* cells (a nonsense mutation), loss of Cut12 function blocks the activation of Plo1 kinase activity that normally accompanies mitotic entry (MacIver, Tanaka et al. 2003). Genetic analysis showed that in a *cut12.1* background, the *cdc25.22* mutation kills the cells at the permissive temperature with an exacerbated *cut12.1* phenotype: all microtubule emanating from just one of the two SPBs to form a monopolar rather than a bipolar spindle (Bridge, Morphew et al. 1998). Conversely, increased levels of Cdc25 suppressed the *cut12.1* defect (Tallada, Bridge et al. 2007). In contrast, in *cut12.s11* cells (a missense mutation), gain of Cut12 function leads to association of Plo1 with the SPB at all steps of the cell cycle. This inappropriate polar recruitment of Plo1 promotes the activity of Plo1 on the SPB and overrides the requirement for Cdc2 activation by Cdc25 in *cdc25.22* background (Mulvihill, Petersen et al. 1999). Therefore, Cut12 might control the timing of SPB recruitment and kinase activity of Plo1, which then, in turn, regulates MPF via Cdc25 to determine the timing of mitotic commitment. Recent electron microscopy serial section analyses showed that during

mitosis, both the activation and insertion of the new SPB into nuclear envelope were blocked in arrested *cut12.1* cells at 36°C (Tallada, Tanaka et al. 2009). The authors suggested that because MPF is recruited to the SPBs of late G2 cells, Cut12 activates MPF at both SPBs, so that the locally activated MPF is able to promote SPB integration into the nuclear membrane. The SPB then becomes activated into a mitotic state that might help to amplify the MPF activation feedback loop, thereby promote global commitment to mitosis.

2). Fin1 regulates mitotic entry through Plo1

The *Aspergillus nidulans* protein kinase NIMA (never in mitosis A) is essential for mitotic entry (Osmani, McGuire et al. 1991). The NIMA related kinase Fin1 in *S.pombe* is not essential for viability, but might still be involved in mitotic controls. Fin1 plays a role in spindle formation with a Cut12 related function (Grallert and Hagan 2002). *fin1* mutations block spindle formation in the manner that is

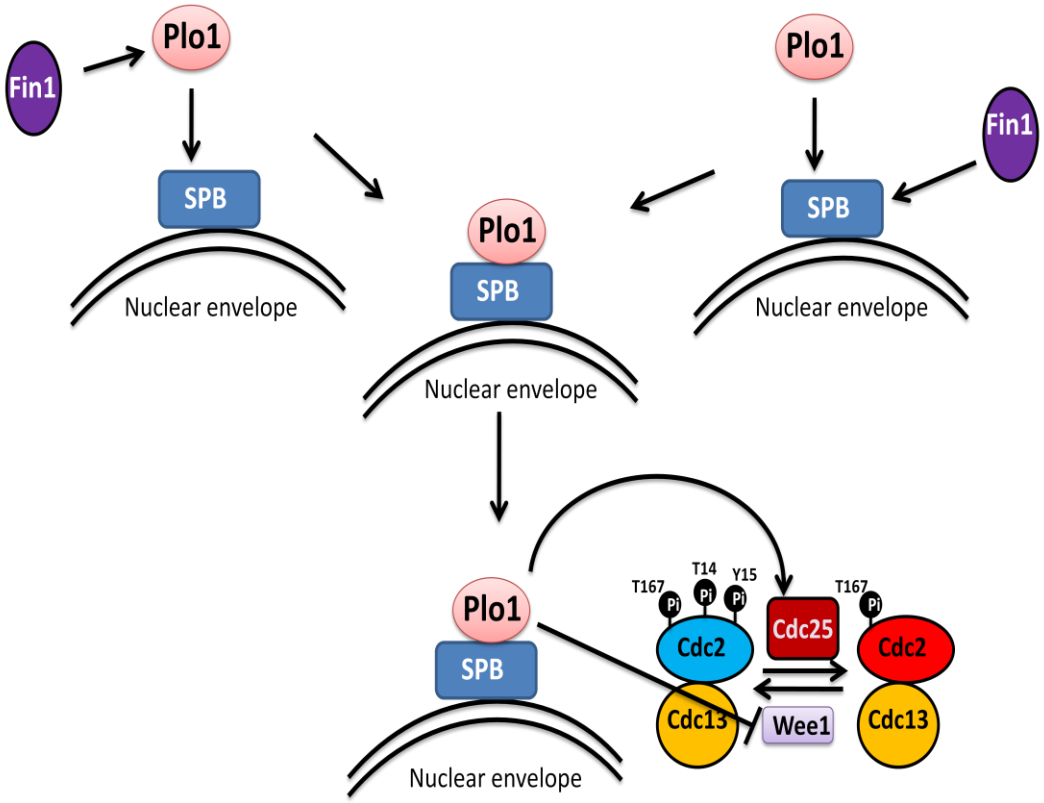


Figure 3 Fin1 regulates mitotic entry through Plo1. Fin1 regulates the positive feedback of MPF through promoting SPB recruitment of Plo1.

indistinguishable from that arising from the *cut12.1* mutation. *cut12.s11* reduced the severity of *fin1.Δ* spindle formation defect. Whereas, *cut12.1 fin1.Δ* double mutation is lethal.

In a manner that is reminiscent of the impact of the *cut12.s11* mutant, *fin1* overexpression suppresses *cdc25.22* and results in the premature recruitment of Plo1 to the interphase SPB. Fin1 regulates the SPB association of Plo1 through an unknown mechanism, Plo1 or SPB may be the downstream targets of Fin1, and Fin1 may phosphorylate either Plo1 or SPB to promote affinity of Plo1 for SPB as a fine tuning control (figure 3) (Grallert and Hagan 2002).

3). Sty1 regulates mitotic entry through Plo1

In late G2, the phosphorylation of Plo1 at serine 402 is increased and it reaches a maximal level during mitosis. Substitution of S402 by alanine (*plo1.S402A*) to block the phosphorylation abolished the inappropriate polar recruitment of Plo1 in *cut12.s11*, and the *cdc25.22* lethality is no longer suppressed in *cut12.s11* background (Petersen and Hagan 2005). This phosphorylation at S402 is regulated through Sty1 stress-response pathway and TOR signaling pathway (Petersen and Nurse 2007) (Petersen and Hagan 2005), suggesting that S402 might serve as a regulatory input into Plo1 that receives the signal from the cell stress response pathway in order to control the affinity of Plo1 for the SPB, thereby coordinating nutrient and stress sensing with mitotic control (detail described in later session)

1.4.2 Greatwall kinase regulates the tyrosine phosphorylation of Cdc2 in the positive feedback loop

Greatwall (GW) kinase is a serine/threonine protein kinase with an unusual and poorly understood split kinase domain. It was first identified in a screen for *Drosophila* mutants defective in chromosome condensation (Yu, Fleming et al. 2004). The fraction of mutant cells in late G2/M-phase transition was increased, indicating a delay in mitotic entry. Those mutants also have a high mitotic index (Yu, Fleming et al. 2004). Although GW mutation causes improper chromosome condensation, cells had normal mitotic levels of chromatin associated condensing regulators and topoisomerase II. Furthermore there was no block to chromosome replication or activation of caffeine-sensitive the G2 checkpoint that restrains mitosis for DNA damage or incomplete replication, suggesting that GW kinase may be involved in chromosome condensation. The main function of GW kinase is to activate cell cycle regulators for entry into mitosis.

Studies in *Xenopus* egg extracts showed that GW depleted cycling Xenopus extracts do not enter M-phase. The cyclin level was unaffected, but the inhibitory tyrosine phosphorylation accumulated on Cdc2. A Cdc2 mutant lacking the tyrosine phosphorylation site was able to bypass the requirement of GW kinase. Furthermore, GW kinase is an *in vitro* substrate of MPF (Yu, Zhao et al. 2006). Therefore, the

function of GW kinase in mitotic commitment is thought to regulate the tyrosine phosphorylation of Cdc2 in the positive feedback loop. However, this hypothesis cannot explain several recent observations, for example, the cell cycle effects of GW depletion and GW addition are substantially independent of other MPF regulator in the feedback loop suck as Polo kinase, and Wee1 (Castilho, Williams et al. 2009), GW kinase must have another role during mitosis.

GW depleted CSF Xenopus extracts caused spontaneous mitotic exit, suggesting GW is also required for M-phase maintenance (Yu, Zhao et al. 2006). It was thought that GW maintains the mitotic state by regulating the MPF activity (Yu, Zhao et al. 2006). However, recent data from immunoprecipitation analysis and phosphatase activity assay of mitotic Xenopus extracts showed that GW kinase promotes inactivation of protein phosphatase 2A (PP2A) (Castilho, Williams et al. 2009). Once mitosis is committed, the activated MPF phosphorylates hundreds of proteins. These phosphorylations are thought to keep the cell in a mitotic state, and they are maintained until mitotic exit, when anti-mitoitc protein phosphatases such as PP2A dephosphorylates the substrates of MPF (Dephoure, Zhou et al. 2008). The anti-mitoitc protein phosphatase must be shut off after the cell enters the next round of mitosis. A model is proposed suggesting that MPF phopshorylates and activates GW kinase after mitotic commitment to negatively regulate the countering antimitotic phosphatases such as PP2A; GW kinase also simultaneously influences the positive feedback loop by perhaps, preventing dephosphorylation of Cdc25. Both mechanisms are required to ensure robust and irreversible mitotic entry (Castilho, Williams et al. 2009).

1.5 Wee1 is inhibited by mitotic inducer Cdr1 and Cdr2

cdr1$^+$ and *cdr2*$^+$ (changed division response) were initially isolated in mutants that were unable to change G2/M size control in response to nitrogen deprivation (Young and Fantes 1987). They were thought to be nutritional sensors that monitored nutrient levels in the environment to regulate G2/M progression. In another genetic screen, *cdr1*$^+$ was also isolated as a multicopy *cdc25-22* suppressor, and named as *nim1* (new inducer of mitosis) (Russell and Nurse 1987). It was then demonstrated that *cdr1*$^+$ does not have a role in nutritional sensing, instead, the defect in responding to nitrogen deprivation by *cdr1*$^+$ mutation was a result of a G2 delay (Belenguer, Pelloquin et al. 1997); and *cdr2*$^+$was cloned from a screen for mutations that become synthetic lethal in a *cdc25-22* background at permissive temperature (Kanoh and Russell 1998). The primary amino acid sequences analysis revealed that Cdr1 and Cdr2 are

two closely related members of SNF1 serine/threonine protein kinase family (Kanoh and Russell 1998). Mutations in either gene results in very similar phenotypes: cell-cycle arrest and the activity of Cdc25 is decreased (Wu and Russell 1993), and the mutations can be suppressed by inactivation of Wee1. Furthermore, Cdr1 and Cdr2 have been shown to directly bind to and phosphorylate Wee1 *in vitro* (Breeding, Hudson et al. 1998). Therefore Cdr1 and Cdr2 are thought to be mitotic inducers that act through Wee1 to regulate MPF.

Several recent findings further our understanding of the roles of Cdr2 in coordinating cell size and mitotic entry in *S. pombe*. During interphase, Cdr2 localizes in a cortical band overlaying the nucleus; and it becomes cytoplasmic at the onset of mitosis (Morrell, Nichols et al. 2004). The localization of Wee1, Cdr1 and another mitotic inducer Blt1 to the medial cortical nodes depended on Cdr2. Cdr2 functions upstream of Cdr1 and Blt1, and together they inhibit Wee1 activity (Almonacid, Moseley et al. 2009). Unlike the GIN4 family kinase (relatives of Cdr1 and Cdr2 in budding yeast), the functions and localizations of Cdr2 are independent of the cytokinesis protein septins (Morrell, Nichols et al. 2004). Instead, a tip protein Pom1 is thought to restrict localization and activity of Cdr2 at the medial cortex through an unknown mechanism Pom1 is the key regulatory factor that coordinates cell polarity and growth with mitotic entry through the Cdr2-Wee1 pathway (details are described in 2.1) (Almonacid, Moseley et al. 2009). Cdr2 also plays role in cytokinesis. In *S. pombe,* the anillin-like protein Mid1 recruits actomyosin-ring components to the medial cortex for medial assembly of the division septum. Cdr2 physically recruits Mid1 exclusively to medial cortex during interphase. At mitotic onset, medial cortical localization of Mid1 is dependent on its nuclear export that is triggered by activated Plo1. Thereby, Cdr2 provides cell polarity information and Mid1 nuclear export provides nuclear positional information; the two overlapping mechanism ensure specification the division plane in the middle of cell and around the nucleus (Almonacid, Moseley et al. 2009). Hence, Cdr2 kinase activity and its localization coordinate mitotic commitment (that takes place in nuclei) with the cytokinesis (that takes places around nuclei).

1.6 mitotic control system functions as binary switch

Simple organisms like fission yeast have evolved such complicated regulatory system to ensure that mitotic commitment is an all-or-none decision. One of the advantages to have Cdc25-Wee1 counteract one another in a regulatory system is that it can from a futile cycle over Cdc2, so the activation of Cdc2 is

rapid and robust. Quantitative input (dosage–dependency of Cdc25 and Wee1) allows the net positive and negative signals to be compared more precisely, so that the decision making can be more flexible; cells can divide even when some conditions are not perfect for division. This positive feedback loop provides a mechanism that gives bistability to the mitotic control system, thereby MPF functions as a binary switch that is able to exist in one of two alternative status (active/inactive), but cannot come to rest in an intermediate state between them (Ferrell 1996).

Protein Kinase and Mitotic Commitment

The MPF complex is the master regulator for M-phase entry. As a checkpoint to ensure faithful cell division in appropriate conditions at the appropriate time, it must be able to sense the signals arising from other cellular processes such as DNA replication, cell growth and environmental stress sensing. One of the great advantages of using fission yeast is that, under appropriate conditions, cell length at division reflects the length of G2 phase. Therefore, any factor that delays mitotic commitment can be detected by measuring cell length at division (Mitchison 2003).

2.1 Cell growth polarity and cell morphology

Cell division has been extensively studied in *S. pombe* since M.J. Mitchison discovered this microbe to be an ideal model organism to look at the biology of cell cycle in the 1950s. This fission yeast has rod-liked cylindrical shape with constant diameter. Cell grows by elongating at the cell ends, this polarized cell growth is coordinated with cell cycle progression, so that cells have very similar shape and size at the end of each cell cycle.

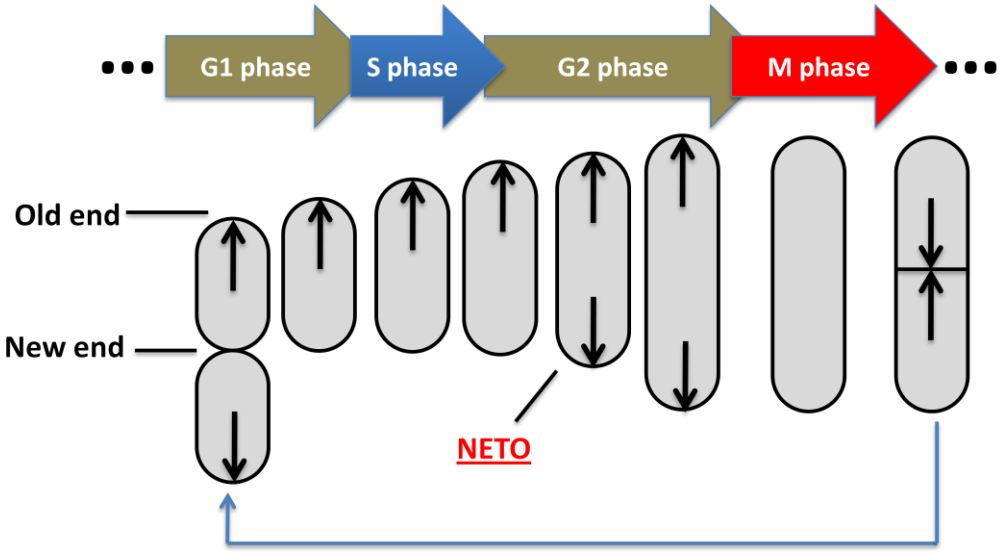


Figure 4 New End Take Off is coordinated with cell cycle progression After cell division, both daughter cells only grow from the old end, which corresponds to the end that existed before cytokinesis, In G2 phase, after cells reach a critical length NETO take place and cell grow in a bipolar manner until M-phase.

After cell division, the polarized elongation of both growing daughter cells is initiated exclusively at the old ends that existed in the mother cell in the previous around of cell cycle; at this stage cell growth is monopolar. In early G2, the new ends that were generated in the preceding cell division also start to elongate; and cell growth becomes bipolar. This is known as new end take off (NETO) (Mitchison and Nurse 1985) (figure 4). In a wild type cell, NETO requires 1) completion of S-phase 2) cell growth must reach a critical length of 9.0-9.5 μm. 3) cells must be at least the first 30-35% of the way through the cell cycle. For small size mutants such as *wee1.Δ*, NETO takes place late in cell cycle (about 80% of the way through) after the cells reach the critical length. The timing of NETO also varies with temperature and nutrient level (Mitchison and Nurse 1985). After NETO, cell size increases more significantly, as growth is now at the both ends. Once cells enter M-phase, they stop growing, so the duration of G2 phase and the timing of mitotic entry determine cell length at division. Wild type cells have a constant cell length at division of about 14.0μm at 25°C in minimal medium. After nuclear division, the polarity machinery is directed to the cell division site. After septation, the polarity machinery is redirected to the old ends to initiate monopolar growth (figure 4) (Martin and Chang 2005). It was not well understood how the cell cycle controls NETO, until recent studies on cell morphology have started to reveal the mechanism of cell cycle regulated polarity growth. Several genetic screens have identified numerous mutants that have defects in cell morphogenesis and/or cell growth polarity. They have been classified into three types 1) protein kinase such as Pom1 (Bahler and Nurse 2001). 2) Microtubule related proteins such as Tea1 (Mata and Nurse 1997) 3) actin binding proteins such as For3 (Nakano, Imai et al. 2002). Those studies have answered a series of questions to reveal how cell growth, cell polarity and cell morphogenesis are coordinated in *S.pombe*.

2.1.1 How is division site determined in the middle of the cell?

As described in session 1.5, the position of nuclei determines the localization of Mid1 hence the site of cytokinesis. The nucleus is always positioned in the middle of the cylinder, so that fission yeast always undergoes binary division and produce daughter cells with the same cylindrical shape. It is still unclear how this nucleus positioning is achieved. One simple explanation for nuclear positioning is that the nucleus is pushed by the forces from microtubule in opposed direction as cytoplasmic microtubules extend toward both cell ends (Tran, Marsh et al. 2001). Florescence microscopy imaging however, shown the interphase nuclei in septation mutants is inconsistent with the pushing mechanism to position nuclei. By monitoring the movement of SPB and the genome during mitosis, the authors suggested that bi-directional microtubule motor proteins provide force for SPB and nucleus movement;

and interphase nuclear positioning via the motor proteins is achieved by the response of the SPB to cell cycle specific signals that remain to be identified (Hagan and Yanagida 1997)

2.1.2 How is cell growth polarity established at the cell tip?

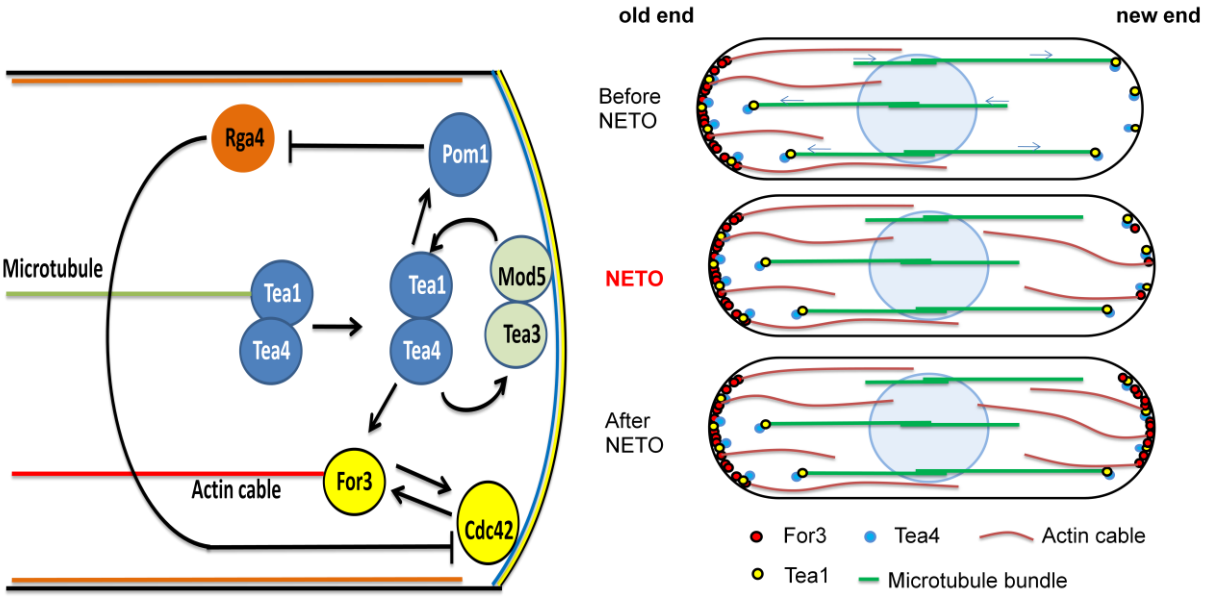


Figure 5 Tip proteins and cytoskeleton are the key regulators of cell growth polarity (**Right**) A model of how polarized growth is established by the tea/microtubule system at the cell tip. (**Left**) a model of NETO, the actin distribution (red lines) is regulated by Tea1 (blue) Tea4 (blue)/microtubule (green lines) via For2 (red), which then determine the growth site.

Microtubules play critical roles in assembly of growth machinery and maintaining cell shape after each division. An interphase *S.pombe* cell has a small number of discrete bundles of microtubules in its cytoplasm (Hagan and Hyams 1988). They are very dynamic and extend along the long axis of the cell (Drummond and Cross 2000). The bundles of microtubules seem to grow from the interphase microtubule-organizing centers (iMTOC), which are tethered to the nuclear membrane. The spindle pole body (SPB) might also contain an iMTOC, and motor proteins rapidly pull away newly formed microtubule from SPB, so asters never form (Hagan 1998). During polarized cell growth, microtubules contribute spatial information to cell morphogenesis by transporting polarity factors to the cell tips. Actin on the other hand provides trackways, on which cargos with material for growth are delivered to the cell tips by actin based motor proteins (myosins). Cytoplasmic microtubules contribute spatial information to cell tip growth by transporting polarity factors such as Tea1 and Tea4 to cell tip (Martin

2009). The *tea1*$^{+}$ mutant was isolated in a screen for mutants that have defective interphase microtubule cytoskeletons but no significant delay in mitosis (Mata and Nurse 1997) (Feierbach, Verde et al. 2004). *tea1*$^{+}$ mutants are curved or tripolar cells. Tea1 forms a complex with Tea4 (figure5 Left) (Martin, McDonald et al. 2005). Tea1-Tea4 complex is then associated with a microtubule +TIP complex. This +TIP complex consists of the EB1 homologue Mal3 that binds growing microtubule plus end and also the microtubule lattice seam (Sandblad, Busch et al. 2006), the CLIP-170 homologue Tip1 that is required for Tea1-Tea4 recruitment (Brunner and Nurse 2000), and the plus end motor Tea2; each of the components promotes growth of microtubule (Bieling, Laan et al. 2007). Therefore, +TIP complex transports Tea1-Tea4 complex to the growing plus ends of microtubules that extend toward the cell tips. Tea1 is tethered at the cell tips by an unknown microtubule-cortex hand-off mechanism, which is thought to be mediated by the Tea1-related protein Tea3 and the prenylated protein Mod5 at cell tips (Snaith and Sawin 2003). By having these membrane associated partners at cell tips; Tea1 is evenly distributed beneath the tip even though Tea1 is only delivered to one part of a tip. Thus, this system allows even extension all parts of the tip.

Actin is also critical for cell growth; it is organized in three types of structure: actin patches, actin cables and actomyosin rings. Actomyosin rings provide cleavage mechanism during cytokinesis, whereas actin patches and actin cables are focused around the areas of cell growth (Marks and Hyams 1985). Actin cables function as trackways along which actin patches (Pelham and Chang 2001) and cargos that contain material for cell growth are delivered to the cell tips. Just after cell division, the cell growth machinery, including Tea1, Tea4 and For3 (a member of the actin nucleating formin family), is only localized at the old end. Thus only the old end has the actin cables for delivering the growth material (figure 5 Right). In G2 phase, the signals for NETO remain unknown, but Tea1-Tea4 complex might form the center of a regulatory network that controls the onset of NETO. Tea1 and Tea2 are delivered and start to accumulate at the new end by the same mechanism described above. The Tea1-Tea4 complex is thought to regulate polarized growth via formation of a large scaffold that modifies membrane domain and recruits the actin assemble machinery at the new end: Both the Tea1-Tea4 complex and Pom1 contribute to the localization and activation of Cdc42 (Tatebe, Nakano et al. 2008). Cdc42 is a small Rho-family GTPase that is essential for most events of cell polarization (Etienne-Manneville 2004). Pom1 inhibits the tip localization of Rga4, the GTPase-activating protein (GAP) for Cdc42. Thus, Cdc42 is only activated at cell tip, where it activates For3's ability to drive actin polymerization at the cell ends only, so growth only takes place at the cell ends (Martin, McDonald et al. 2005) (figure 5). Actin patches are dense membrane-associated structures that are important in cell wall synthesis. In regenerating

protoplasts, the active site of the cell wall is overlapped precisely with actin patches (Kobori, Yamada et al. 1989). Sla2 localises to actin patches (Kaksonen, Sun et al. 2003). In mammalian cells, Sla2 homologue (HipR) connects clathrin coat of endosomes to the actin cytoskeleton (McPherson 2002). In *S.pombe*, deletion of the C-terminal talin-like domain of Sla2 causes a specific NETO defect (Castagnetti, Behrens et al. 2005), Sla2 is thought to organize actin patches and link NETO to actin patches recruitment. Actin transiently disappears from both cell tips upon the onset of mitosis, and polarized cell growth stops. At the post-mitotic stage, cortical actin reappears in the central region, septum formation and cytokinesis then takes place (Marks and Hyams 1985).

In conclusion, the Tea1-Tea4 / microtubule system forms a positive feedback between cell shape and polarised cell growth. The rod-liked cell shape guides the longitudinal organization of microtubules, which in turn determines the tip localization of the Tea1-Tea4 complex, which promotes actin polymerisation at the cell tips only to guide polarised cell growth, which defines cell shape, and this cycle of regulation goes on. Thereby, the Tea1/microtubule system coordinates the sites of cell growth with cell morphogenesis.

2.1.3 How does cell sense and control its size

As described above the *wee* mutants and some *cdc* mutants can divide at cell lengths that are uniformly shorter or longer than wild type cells. *S. pombe* must have a G2/M cell size control mechanism that can sense cell length and initiate mitosis once the cell reaches a certain length. For many years it was unclear how fission yeast coordinates polarized cell growth with cell division. Several recent reports have shown a tip protein Pom1 is the key factor that regulates this coordination. It does so through the Cdr2-Wee1 pathway.

Pom1 plays important roles in driving polarized cell growth at NETO (Bahler and Pringle 1998). Pom1 was the first identified protein shown to play a critical role to distinguish the old end of a newborn cell from its new end (Bahler and Pringle 1998): A high concentration of Pom1 is found at the new end after cell division. *pom1.Δ* mutants continue to grow at a randomly chosen end until division, 5% of *pom1.Δ* mutant cells show an aberration in the orientation of the growth axis relative to the ends and sides of the cells. Similar and more frequent phenotypes have been observed in mutants lacking the cell tip protein Tea1 (Mata and Nurse 1997), suggesting that Tea1/ microtubule system is the major factor that defines the ends of cells. Pom1 may act as the mediator that transmits positional information from the microtubule-Tea1 system to the actin cytoskeleton (Bahler and Pringle 1998). Tea1 may act upstream of

Pom1 in the same pathway, and Tea1 is important for the localization of Pom1 to the cell ends but not for its activation (Martin and Berthelot-Grosjean 2009). Like *tea1.Δ*, *pom1.Δ* mutant cell has long and curved microtubules; *pom1*$^{+}$ overexpressing cells have short and aberrant microtubules. It has been proposed that Pom1 regulates cell polarity through its effect on microtubules (Mata and Nurse 1997), (Bahler and Nurse 2001).

Pom1 is a member of the dual –specificity tyrosine-phosphorylation regulated kinase (DYRK). The kinase activity of Pom1 is regulated during the cell cycle in correlation with the state of cell symmetry: it has high activity in cells undergoing symmetrical growth and division; and lower activity in newborn cells that grow asymmetrically (Bahler and Nurse 2001). After cell division, Pom1 is highly concentrated at the new end and lower at the old end. Thus, it might provide a negative signal for assembly of the tip growth machinery at the new end. The concentration of Pom1 at the new end remains high long after NETO, suggesting that the kinase activity of Pom1 might be inhibited at the appropriate stage in G2 to allow bipolar growth. The kinase activity of Pom1 is essential for cell symmetry during both division and growth (Bahler and Nurse 2001). The mechanism of Pom1- regulated growth at the cell ends remains unclear. *pom1.Δ* mutant cells are significantly shorter than wild type cells, and the temperature-sensitive *cdc25-22* mutant can be suppressed by *pom1*$^{+}$ deletion. Furthermore, cell size at division increases with increased levels of *pom1*$^{+}$ expression in both *cdc25-22* mutants and wild type cells. All these data indicate that Pom1 is a dose-independent inhibitor of mitotic entry (Martin and Berthelot-Grosjean 2009; Moseley, Mayeux et al. 2009). It was also demonstrated that inhibitory phosphorylation of Cdr2 is dependent on *pom1*$^{+}$ *in vivo*. Pom1 forms a large complex with other tip proteins at cell ends, and it might be anchored to a membrane protein such as Pck2 (Bahler and Nurse 2001). Quantitative fluorescent microscopy image of Pom1-GFP reveals a concentration gradient emanating from cell tips (Padte, Martin et al. 2006). The concentration of Pom1 in the middle of the cell decreases as the cell grows. In 2009, two research letters (Martin and Berthelot-Grosjean 2009; Moseley, Mayeux et al. 2009) proposed the same model for the role of Pom1 in G2-M transition: In small cells, the Pom1 polar gradient overlaps with the interphase nodes, and Cdr2 in medial cortical nodes is phosphorylated and inactivated by Pom1. Thus Wee1 can phosphorylate Cdc2 and block mitotic entry. After cell growth has exceeded the critical length, the concentration of Pom1 in the middle of the cell dips below a threshold level such that Cdr2 is no longer inhibited, and Cdr2 is free to inhibit Wee1 resulting in mitosis (figure 6). Thereby, Pom1 at the cell tip is the cell size sensor that coordinates cell growth with cell division. Pom1 also inhibits Mid1 localization to cell ends, and so helps to restrict the division plane to the midzone

(Padte, Martin et al. 2006). Pom1 is the first identified regulatory factor that has been shown to link cell morphogenesis and tip growth with cell division.

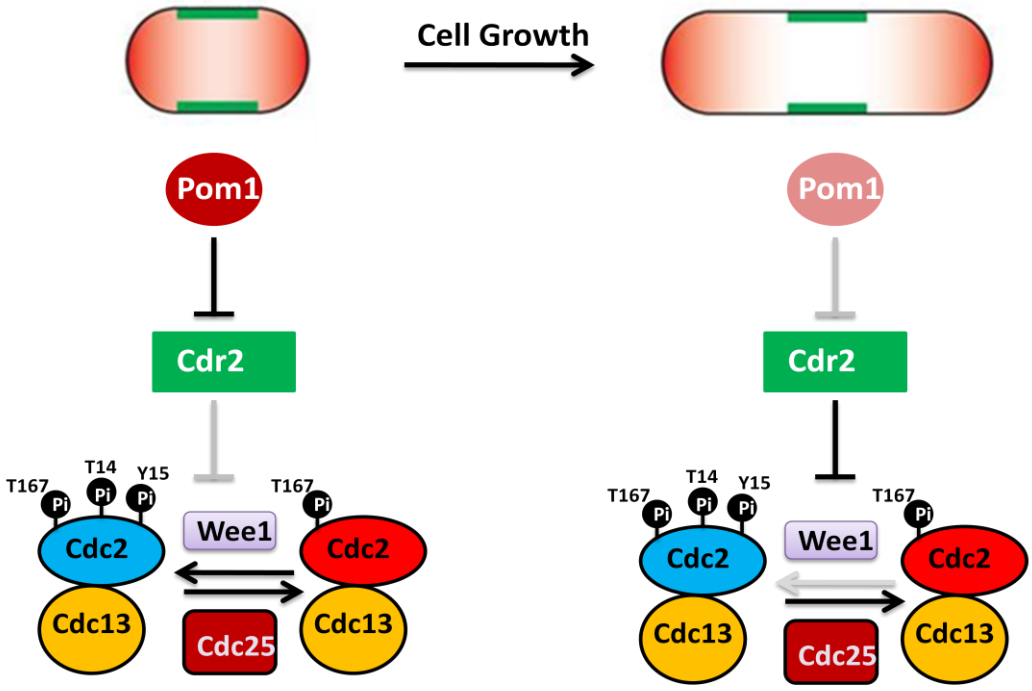


Figure 6 Pom1 is a cell size sensor. In early G2 phase, cells are short and tip protein Pom1 inhibits mitotic entry through the Cdr2-Wee1-Cdc2 pathway, this inhibition is released as the cell is growing longer and Pom1 is moving away from the medial cortex where Cdr2 is localized.

Although the molecular mechanism of polarized cell growth is slowly becoming clear, one of the major unanswered questions is how this process is controlled through the cell cycle. It has been proposed that in G2 phase, unknown molecules that monitor the completion of DNA replication and cell size initiate a NETO signal that triggers the assembly of polarized cell growth machinery such as Tea1 and Tea4 to the new end. The NETO signal also facilitates the formation of the complex through protein phosphorylation events that increase the affinity of these polarity proteins for one another. After NETO, cell length increases significantly, which takes away the mitotic inhibitor Pom1 that is anchored on the cell tips from the MPF, then cell enters M-phase via MPF activation.

2.2 Environmental control

In order to survive, all organisms have to be able to respond to changes in the environment such as stress insults and nutrient availability. *S.pombe* is able to sense the environmental factors and transmit the signal to control the rate of cell growth and division, so that cells can maintain a constant size in a particular environment. Mitogen-activated-protein-kinase (MAPK) pathways are protein phosphorylation mediated signal transduction mechanisms that serve to amplify a small signal initiated at the cell surface and to convert a graded input into highly sensitive responses (Ferrell 1996). MAPK pathways regulate many cellular processes such as cell cycle control, cell proliferation and cell differentiation. MAPK cascades are ancient and conserved from yeast to mammals. The multiple MAPK pathways found in eukaryotes such as budding yeast and fission yeast, control gene expression and posttranscriptional regulation of the target proteins in response to various extracellular stimuli. Three distinct MAPK cascades have been identified and studied so far in *S.pombe*. These include the mating pheromone responsive Spk1 MAPK pathway, the stress-activated Sty1/Spc1 MAPK pathway and the cell integrity Pmk1 MAPK pathway whose function is less clear.

2.2.1 MAPK and Plo1 in stress response

The stress-activated Sty1/Spc1 MAPK pathway senses different external stimuli such as osmotic and oxidative stress, and activate transcriptional factors such as Atf1 (Wilkinson, Samuels et al. 1996). Atf1 activation by SRP then activates transcription of core environmental stress response genes to cope with the stresses. The mutation that results in constitutive activation of the MAPK kinase Wis1 (*wis1.DD*) advances cells into mitosis at reduced cell size (Shiozaki, Shiozaki et al. 1998). Whereas the mutation in the MAPK kinase kinase (MAPKKK) gene *win1*$^{+}$ (*win1.1*) or deletion of the MAPK gene *sty1*$^{+}$ (also known as *spc1*$^{+}$) delay entry of mitosis. Furthermore, hyperactivation of Sty1 in *wis1.DD* background suppresses *cdc25.22* (Shiozaki, Shiozaki et al. 1998), suggesting that Sty1 is a mitotic inducer. As described above, Phosphorylation of S402 of Plo1 regulates the recruitment of Plo1 to SPB, which then regulates mitotic commitment. S402 was found to be phosphorylated in a heat shock experiment (Petersen and Hagan 2005), suggesting that phosphorylation of S402 is controlled through MAPK SRP. Deletion of *sty1*$^{+}$ blocks phosphorylation of S402 and significantly delays mitotic entry, while *plo1.S402A* abolishes the ability of hyperactivated Sty1 to suppress *cdc25.22* in *wis1.DD* background. Furthermore, the *plo1.S402E* mutation, which mimics phosphorylation of S402, reduces cell length at division of *sty1*$^{+}$ deleted cells, whereas, *plo1.S402A* mutation has no effect on the cell length (Shiozaki and Russell 1995; Petersen and

Hagan 2005). Thus the MAPK SRP regulates mitotic commitment by modulating SPB recruitment of Plo1 through Sty1 (figure 7).

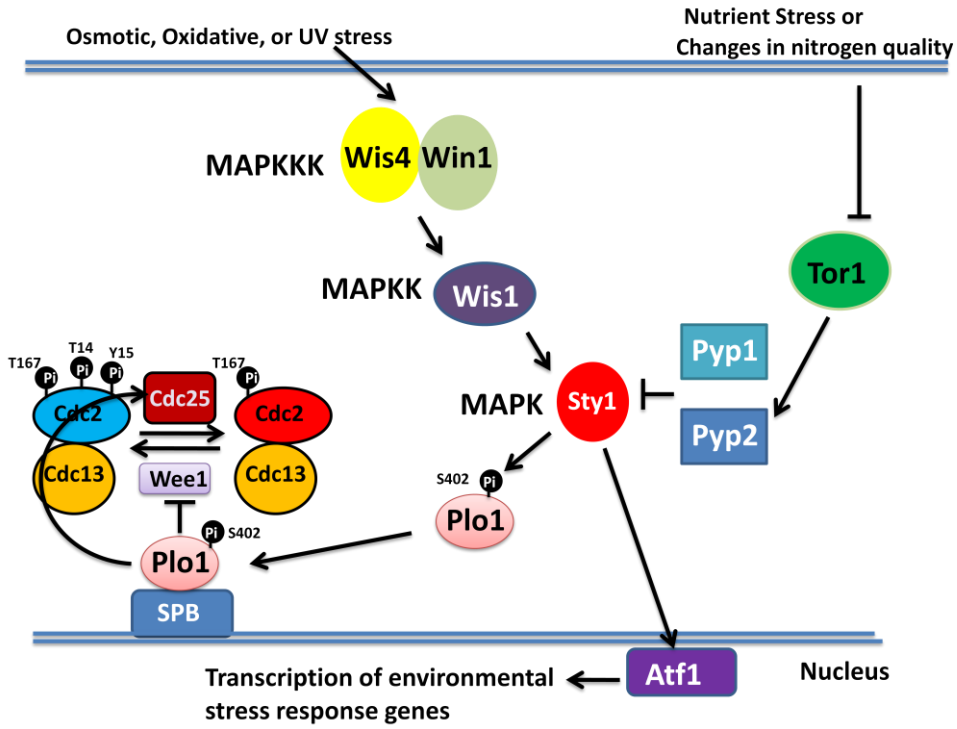


Figure 7 Sty1/Spc1 MAPK pathway regulates mitotic commitment through SPB recruitment of Plo1. environmental stress signal from MAPK pathway and nutrient sensing signal from Tor1 pathway are passed to Sty1, which in term regulate SPB recruitment of Plo1 via phosphorylation of S402 on Plo1

2.2.2 Tor1-Sty1andPlo1 in nutrient sensing

The size of *S.pombe* at division is modulated by changes in nutrient availability; cells were able to adjust to a new environment with a higher rate of cell division after a shift from a rich to poor nitrogen source (nutrient stress) (Fantes and Nurse 1977; Petersen and Nurse 2007). This environmental regulation of growth control goes through TOR (target of rapamycin signaling) (Petersen and Nurse 2007).

Unlike mammalian cells that only contain one TOR kinase, fission yeast contains two, Tor1 and Tor2, and only Tor1 has essential role in nutrient stress pathway (Alvarez and Moreno 2006). Tor1 kinase activity was reduced following a nutrient stress. This reduction of TOR signaling is responsible for the increased cell division rate. Furthermore, rapamycin- and nutrient stress-induced inhibition of TOR signaling had the same impact (Weisman R. 2004) on mitotic commitment; both reduced cell size at division by the same degree. In contrast, a shift from poor to rich nutrient source delayed mitotic entry, in a TOR

dependent manner. Both rapamycin- and nutrient- induced inhibition of TOR signaling lead to activation of Sty1, and the TOR –dependent MAPK activation is mediated through inhibition of SRP protein phoshatase Pyp2, as Pyp2 dephosphorylates Sty1 (Shiozaki and Russell 1995; Petersen and Nurse 2007). Activation of Sty1 leads to SPB recruitment of Plo1 to control mitotic commitment (figure 7).

2.2.3 Pmk1 MAPK pathway.

The Pmk1/Spm1 MAPK pathway was first studied independently by two research groups as a structural homolog of the budding yeast Mpk1 MAPK (Toda, Dhut et al. 1996; ZaitsevskayaCarter and Cooper 1997; Loewith, Hubberstey et al. 2000). Mkh1 and Pek1 were identified as the MAPKKK and MAPKK of the Pmk1 MAPK pathway (Sengar, Markley et al. 1997). Yeast two hybrid experiments showed that Mkh1, Pek1 and Pmk1 form a ternary complex (Loewith, Hubberstey et al. 2000). In the absence of stimuli, unphosphorylated Pek1 in its inactivated state binds Pmk1 as a potent inhibitor. Stimuli triggers phosphorylation and activation of Pek1 which then promotes its activity toward Pmk1 at conserved threonine (T186) and tyrosine residues (Y188) to activate it (Sugiura, Toda et al. 1999). These phosphorylations were not observed under different stress stimuli in $mkh1.\Delta$ or $pek1.\Delta$ cells, suggesting that Pmk1 is activated only through Mkh1-Pek1. Genetic data showed that *wis1*$^{+}$, the MAPKK of the Sty1/Spc1 MAPK pathway does not interact with *pmk1*$^{+}$ (Toda, Dhut et al. 1996; ZaitsevskayaCarter and Cooper 1997; Loewith, Hubberstey et al. 2000). However, deletion of *sty1*$^{+}$ or its downstream effector *atf1*$^{+}$ elicited an increased activation of Pmk1 by Sorbitol or KCl, and this activation was maintained for longer than in wild type cells. This indicates that Pmk1 deactivation under osmostress is dependent upon Sty1-Atf1 function (Madrid, Soto et al. 2006), in a cross-talk between two MAPK pathways in fission yeast. The protein phosphatase Pmp1 is able to dephosphorylate Pmk1 in *vitro* and in *vivo*, and physically associate with Pmk1 in *vivo* (Toda, Dhut et al. 1996), strongly suggesting that it acts as a negative regulator of Pmk1 MAKP pathway.

In budding yeast, Mpk1 MAPK pathway is activated by a variety of conditions such as heat shock, hypertonic shock, oxidative stress and cell wall damage (Martin, Rodriguez-Pachon et al. 2000). In animal cells, extracellular signal regulated kinase 1 and 2 (ERK1/2) have MAPK domains similar to that of Pmk1, and they are strongly activated in response to phorbol esters and growth factors, or by cytokines and stress in less extent (Roux and Blenis 2004). In *S.pombe*, Pmk1 is activated in multiple cell stress conditions. The degree and kinetics of this phosphorylation depends on the type of triggering stimulus (Madrid, Soto et al. 2006): the phosphorylation level of Pmk1 increasing rapidly after hypertonic shock (sorbitol), salt-induced osmostress (NaCl or KCl), addition of $CaCl_2$, or after oxidative stress induced by

hydrogen peroxide and other pro-oxidants. In contrast, depletion of glucose triggered a delayed Pmk1 activation; a delayed Pmk1 activation was also evident after heat shock or the addition sodium vanadate, caffeine or Calcofluor, all of which induce changes in the biosynthesis and architecture of the yeast cell wall (Perez and Ribas 2004). *pmk1.Δ* cells are hypersensitive to KCl and hypertolerant to NaCl. They have reduced growth in the presence of sodium vanadate, Calcofluor, pro-oxidants or $CaCl_2$, or at the high temperature, whereas they showed no growth defects in the presence of caffeine, sorbitol or hydrogen peroxide, suggesting Pmk1 is important in maintaining cell viability against most of the stressors that activated MAPK (Madrid, Soto et al. 2006). Pmk1 MAPK and calcineurin might play antagonistic roles in ion homeostasis in *S.pombe*. Mutations in the *S.pombe* calcineurin gene *ppb1*$^+$ lead to hypersensitivity to Cl^-, which is suppressed by *pmk1*$^+$ deletion (Toda, Dhut et al. 1996). There is some evidence for two distinct calcium-dependent pathways for calcineurin activation in fission yeast, one being via Yam8/Cch1 mediated Ca^{2+} influx, under the control of Pck2-Pmk1 MAPK pathway but not Pck1 (Deng, Sugiura et al. 2006).

The Pmk1 MAPK pathway also regulates cell morphology and integrity. *pmk1.Δ* cells are hypersensitive to cell wall digestion enzyme β-glucanase treatment, suggesting that Pmk1 is involved in cell wall integrity (Toda, Dhut et al. 1996). Under nutrient, temperature or hypertonic stress conditions, the majority of *pmk1.Δ* cells grow as short branched filaments, those cells are joined end to end like short trains; unseptated cells are often shorter and more rounded than wild type cells. Electron microscopy showed that these branched cells remained attached across the entire division plate, and that their cell walls and septa were dramatically thickened, suggesting defects in polarised cell growth and cell wall remodelling during cell division (Toda, Dhut et al. 1996; ZaitsevskayaCarter and Cooper 1997; Loewith, Hubberstey et al. 2000).

2.3 DNA damage and DNA replication checkpoint

The G2/M checkpoint ensures that cells only enter mitosis after their DNA has been completely replicated and repaired (if damage has occurred). Entry into M-phase is delayed in response to DNA damage or incomplete DNA synthesis (Shiloh 2003). In fission yeast both the DNA damage and replication checkpoints regulate the inhibitory tyrosine phosphorylation of Cdc2 through Cdc25 and Mik1 pathways (OConnell, Raleigh et al. 1997)

2.3.1 DNA Damage Checkpoint

When DNA damage occurs in S or G2 phase, the DNA damage response pathway is activated to block mitotic entry until the damage has been fully repaired. All the DNA damage pathways comprise three essential components: damage sensors that detect different types of DNA damage; signal transducers that pass the signal from different sensors to the effectors that halt the cell cycle progression and activated DNA repair mechanism (Zhou and Elledge 2001).

The DNA damage checkpoint protein kinase Chk1 was discovered in fission yeast (Walworth, Davey et al. 1993). It acts as signal transducer downstream of a group of DNA structure sensors known as "checkpoint Rad proteins" that includes Rad1, Rad3, Rad9, Rad17, Rad26 and Hus1 (Abraham 2001). These molecules are thought to be involved in the monitoring and signaling events that detect both incomplete DNA replication and DNA damage (Saka and Yanagida 1993; Walworth and Bernards 1996) . The molecular mechanisms and functions of Rad proteins are not fully understood, however, it is known that the checkpoint Rad proteins Rad4/cut5 and Rhp9/Crb2 are required for the Chk1 phosphorylation that is triggered following DNA damage treatment (Walworth and Bernards 1996; Saka, Esashi et al. 1997; Willson, Wilson et al. 1997).

Cdc25 was identified as the first downstream effector of Chk1 (Kommajosyula and Rhind 2006). As described above, Cdc25 is a tyrosine phosphatase that removes the inhibitory phosphorylation of Y15 on Cdc2 by Wee1 and Mik1. The rate of Cdc2 tyrosine dephosphorylation is reduced by irradiation (Kommajosyula and Rhind 2006). Chk1 regulates Cdc25 by two mechanisms 1). It has been demonstrated that Chk1 directly inhibits the phosphatase activity of Cdc25 in mitotic control *in vivo*. (Furnari, Blasina et al. 1999). 2). In addition, Chk1 phosphorylates Cdc25 to create a binding site for fission yeast 14-3-3 protein Rad24, and Rad24 enhances the nuclear export of Cdc25 from the nuclear pool of MPF in response to DNA damage (Lopez-Girona, Furnari et al. 1999).

Later, a careful analysis of synchronous cultures showed that DNA damage causes a substantial mitotic delay in *cdc2-3w*; this delay was abolished by *chk1*$^+$ or *mik1*$^+$ deletion mutations (Sanchez, Wong et al. 1997). Furthermore, GST-Chk1 overexpression arrests division in *wee1-50 cdc25.Δ* cells, and this arrest was suppressed by *mik1*$^+$ deletion (Baber-Furnari, Rhind et al. 2000) but not *wee1*$^+$ deletion (Furnari, Rhind et al. 1997), suggesting that Chk1 might also regulate Mik1 to activate the DNA damage checkpoint. The damage checkpoint defect caused by *mik1*$^+$ deletion however, is modest relative to the

absence of checkpoint defect exhibited by *chk1.Δ* cells, hence Mik1 might be secondary importance and functions primarily to enforce that DNA damage checkpoint. Mik1 protein levels were significantly increased in cells treated with bleomycin that induces Chk1 mediated DNA damage checkpoint (Boddy, Furnari et al. 1998)(Baber-Furnari, Rhind et al. 2000). Chk1 might therefore regulate MPF tyrosine phosphorylation by stabilizing Mik1 to enforce the DNA damage checkpoint (figure8 left).

2.3.2 DNA replication checkpoint

In eukaryotic cells, perturbations of DNA replication prevent the subsequent mitosis (Enoch and Nurse 1990). Like the DNA damage checkpoint, there must be checkpoint controls for linking completion of S-phase with mitotic commitment. In fission yeast, the DNA replication checkpoint regulates mitotic commitment via the control of the tyrosine phosphorylation status of Cdc2.

When S-phase is inhibited by hydroxyurea (HU) treatment, mitosis was blocked in *wee1*$^+$ loss-function mutant cells. *mik1*$^+$ loss-function mutations had a partial, yet significant, mitotic arrest defect, while temperature sensitive *wee1-50 mik1.Δ* cells were completely unable to arrest mitotic commitment at the permissive temperature (Sheldrick and Carr 1993), suggesting that Wee1 and Mik1 have overlapping roles in the control of mitosis during DNA replication arrest. Furthermore, *wee1.Δ cdc25.Δ* double mutants were still able to delay mitosis when exposed to HU, suggesting that Mik1 alone is able to link S-phase to mitosis (Enoch, Carr et al. 1992). Mik1 is S-phase specific and the up-regulation of *mik1*$^+$ transcript is maintained in S phase-arrested cells (Christensen, Bentley et al. 2000). Therefore, the mitotic inhibitor Mik1 is produced and stabilized in S-phase only to prevent S-phase cells from entering mitosis; it serves as an intrinsic link between S-phase and mitosis. The checkpoint Rad proteins are essential for cell survival during HU induced S-phase arrest. HU treatment results in rapid cell death during S-phase in cells lacking Rad proteins cells (Enoch, Carr et al. 1992). The checkpoint Rad proteins are monitoring the presence of specific DNA structures or DNA binding proteins and activate different signal transducers as appropriate. HU treatment activates the *S.pombe* Rad3-dependent checkpoint pathway (Alkhodairy and Carr 1992). Rad3 is required for phosphorylation and activation of the protein kinase Cds1 that is a signal transducer in DNA replication arrest (Matsuoka, Rotman et al. 2000). Cds1 was identified as a multicopy suppressor of a temperature sensitive DNA polymerase α mutation (Lindsay, Griffiths et al. 1998). It is a structural homolog of budding yeast Rad53 that is involved in both DNA damage and replication checkpoint (Weinert, Kiser et al. 1994). Cds1 is also activated by DNA damage but this is only seen in S-phase suggesting that the Cds1 response is specific ally activated by particular replication structures. Cds1 may interact with replication proteins and stabilize this replication

structure to maintain S-phase, and inhibit mitosis. It is thought to do the latter by phosphorylating Cdc25 to promote it's nuclear export, and possibly to phosphorylate Wee1 (Boddy, Furnari et al. 1998; Lindsay, Griffiths et al. 1998). Cds1 remains inactivated in S- phase during unperturbed cell cycles, suggesting that it links mitosis to S-phase specifically during perturbed replication. Therefore, Rad3-Cds1 is an S/M-phase checkpoint that links S-phase progression with mitotic commitment when replication problems arise. Alternatively, it can be also considered as an S- phase Specific DNA damage checkpoint that blocks mitosis to ensure DNA replication is correctly completed in S-phase.

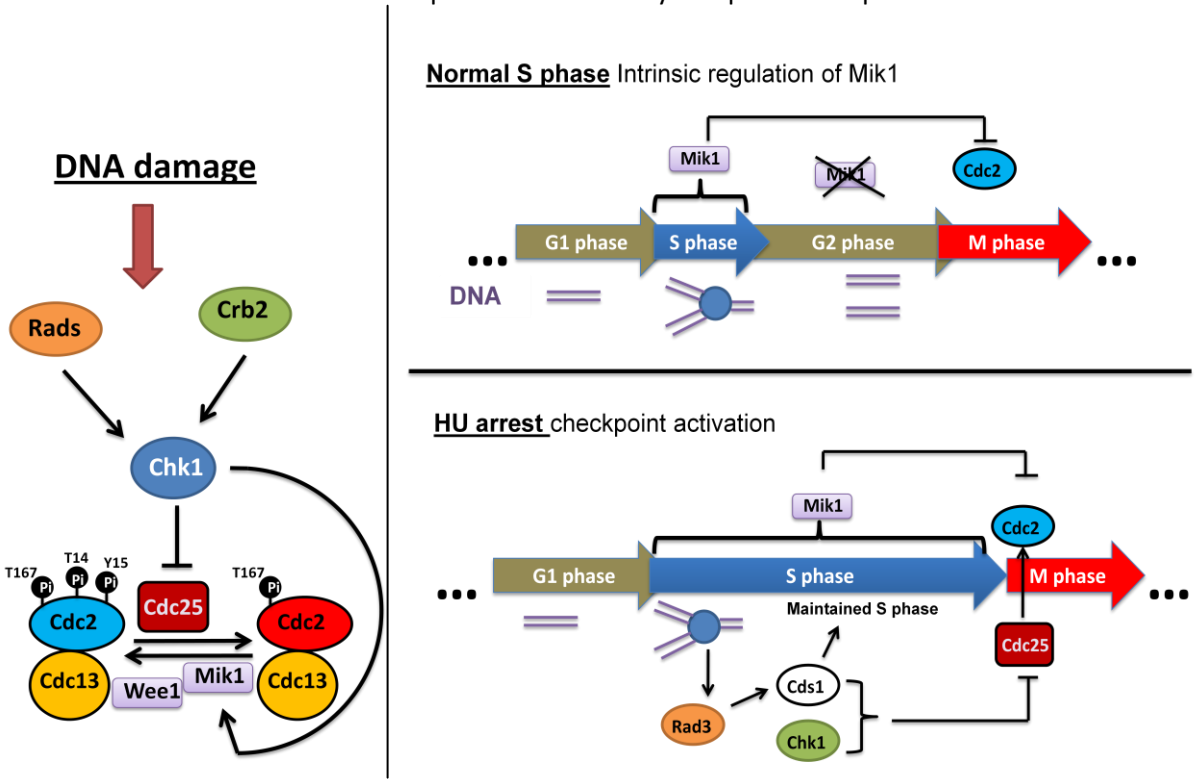


Figure 8 DNA damage and DNA replication checkpoint can prevent mitotic entry through Rad proteins that can monitor DNA integrity. Left: Chk1 mediated DNA damage checkpoint regulates MPF activity through Mik1 and Cdc25. **Right:** Mik1 is S-phase specific mitotic inhibitor; providing an intrinsic link between S-phase and mitosis. Rad3 can sense the replication structure in S phase and inhibit MPF through Cdc25.

Protein kinase C

3.1 Introduction

Protein Phosphorylation/dephosphorylation is one of the major mechanisms through which cellular information can be reversibly passed on between different molecules within the cell. The addition of a phosphate to a molecule can induce a conformational change of the target protein to alter it's function and property; alternatively, protein phosphorylation can create a new binding site for protein -protein interaction and recognition. Members of protein kinase C family are the major players in cellular signal transduction; their biological functions depend on a series of ordered phosphorylation/dephosph-orylation reactions (Mellor and Parker 1998; Newton 2003; Roffey, Rosse et al. 2009). Protein kinase C (PKC) was one of the very first Serine/threonine protein kinases to be discovered. It plays a variety of roles in different cellular processes including cell proliferation and differentiation, apoptosis and tumorigenesis.

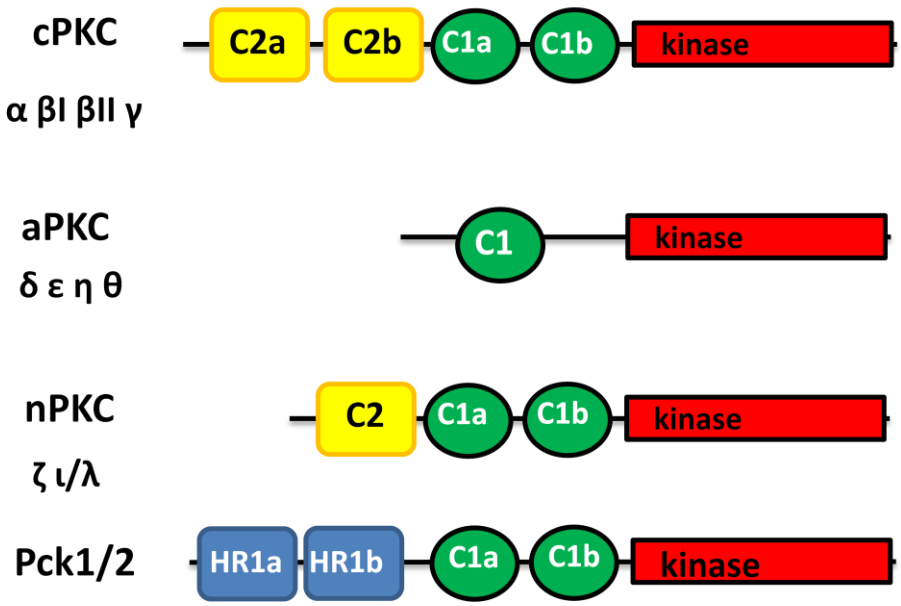


Figure 9 Domain composition of protein kinase C in mammalian cells and *S. pombe.* All protein kinase C isoforms have a highly conserved C terminal kinase domain (red) Pck1/2 contain two C1 domains (green) that can be found in cPKC and nPKC (see results session); and two HR1 domains (blue) that can be found in protein kinase C related kinase (PRK/PKN). Pck1/2 don't contain the C2 domain that is found in some PKC isoforms of higher systems such as the cPKC of man (yellow).

The greatest challenge faced in understanding the functions of PKC is the number of PKC isoenzymes in existence: a superfamily of 10 distinct mammalian PKC isoenzymes has been defined. The domain composition of the regulatory moiety of each isoenzyme determines its cofactor-dependence, which, in turn, determines the property and function of the kinase (Newton 2001). Based on all these parameters, the 10 PKC isoenzymes are grouped into 3 subclasses conventional (cPKC α, βI, βII and γ), novel (nPKC δ, ε, η and θ) and atypical (aPKC ζ ι/λ) (Mellor and Parker 1998) (figure 9).

3.2 Structure of Protein Kinase C

PKCs have a core kinase domain that recognizes specific motifs on target proteins and transfers a phosphate group to the hydroxyl group of a serine/ threonine residues. The regulatory domains of PKCs control their kinase activity, localization and protein-protein interaction.

3.2.1 Core Kinase domain

The kinase core domain of ABC protein kinases are conserved with more than 40% sequence identity (Newton 2003). More than 30 protein kinase crystal structures have been solved. All of their core domains share the same bilobal structure: an N-terminal lobe that is rich in β-sheets, and a C-terminal lobe rich in α-helices (Taylor and Radzioandzelm 1994; Johnson and Lewis 2001). The substrate and ATP binding site is located in a cleft between the two lobes.

All PKCs have three highly conserved phosphorylation motifs that are critical regulatory sites for kinase function. They serve as phosphorylation controlled switches to regulate inter and intra-molecular interactions. Phosphorylation/dephosphorylation on one or more of these sites is a pre-requisite step to allow substrate phosphorylation.

1) The Activation loop

The first phosphorylation activating motif is on a segment near the entrance to the active site within the core domain, known as the activation loop. A threonine residue (Thr308 in cPKC βII) on this loop must be phosphorylated before the kinase can phosphorylate its substrate. The modification of the threonine residue on activation loop is achieved via autophosphorylation in PKA (Yonemoto, McGlone et al. 1997). In the case of PKB and PKC, this role is fulfilled by an upstream novel kinase named 3-phosphoinositide-

dependent protein kinase (PDK-1). In PDK-1 deficient mice embryonic stem cells, levels of PKC isoenzymes are decreased significantly (Edwards, Faux et al. 1999), suggesting that these PKCs are unstable in their non-phosphorylated form (Edwards, Faux et al. 1999).

2) Turn motif

A segment of the PKC C-terminus contains a highly conserved phosphorylation site. In PKC βII, phosphorylation of Thr641 results in compensating phosphorylation of adjacent residues to produce a functional kinase (Shibata, Mukai et al. 1996). Biochemical evidence also established that phosphorylation at the turn motif is critical to stabilize the kinase core structure.

3) Hydrophobic motif

PKCs have a conserved phosphorylation site in their C terminal regions that contains a serine or threonine residue flanked by hydrophobic residues. The hydrophobic motif in PKB is important for its activation (Yang, Cron et al. 2002). However, mutation of the hydrophobic site in PKC to an alanine residue showed that this site is not essential for PKC function; rather it stabilized the enzyme (Edwards and Newton 1997).

3.2.2 Regulatory domains.

The function of the kinase domain is allosterically regulated by a corresponding regulatory domain. PKA has its regulatory moiety on a separate polypeptide, whereas the regulatory domain of PKB and PKC are on the same polypeptide as the kinase domain (Newton 2003). The regulatory domains have two functions: first they serve as an autoinhibitory module that regulates kinase activity; secondly they target the protein to the appropriate cellular location (Newton 2003).

C1 domain

All PKC isoenzymes contain at least one C1 domain. It was first defined by two repeated zinc-finger motifs (C1a and C1b) in the cPKCs and nPKCs. Each of these has a conserved pattern of histidine and cysteine residues that co-ordinate Zn^{2+} ions, and folded into a small globular structure (Hubbard, Bishop et al. 1991); two separated β-sheets within the C1 domain form a binding site for diacylglycerol (DAG) on the plasma membrane (Zhang, Kazanietz et al. 1995) and phorbol ester (Kaibuchi, Fukumoto et al. 1989). Therefore C1 is thought to target PKC to the cell plasma membrane. It also specifically binds to

phosphatidylserine (Johnson, Giorgione et al. 2000). The C1 domain can bind to phorbol esters *in vivo* (Kazanietz 2002). Phorbol esters are widely used as tumour promoting agents in the animal models of carcinogenesis. In atypical PKCs, there is only one C1 repeat; they respond to neither phorbol ester nor DAG.

C2 domain

The C2 domain binds to phospholipids in a Ca^{2+} dependent manner; thereby targeting PKC to the plasma membrane in the presence of Ca^{2+} ions. Conventional and novel PKCs contain a C2 domain (Nalefski and Falke 1996). Like the C1 domain, the C2 domain is also found in many other signaling molecules such as phospholipases, GAPs and rabphilin-3A (Ponting and Parker 1996). The C2 domain is not found in the Ca^{2+} independent aPKCs.

HR1 domain

This domain was initially indentified as a region of homology between PRK1 and PRK2 (Palmer and Parker 1995). Like other aPCKs, both PRKs are insensitive to Ca^{2+}, DAG and phorbol esters (Palmer, Ridden et al. 1995). However, both are able to bind to the activated RhoA GTPase through their HR1 domain. It is thought that an unidentified pseudosubstrate motif might be near the HR1a of PRKs; and binding of RhoA can keep the pseudosubstrate motif away from the activate site of the kinase domain (Flynn, Mellor et al. 1998). PRK1 is targeted to the endosomal compartment by RhoB via HR1 (Mellor, Flynn et al. 1998). Thus, HR1 is also thought to act as a localization signal to target the PKC activity to specific cellular compartments.

pck1^{+} and *pck2*$^{+}$ are the two protein kinase C like genes found in fission yeast (Toda, Shimanuki et al. 1993). Both contain a common C-terminal kinase core domain that is highly conserved between the two isotype (71% identity) and Pkc1 of budding yeast (64% identity). Both kinases also have a C1 domain. The tandem repeats of the cysteine-rich motif within the C1 domain contain characteristic pattern of C1 sub domains found in other PKC molecules: H-X_{12}-C-X_2-C-$X_{11,13}$-C-X_2-C-X_4-H-X_2-C-X_7-C (Hubbard, Bishop et al. 1991). The C1 domain is thought to form the binding site for DAG and phorbol ester (Ono, Fujii et al. 1988). Both Pck1 and Pck2 lack a C2 domain, suggesting that they are not regulated by ion Ca^{2+} ions. Pck1 and Pck2 have the N-terminal HR1 that binds to the small GTPase Rho (Lane and Nigg 1996; Sayers, Katayama et al. 2000).

3.3 function of protein kinase C

In mammalian cells, PKC is thought to transmit the DAG/phorbol ester signal into the Raf/MEK/ERK signaling pathway, however, the exact mechanism remains unknown, with conflicting results as to whether PKC activates c-Raf-1 or not (Schonwasser, Marais et al. 1998). PKCs also play an important but not well understood roles in integrin signaling (Kolanus and Seed 1997). Budding yeast and fission yeast are simple and genetically amenable model organisms; they have been extensively used for characterising a large variety of protein kinases in signaling transduction.

3.3.1 Protein kinase C in budding yeast

The budding yeast *Saccharomyces cerevisiae* has only one PKC, Pkc1 (Antonsson, Montessuit et al. 1994) which has been extensively studied (Mellor and Parker 1998). *pkc1.Δ* cells showed an arrest of protein synthesis and cell growth after DNA synthesis and before mitosis. This arrest can be reversed by an osmotic stabilizer, suggesting that Pkc1 is involved in cell wall formation (Levin, Fields et al. 1990). Electron-microscopy showed that *pkc1.Δ* cells have thinned cell walls and holes at the bud tip (Levin, Bowers et al. 1994). Pkc1 is activated by hypotonic osmotic cellular stress (Davenport, Sohaskey et al. 1995), and also by cell cycle dependent establishment of polarized growth (Kamada, Jung et al. 1995), as dynamic cell wall remodeling is required during growth.

Pkc1 is immediately downstream of the Rho GTPase Rho1 (Nonaka, Tanaka et al. 1995). Rho1 is activated by its guanine nucleotide exchange factors (GEF) Rom1, Rom2 and Tus1 (Ozaki, Tanaka et al. 1996; Schmelzle, Helliwell et al. 2002); and inhibited by its guanine nucleotide activating proteins (GAP) Sac7, Bem2, Lrg1 and Bag7 (Peterson, Zheng et al. 1994; Schmidt, Bickle et al. 1997). Rom2 and Tus1 function in Rho1 activation and localisation during cytokinesis; the regulation of both Rho1 GEFs is mediated by Cdc5 (Polo like kinase in budding yeast) (Yoshida, Kono et al. 2006). Yeast PI3-kinase related protein Tor2 activates Rom2 (Schmidt, Bickle et al. 1997). Pkc1 activates the Bck1-Mkk1/2-Mpk1 MAPK pathway that then regulates the activity of a group of transcription factors (Lee and Levin 1992) (figure 10 right). *pkc1.Δ* cells showed down regulation of genes that are involved in cell wall formation, such as those encoding a subunit of β(1,3)-glucan synthase (FSK1) and α-1,3-mannosyltransferase (MNN1) (Igual, Johnson et al. 1996). It has been suggested that the DNA binding protein Swi4 acts

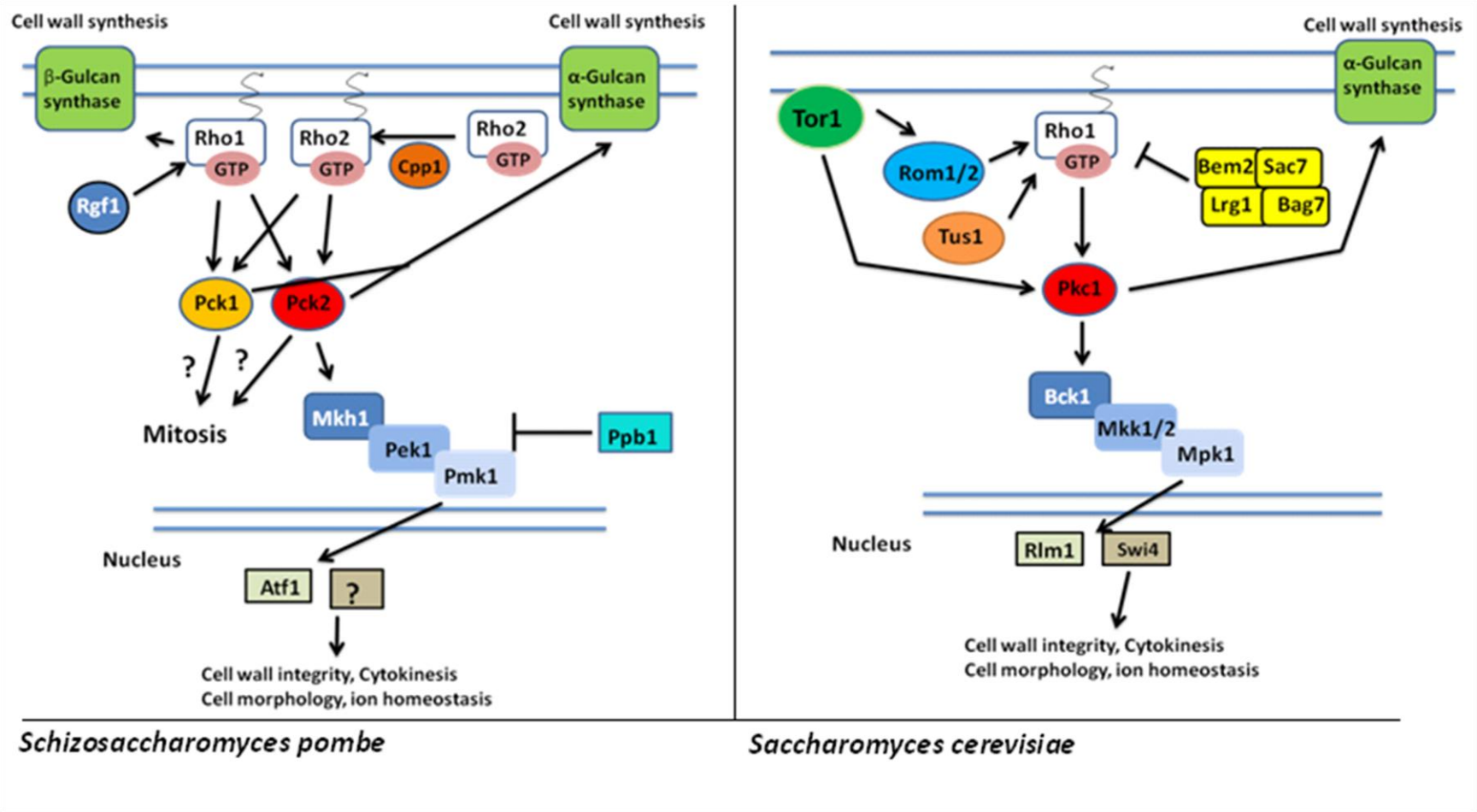


Figure 10 protein kinase C regulates cell wall integrity and cell morphology in fission yeast and budding yeast Left: cell wall integrity pathway in budding yeast. **Right**: Pck2, Pck1 signaling pathway in fission yeast.

downstream of Pkc1 (Madden, Sheu et al. 1997). Swi4 controls the expression of a group of genes involved in cell wall formation at G1/S-phase boundary (Igual, Johnson et al. 1996).

Mpk1 also phosphorylates and activates the transcription factor Rlm1 in response to cell wall stress, Rlm1 regulates a group of genes that are implicated in cell wall biogenesis (Watanabe, Irie et al. 1995). Other than transcriptional regulation, the Pkc1 activated Mpk1 pathway might be involved in polarised growth that requires transport of secretory vesicles along actin cables to the site of cell wall formation. Conditional Mpk1 mutations showed delocalisation of cortical actin structure and accumulation of vesicles in the cytoplasm (Mazzoni, Zarzov et al. 1993).

3.3.2 Protein kinase C in fission yeast

The *S. pombe* protein kinase C homologues Pck1 and Pck2 were first isolated and characterized in Mitsuhiro Yanagida's lab (Toda, Shimanuki et al. 1993). The group showed that, collectively, *pck1*$^+$ and *pck2*$^+$ have essential roles in cell viability as *pck1.Δ pck2.Δ* double mutant is lethal, even in the presence of an osmotic stabilizer. *pck2.Δ* cells displayed defects in cell morphogenesis. Some cells had an irregular, round, pear-like shape rather than the cylindrical shape of wild type cells. *pck2.Δ* cells also have an abnormal calcofluor staining pattern. In contrast, overexpression of *pck2*$^+$ is lethal, giving elongated, multiseptated and branched cells. All these observation suggest that *pck2*$^+$ is involved in the control of cell growth polarity.

In fission yeast, tip growth requires highly dynamic cell wall formation; the cell must be able to generate cell wall during cell growth. Pck2 is important for cell wall formation. *pck2.Δ* cells have less cell wall than wild type cells and overexpression of *pck2*$^+$ increases β-glucan content and β-glucan synthase activity (Arellano, Valdivieso et al. 1999), and Pck2 but not Pck1 is essential for protoplast regeneration (re-formation of the cell wall after it is completely removed by enzymes) (Mizuta, Sawada et al. 1985). The enzyme α(1,3)-glucan synthase that catalyses the biosynthesis of an essential component of the cell wall is a downstream effector of Pck1 and Pck2. Furthermore, Rho1 is a regulatory component of β(1,3)-glucan synthase (Arellano, Duran et al. 1996). Rho1 binds to the HR1 domain of Pck1 and Pck2 and activates their kinase activity (Sayers, Katayama et al. 2000), suggesting a link between Pck1 and Pck2 signaling and cell wall biosynthesis. Cells that lack Pck2 are hypersensitive to staurosporine, which is an ATP analogue that inhibits a subset of protein kinases. Recent research on Pck2 and Pck1 has mainly focused on their involvement in cell integrity through the Pmk1 MAPK pathway (figure 10 left).

3.3.3 Pck1 and Pck2 is upstream of Pmk1 MAPK pathway

Because Pkc1 activates Mpk1 pathway in budding yeast (Errede and Levin 1993), Pck1 and Pck2 were first tested for being candidates that interact with Pmk1 in cell wall integrity control; it is still unclear how exactly Pck1 and Pck2 regulate the Pmk1 pathway, two plausible models were proposed after the initial studies of Pmk1 (Toda, Dhut et al. 1996):

1). Pck1 and Pck2 act on separately regulated pathways from the Pmk1 pathway, but both pathways are functionally related to cell integrity. The *pck2.Δ pmk1.Δ* cells have synergistic cell wall defect phenotypes. Some phenotypes of *pck2.Δ* cells and *pmk1.Δ* cells are different, for example, *pmk1.Δ* cells are not sensitive to staurosporine. However, Pck1 might have a minor role in minor staurosporine sensitivity in *pmk1.Δ* cells.

2). If Pck1 and Pck2 act upstream of Pmk1, Pck1 and Pck2 must act through a pathway in cell wall integrity with at least two branches, with one of them acting through Pmk1. The initial evidence supporting this model was that *pmk1.Δ* is viable whereas *pck1.Δ pck2.Δ* is lethal.

In both models, Pck1 and Pck2 must play another essential role that is independent from the Pmk1 pathway. A number of recent publications demonstrated that the genetic interaction between Pck2 and Pmk1 is likely to follow the second model. Deletion of the Rho1 GEF *rgf1*$^{+}$ (Garcia, Tajadura et al. 2009), *pck2*$^{+}$ or the components of Pmk1 MAPK pathway (Ma, Kuno et al. 2006) are hypersensitive to high concentrations of Cl^{-} and cell wall-damaging agents such as micafungin or an inhibitor of (1,3)-β-D-glucan synthase (Carver 2004). *sty1.Δ* or *spk1.Δ* in contrast don't have those phenotypes (Ma, Kuno et al. 2006). The Cl^{-} hypersensitivity of *ppb1.Δ* cell (presumably caused by constitutive activation of Pmk1 pathway) can be suppressed by deleting *pmk1*$^{+}$, *pck2*$^{+}$, *cpp1*$^{+}$ (Ma, Kuno et al. 2006) or *rgf1*$^{+}$ (Garcia, Tajadura et al. 2009), this is known as the 'viable in the presence of immunosuppressant and chloride ion' (*vic)* phenotype (Ma, Kuno et al. 2006), suggesting interactions between those genes in the Pmk1 pathway that regulate cell integrity and ion homeostasis.

3.3.4 Rho GTPases regulate Pck2

To produce a cylindrical cell shape and maintain intracellular osmolarity in fission yeast, cell wall material must be continually synthesised and remodified only at the cell tips during cell growth. This highly dynamic process is mediated by Tea1/microtubule and actin cytoskeleton (details are described above in the polarised growth section) (Martin 2009). Rho1 is found in cell poles, sites of polarity growth

and septum, and it is thought to coordinate polarized growth and cell wall biosynthesis (Arellano, Duran et al. 1997). Rho1 signalling regulates β-(1,3)-glucan biosynthesis and cell wall synthesis in general. It is required to maintain cell wall integrity and actin polymerisation. β-(1,3)-glucan synthase (GS) catalyses the biosynthesis of one of the main polysaccharides in fission yeast cell wall, β-(1,3)-glucan. GS is composed of two molecules: a catalytic subunit and a regulatory subunit. Rho1 is one of the essential regulatory subunits of GS (Arellano, Duran et al. 1996). It acts as a binary switch by cycling between an active GTP bound state and an inactive GDP bound state. Rho1 activates GS in its GTP-bound prenylated state. Thereby, GS can be turned on/off in response to Rho1 GTP/GDP bound state that is regulated by its GEF/ GAP, respectively. One of the advantages of having multiple GEF/GAP molecules for Rho1 is that each GEF determines a specific downstream signalling of Rho GTPase for example Rgf3 accumulates at the contractile ring where it activates Rho function to maintain cell integrity specifically during cytokinesis (Tajadura, Garcia et al. 2004). Rho1 also regulates the organization of F-actin patches (Arellano, Duran et al. 1997).

Rgf1 is one of three Rho GEFs found in *S.pombe*. It binds to and activates Rho1 by promoting the stability of its GTP bound state. Rho1 in its GTP bound state binds to Pck1 and Pck2 through their N-terminal HR1 domains and activates their kinase activity (Sayers, Katayama et al. 2000), resulting in increased concentrations of Pck1 and Pck2 precisely in the areas of growth (Arellano, Valdivieso et al. 1999). Fluorescence microscopy imaging showed that Rgf1 is localised at the growing region (one or both cell tips), the media ring and septum, and all along plasma membrane. *tea1.Δ rgf1.Δ* cells have defects in actin organisation and in β-glucan biosynthesis that are similar to those of cells from which *rho1*$^{+}$ has been deleted (Arellano, Duran et al. 1997); cells fail to initiate NETO and lyse. A possible explanation for this phenotype is that the Rho1-β-GS complex is not properly activated in *Δrgf1* cells during biopolar cell growth. *tea1.Δ* cells also have defects in NETO. In *tea1.Δ* cells, Rgf1 is not maintained at one of the new cell ends, and cells did not grow at that end. Therefore, Rgf1 regulates Rho1 for its function in polarized cell growth (Arellano, Duran et al. 1997).

Rgf1 appears to play a role that is antagonistic to that executed by calcineurin. Biochemical evidence also showed that Pmk1 phosphorylation and activation in response to osmotic stress or cell wall damage depends on Rgf1 signalling through Rho1 and Pck2 (Garcia, Tajadura et al. 2009). Thus Rgf1 may positively regulate a subset of activators toward the Pck2-Pmk1 MAPK cell integrity pathway during polarised cell growth.

3.3.5 FTase-Rho2-Pck2-Pmk1 MAPK

Genetic analysis showed that Rho2 GTPase regulates the synthesis of α-D-glucan and other main structural polymer of fission yeast cell wall. Rho2 transmits signalling through the Pck2-Mkh1-Pek1-Pmk1 MAPK pathway: Overexpression of Rho2 is toxic to wild type cells but not to *pck2.Δ pmk1.Δ*, *mkh1.Δ* or *pek1.Δ* cells (Calonge, Nakano et al. 2000)(Ma, Kuno et al. 2006). *pmk1*$^{+}$, *mkh1*$^{+}$ or *pek1*$^{+}$ deletion can rescue the lethality of *pck2*$^{+}$ overexpression. Biochemical analysis further confirms that Rho2 and Pck2 act upstream of the Pmk1 pathway: Overexpression of *rho2*$^{+}$ and *pck2*$^{+}$ significantly increased Pmk1 phosphorylation. In contrast, overexpression of *pck1*$^{+}$ had no impact on the Pmk1 phosphorylation level of Pmk1. Furthermore, Pck2, but not Pck1 coimmunoprecipitated with Mkh1 (Ma, Kuno et al. 2006).

cpp1^{+} was isolated in a genetic screen for the *vic* phenotype (section 3.3.4.). It encodes a subunit of the farnesyltransferase (FTase) (Ma, Kuno et al. 2006). In mammalian cells, FTase targets the Ras family of small GTPase to the cell membrane (Glomset and Farnsworth 1994). In Fission yeast Cpp1 has a critical function in morphogenesis and sexual differentiation through Ras, and is also important in cell cycle progression through Rheb (Yang, Urano et al. 2000; Yang, Tabancay et al. 2001). Fluorescence microscopy imaging showed that Rho2 is localised to the plasma membrane, but becomes cytoplasmic in *cpp1.Δ* cell. In contrast, the geranylgeranylated mutant form of Rho2 (Rho2CIIL) bypasses the requirement for FTase, and localises to the plasma membrane of both wild type and *cpp*1.*Δ* cells. Rho2CIIL fully suppressed the temperature-sensitive and Cl^{-} hypersensitivity of *rho2*$^{+}$ deletion. Therefore, the FTase encoded by *cpp1*$^{+}$ is essential for the membrane localisation of Rho2 which , in turn, is critical for its function in the Pmk1 pathway due to its impact on Pck2 (Ma, Kuno et al. 2006).

Both *pck1.Δ* and *pck2.Δ* cells have cell wall integrity defects; *pck1.Δ* cells have weaker sensitivity to micafungin (drug that inhibits β-(1,3)-glucan synthase) than *pck2.Δ* cells. The sensitivity to micafungin is synergistic for the *Pck1.Δ rho2.Δ* double mutant. In contrast, *pck2.Δ rho2.Δ* double mutants showed similar sensitivity to micafungin as that of *pck2.Δ* cells. The sensitivity of *mkh1.Δ* cell and *pmk1.Δ* cell to micafungin can be compromised by *pck1*$^{+}$ but not *pck2*$^{+}$ overexpression (Ma, Kuno et al. 2006). Furthermore, Yam8/Cch1- mediated Ca^{2+} influx stimulate calcineurin through Pmk1; this activation of calcineurin is suppressed by *pck2*$^{+}$ deletion but not by *pck1*$^{+}$ deletion (Deng, Sugiura et al. 2006). Therefore, Pck1 might regulate cell integrity in a Pmk1 independent manner.

So far, the only downstream effector of the Pmk1 MAPK pathway that has been confirmed is a transcription factor Atf1. *atf1.Δ* cells exhibited the *vic* phenotype and are hypersensitive to micafungin, Pmk1 phosphorylates Atf1 in response to micafungin induced cell wall damage (Takada, Nishimura et al. 2007). Mbx2, the homolog of the budding yeast Mpk1 downstream effector Rlm1, might also be regulated by Pmk1, but *mbx2.Δ* cells only have a weak *vic* phenotype (Takada, Nishimura et al. 2007). The effect of Atf1 phosphorylation by Pmk1 is unclear; a putative ATF/CREB-binding site has been identified in the Ecm33 promoter region. It is thought that $ecm33^{+}$ is involved in the negative feedback regulation of the Pmk1 pathway and is linked to Ca^{2+} signalling (Takada, Nishimura et al. 2007). Atf1 is also regulated by Sty1 (Wilkinson, Samuels et al. 1996; Reiter, Watt et al. 2008), it would be interesting to find out if Sty1 and Pmk1 phosphorylate Atf1 at the same or different site(s), and if there is cross talk between two MAPK pathways in fission yeast.

3.3.6 Protein kinase C and Cancer

Inappropriate cell division is a major hallmark of cancer. In the last fifteen years a large number of protein kinases have been associated with mitotic control. Understanding these protein kinases has not only furthered our knowledge of cancer biology, but also helped to develop anti-tumour drugs.

In human cells, the downstream events after PKC activation are not well known; the MEK-ERK pathway is thought to be activated by PKCs. PKCα, δ and ε can phosphorylate and activate Raf1 both *in vitro* and *in vivo* (Kolch, Heidecker et al. 1993; Ueda, Hirai et al. 1996; Cai, Smola et al. 1997). Activated Raf1 phosphorylates and activates MEK1/ME11K2 which then activates the mitogen-activated protein kinases, resulting in up-regulation of genes involved in cell proliferation (Marshall 1996). PKCα, βI and γ can specifically inhibit GSK-3β and lead to down-regulation of the cJun transcription factor (Goode, Hughes et al. 1992). PKCθ acts with the $Ca2^{+}$-dependent phosphatase calcineurin to stimulate JNK1 via Rac1 (Werlen, Jacinto et al. 1998). PKCs are involved in carcinogenesis, neoplastic transformation and metastasis (reviewed in (Mackay and Twelves 2003)). A number of PKC inhibitors Enzastaurin and Ruboxistaurin are currently being used in oncology clinical trials (reviewed in (Roffey, Rosse et al. 2009)).

Aims of the project

An *S. pombe* mutant allele of Pck2 (Pck2.as) has been generated in the lab, which can be reversibly inhibited by adding a non-hydrolysable analogue of ATP to the cell culture (Bishop, Buzko et al. 2001). Manipulation of this allele to provide a burst of Pck2 activity was able to promote a burst of mitosis (Dr Agnes Grallert unpublished work). The goal of my MPhil project is to investigate how the Pck2

dependent signal transduction pathway regulates mitotic commitment. My work demonstrates that Pck2 regulates mitotic commitment via Wee1 dependent mechanism, and that Pck2 might also regulate cell growth polarity.

Materials and methods

Yeast strain

Strains used in this study are listed in the table below

Strain	*Genotype*	*source*
IH5974	*Wild type*	Fantes P
IH6113	*h⁻ pku80::ura4⁺ ura4.d18 leu1.32*	Lab stock
IH1231	*h⁻ pck1:: ura4⁺.dis ura4.d18*	Toda T
IH1321	*h⁺ pck2::LEU2+.dis ura4.d18*	Toda T
IH6517	*h⁻ pck2::ura4 ⁺ura4.d18*	Bimbo A
IH9399	*h⁻ pck2::kanMX6.del*	This study
IH6756	*h⁻ Pck2.as:kanMX6*	Lab stock
IH6715	*h⁺ Pck2.as:kanMX6 pck1::ura4⁺.dis ura4.d18*	Lab stock
IH8293	*h⁻ cdc2.1w Pck2.as:kanMX6 pck1::ura4⁺.dis ura4.d18*	Lab stock
IH8375	*h⁻ cdc2.3w Pck2.as:kanMX6 pck1::ura4⁺.dis ura4.d18*	Lab stock
IH8610	*h⁺ pmk1::ura4⁺ Pck2.as:kanMX6 pck1::ura4⁺.dis ura4.d18*	Lab stock
IH8686	*h⁻ sty1::kanMX6 Pck2.as:kanMX6 pck1::ura4⁺.dis ura4.d18*	Lab stock
IH8924	*h⁻ plo1.S402A Pck2.as:kanMX6 pck1::ura4⁺.dis ura4.d18*	Lab stock
IH9232	*h⁺stf1.1 Pck2.as:kanMX6 pck1::ura4⁺.dis ura4.d18*	Lab stock
IH9404	*h⁻ wee1::ura4⁺ Pck2.as:kanMX6*	Lab stock
IH8595	*h⁻ cdr1::kanMX6 Pck2.as:kanMX6 pck1::ura4⁺.dis ura4.d18*	Lab stock
IH8349	*h⁺ cdr2::kanMX6 Pck2.as.kanMX6 pck1::ura4⁺.dis ura4.d18*	Lab stock
IH8348	*h⁺ pom1-D1 Pck2.as.kanMX6 pck1::ura4⁺.dis ura4.d18*	Lab stock

"dis": gene disruption. "del": gene deletion.

Cell culture

Cell culture and maintenance were carried according to (Moreno, Klar et al. 1991). EMM2 liquid media was used in the main culture of cell length measurement and Shokat washout approach:

Edinburgh Minimal Medium 2

20g/l Glucose (2% w/v)

3g/l Potassium hydrogen phthallate (14.7mM final)

2.2g/l Na_2HPO_4 (15.5mM final)

5g/l NH_4Cl (93.5mM final)

20ml/l SALTS (x50)

- 52.5g/l $MgCl_2.6H_20$ (0.26M)
- 0.735mg/l $CaCl_2.2H_20$ (4.99mM)
- 50g/l KCl (0.67M)
- 2 g/l Na_2SO_4 (14.lmM)

1ml/l VITAMINS (x1000)

- 1g/l pantothenic acid (4.20mM)
- 10g/l nicotinic acid (8l.2mM)
- 10g/l inositol (55.5mM)
- 10mg/l biotin (40.8µM)

0.1ml/l MINERALS (x10,000)

- 5g/l boric acid (80.9mM)
- 4g/l $MnSO_4$ (23.7mM)
- 4g/l $ZnSO_4.7H_2O$ (13.9mM)
- 2g/l $FeCl_2.6H_2O$ (7.40mM)
- 0.4g/l malonic acid (2.47mM)
- 1g/l KI (6.02mM)
- 0.4g/l $CuSO_4.5H_2O$ (1.60mM)
- 10g/l citric acid (47.6mM)

Genetic crosses-mating of *S.pombe*

Fresh single colonies from rich Yeast Extract with Supplements (YES) plates of partner cells of opposite mating types were mixed on Minimal Sporulation Agar (MSA) plate for random spore, or on Sporulation Agar (SPA) plate for tetrad dissection plates. Both MSA and SPA were supplemented with 0.8mg histidine, 0.8mg leucine, 0.8mg uracil and 0.4mg adenine per plate with (contains 20ml media). Samples were incubated at 25°C until asci appeared (approximately 36 hours).

Random spore analysis

Asci from successful mating on MSA plates were treated with 2µl β-glucuronidase for at least 4 hours at room temperature to digest the cell walls and release spores from the asci. The samples were then treated with 30% ethanol for 30 mins to kill any remaining vegetative cells, and diluted and plated on YES to give approximately 200 spores on each plate. Once colonies were formed from the spores, appropriate selective plates were used to replicate the original plates and select the strains containing the appropriate genotypes.

Tetrad analysis

A single ascus from a successful mating on a SPA plate was separated on a YES plate via an AU tetrad microscope (Micro Instruments Ltd.), the asci were then incubated at 36°C to release their spores. Individual spores from a single ascus were separated, so that each spore formed an individual colony after germination (4-5 days at 25°C). Appropriate selective plates were used to replicate the original plates with spores for selecting the strain containing the desired genotypes.

Bioinformatics

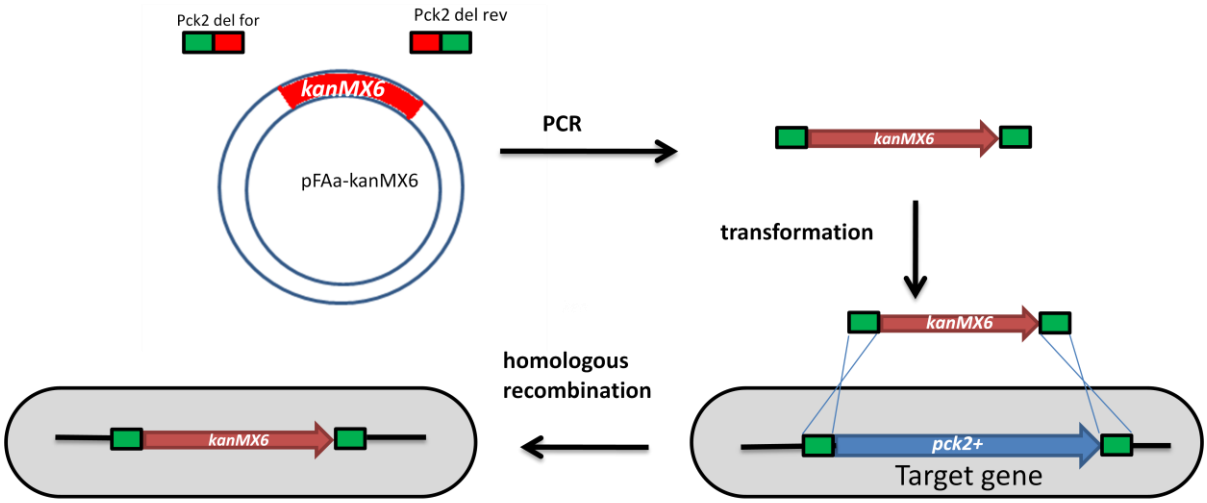
Protein primary sequences were aligned by Clustal W Method. For the study of the protein domain architecture, the primary sequence of Pck2 was aligned against the sequences within the Pfam 24.0 database (updated in October 2009; 11,912 families in total) on the Wellcome Trust Sanger institute website http://pfam.sanger.ac.uk. The multiple sequence alignments were generated by hidden Markov models (HMM). The domains were defined by the highly conserved residues.

S.pombe transformation using lithium acetate

pku80::ura4^{+} *ura4.d18 leu1.32* cells (IH6113) were grown to the late log phase (4-5×10^{6} cells/ml) in YES rich media and centrifuged at 1600 rpm for 3mins. The pellet was washed in sterile water and resuspended in appropriate volume of lithium acetate (0.1M, pH 4.9) to give 2×10^{8} cells/ml. samples

were incubated at 32°C for an hour then mixed with 1-2µg of DNA and 2.9 times volume of fresh polyethylene glycol-4000 (50% (w/v) in 0.1M, pH 4.9 lithium acetate). Samples were again incubated for an hour at 32°C before heat shock for 15 mins at 42°C. The cells were then washed in sterile water and plated on selective plates.

Gene deletion in *S.pombe*.



List of primers

***Pck2⁺* deletion**

pck2 del for

5′-CACATTAATATTAAAAACGGAAGCGAAAAATTGCAGATAAGAAGTTAAGCGGA AAAATCAGGATTAGGCCATTTAAAGTACGGATCCCCGGGTTAATTAA -3′

pck2 del rev

5′-AAAAGTCGAAATTAGAATAATTTATCAATGCAATGAAAGATTAAGAAAATGAG AGTAACTTTATGCTCAATTTAAGGTGGAATTCGAGCTCGTTTAAAC -3′

PCR checking for gene deletion

Pck2up

5′-GCGGCCGCCAATATATTCGCACAAGAT -3′

Pck2dsR

5′-GCGGCCGCTACCTTACTCAGTTCATTGTC-3′

uniMarkerCheck

5′- AAGCAGCCTTCACGAAAC AAC AGG CCACT-3′

Figure 11 the strategy for deletion allele construction

Synthetic primers containing 80 nucleotides complementary to the flanking regions of Pck2 followed by 20 nucleotides homologous to the Genectin selection marker cassette were used for PCR with the template plasmid pFA6a-MXkan6, to produce an oligomer that contains *kanMX6* flanked by *pck2*'s flanking region (figure 11). The oligomer was then transformed into *pku80::ura4⁺ ura4.d18 leu1.32* cells

(IH6113) via the lithium acetate method described above. Colonies were selected on EMM plates containing Genectin. The generated mutant strain was then back crossed with wild type cells three times to eliminate unwanted mutations.

Shokat washout

The principle of the Shokat washout approach is illustrated in figure 12. Mutated version of Pck2 has been generated (Pck2.as). Pck2.as has the similar ATP affinity and kinase activity as that of the endogenous Pck2, but a point mutation (Methionine to Alanine at amino acid 763) near the native ATP-

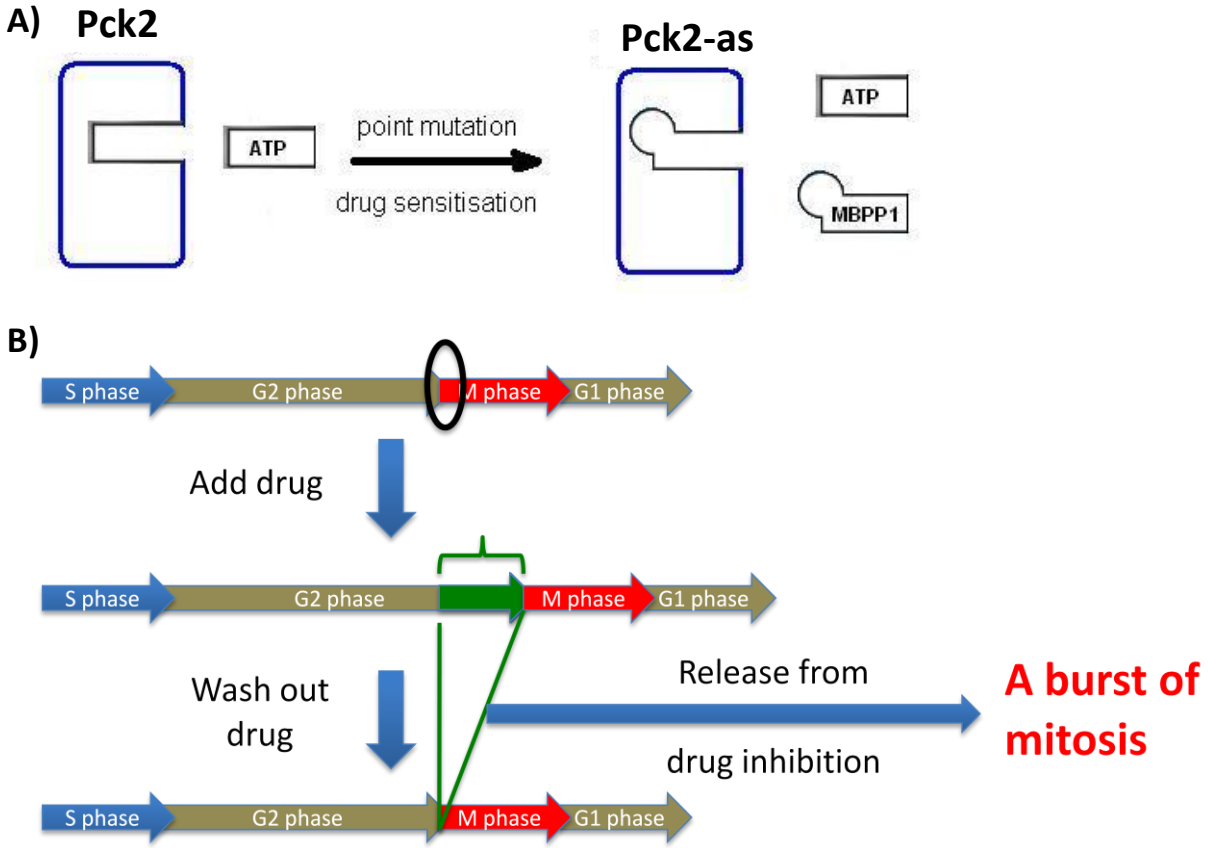


Figure 12 Procedure of Shokat washout approach for study the kinases that are involved in mitotic entry. A) the mutation M763A expands the ATP binding pocket of Pck2, which allows the ATP analogue 3MBPP1 to bind into the pocket and inhibit the kinase activity of Pck2.as **B)** Adding 3MBPP1 that can specifically inhibit the activity of kinase of interest, if the kinase activity is required for mitotic entry, cells will accumulate in G2 phase. Removal of the ATP analogue leads to a burst of mitosis in the culture as the accumulated cells now regain the kinase activity that can then promote mitotic entry.

binding pocket of Pck2 has expanded the pocket to create a new binding site for 3MBPP1 (Figure 12 A). This small molecule is a non-hydrolysable analogue of ATP, which acts a reversible competitive inhibitor of Pck2.as. Therefore, in the presence of 3MBPP1, the kinase activity of the Pck2.as is thought to be dramatically reduced (Bishop, Buzko et al. 2001). This method has been applied to the study of the Cdc28p CDK in *S.cerevisiae* (Bishop, Ubersax et al. 2000). In the Shokat washout approach, 3MBPP1 is added to cells in their exponential growing phase in liquid minimal culture. After 5 hours drug incubation (fission yeast has generation time of 4.5 hours at 25^{o}C in EMM2), the ATP analogue is removed by filtration. The culture is then sampled every 20 mins over next 3 hours to measure the mitotic index (the percentage of cells that undergoing mitosis). Wild type cells will give stable mitotic indexes after the ATP analogue washout as the addition of 3MBPP1 at this concentration has no impact on unmodified kinases. If the kinase is not involved in mitotic control at all, the mitotic index will also remain constant throughout the time course. In contrast, if the kinase can directly or indirectly induce mitosis, adding the ATP analogue would lead to the accumulation of a large number of cells in late G2 phase, and a burst of mitosis should be observed after removal of the ATP analogue. The concentration of the ATP analogue used is determined by the sensitivity of Shokat inhibitable protein to the ATP analogue. 20μM 3MBPP1 was used for Pck2.as (determined in Dr Agnes Grallert unpublished work).

Microtubules fixation and staining for immunofluoresence

Formaldehyde fixation was done according to (Hagan and Hyams 1988): 30% (w/v) paraformaldehyde was freshly prepared in PEM (1mM EGTA, 1mM $MgSO_4$, 100mM PIPES pH 6.9). The solution was incubated at 65°C for 5 mins, NaOH to a final concentration of 80mM was then added and the solution was further incubated at 65°C till almost all of the solid was dissolved. The solution was cooled on ice to room temperature, and then centrifuged at 4400g to pellet the undissolved Formaldehyde. One volume of sample was vigorously mixed with 0.125 volume of 30% paraformaldehyde for 30-60 sec. 50% (v/v) glutaraldehyde was added to get final concentration 0.2% (v/v). The mixture was incubated on the shaker for 30 – 90 mins. The fixed sample was washed with PEM for three times, and resuspended in 0.5mg/ml zymolase (dissolved in PEMS: PEM + 1M sorbitol) and incubated at 37°C for 70 mins to digest the cell wall. The sample was then resuspended in 0.1% triton X100 (in PEMS) to remove the membrane. The sample was washed with PEM for three times followed by 5 mins incubation in fresh sodium borohydride solution to quench the unreacted glutaraldehyde. The sample was then washed with PEM

twice and resuspended in PEMBAL (0.1M L-lysine hydrochloride, 1% globulin free bovine serum albumin, 0.1% sodium azide in PEM) and incubated for 30 mins.

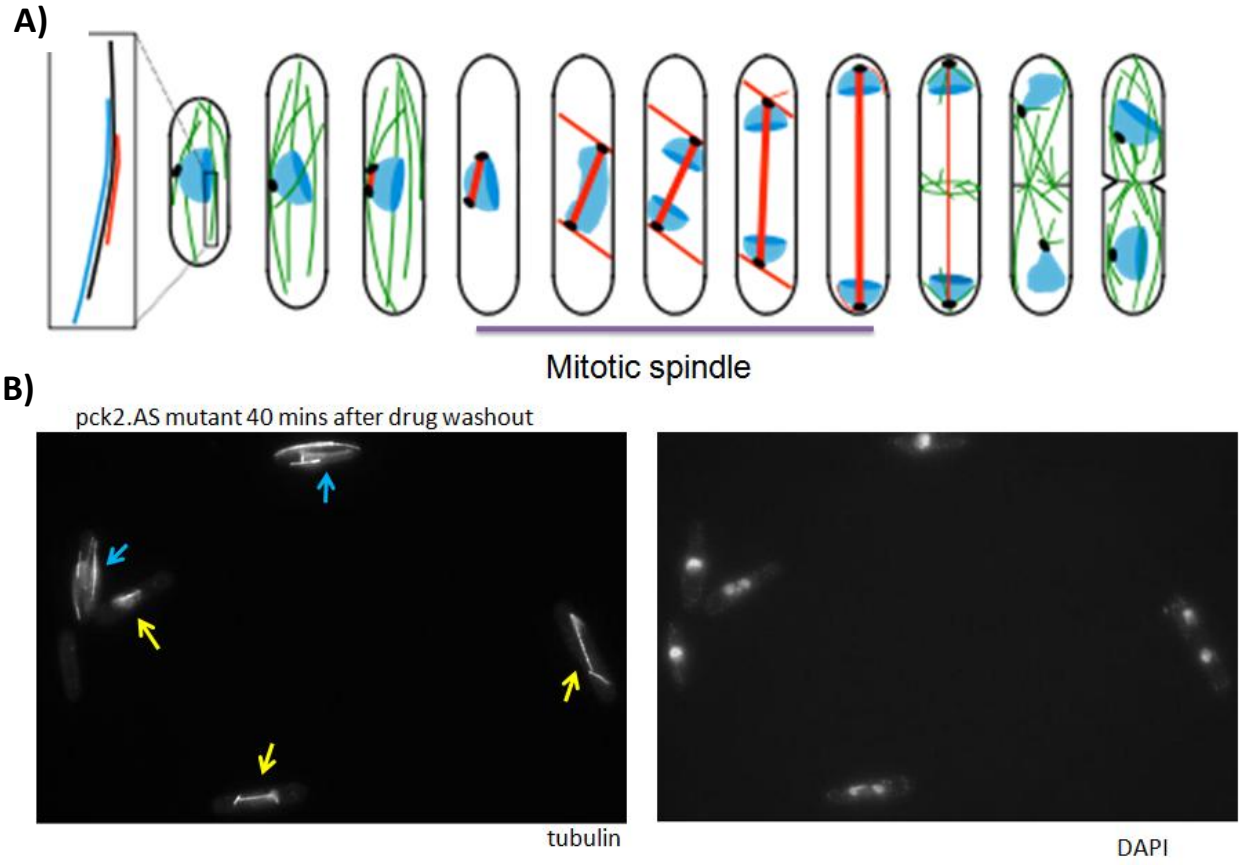


Figure 13 mitotic spindle were used as marker to measure the mitotic index of a sample. A) A illustrating the distribution of microtubule through a cell cycle (Hagan 1998) (this figure is given by Prof Iain Hagan with permission to be used in my thesis). **B)** Immunostaining images illustrating how mitotic cells (yellow arrows) can be distinguished from interphase cells (blue arrows). Each M phase cell has a single mitotic spindle across the dividing nucleus in the middle of the cell. In contrast, each interphase cell has several microtubules; often lay along the periphery of the cell, and those microtubules end on the tips of the cells

The primary mouse antibody, TAT-1 was used for α-tubulin staining at a dilution of 1:80 in PEMBAL. Secondary FITC anti-mouse antibody (Sigma Aldrich) was used at a dilution of 1:100 in PEMBAL, DNA was stained with 0.2µg/ml DAPI. Mounting media contains 1% paraphenylene diamine with glycerol. Slides were viewed with an inverted Zeiss axioplan two system with a coolsnap camera (Photometrics) driven by Meta- morph software (Universal Imaging).

S.pombe has characteristic patterns of microtubule distribution during mitosis, which can be distinguished from microtubule of interphase cells (illustrated in the figure 13). At least 200 cells were

counted for each time point, and the mitotic indexes were calculated by dividing the number of M-phase cells by total number of cells counted.

DAPI/Calcofluor-Staining and Cell length measurement

The strain of interest was grown in EMM2 liquid media for at least 18 hours to reach the early log phase (1-1.5×10^6 cells/ml). 900µl of this culture were fixed by mixing with 100µl of 37% formalin and incubated on ice for 10 mins. The fixed cells were then washed three times with PBS and resuspended in 40µl PBS with 1% of NaN_3. The sample was mounted with fresh mounting media (3.5µl saturated calcofluor, 40µl water, 10µl of 10mg/ml paraphenylene diamine and 38µl 100% glycerol) that stains the septa and cell wall. Slides were viewed (immediately after mounting) with an inverted Zeiss axioplan two system with a coolsnap camera (Photometrics) driven by Meta morph (Universal Imaging).

Cells with stained septa that were yet to initiate the contractions of actomyocin ring were counted as dividing cells. The length from one cell tip to another was measured by imageJ. At least 50 cells were measured for each sample to give estimation of the average cell length in the population.

Results

1. Pck1 and Pck2 contain two HR1 and two C1 domains in its N-terminal regulatory region.

Bioinformatics not only allows us to link the function of an evolutionally conserved protein of interest in different species, but also helps us to predict the functions of a protein from the information available on the characterised homologues in the same or other species. Protein domains are functional and structural units on a protein molecule. The primary sequence of protein domains can often reveal functions and means of control of the protein of interest.

The primary sequences of Pck1 and Pck2 were aligned with 11,912 families within the Pfam database for searching the highly conserved sequences and patterns that define different domains. The alignment results are summarised in figure 14: both Pck1 and Pck2 contain a highly conserved C-terminal kinase domain, and much less conserved N-terminal regulatory regions. Both have two HR1 domains followed by two C1 domains in their N-terminal regulatory region. Both also contain conserved small motifs between the HR1 domains and C1 domains; however Pfam did not define this region as being similar to any previously characterised domains in its database. It has been suggested that this region contains a Pseudo-substrate site in Pck2 (Sayers, Katayama et al. 2000).

The HR1 domains of Pck1 and Pck2 were aligned with 445 HR1 domains from different molecules within the Pfam database. The primary sequences of HR1 from 7 species are presented here (figure 15), 81% HR1 sequences in the database contain one $\psi–x_3–\psi–x_2–E–x_5–G$ motif and 97% contain one $\psi–x_2–L–x_3–\psi$ motif (here x can be any amino acid and ψ is a hydrophobic residue, most frequently Leucine or Isoleucine). These two motifs are likely to play functional or structural roles. 2.2Å crystal structure of human RhoA bound to HR1a domain of PKN1 has been reported (Maesaki, Ihara et al. 1999). The HR1 domain folds into an antiparallel coiled-coil (known as ACC finger); a number of interhelical interactions hold the two long α helices together: E45 of the $\psi–x_3–\psi–x_2–E–x_5–G$ motif forms an interhelical hydrogen bond with S81; and hydrophobic residues such as L83, L87 and L91 of $\psi–x_2–L–x_3–\psi$ motif form an interhelical hydrophobic core. Interestingly, two contacts were observed between RhoA and HR1a (figure 16). The surface of the ACC finger is amphipathic. In contact 1, the residues on the hydrophilic surface of the ACC finger forms hydrogen bonds with the acidic/basic residues of RhoA. In contact 2, the residues on the hydrophobic surface of the ACC finger interact with different residues on RhoA. Figure 16 show that although the key residues on both the AAC finger and RhoA for contact

1. Pck1 2. Pck2 *in S.pombe*

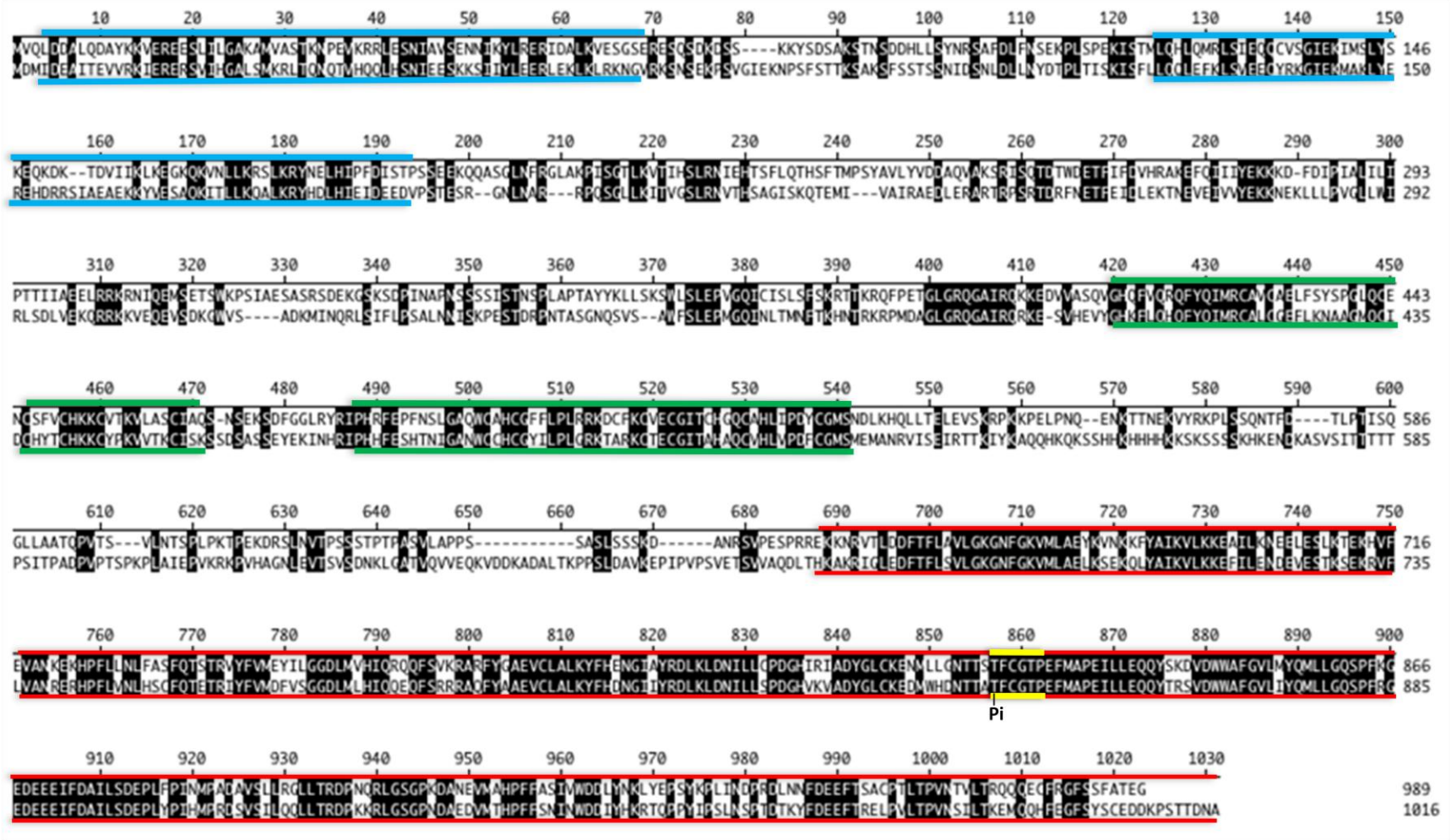


Figure 14 Domain composition of protein kinase C *S.pombe.* both isotypes contain a highly conserved N-terminal kinase domain (red), two HR1 domains (blue), and two C1 domains (green). A phosphorylation site was found on the activation loop (yellow)within the kinase domain (Sayers, Katayama et al. 2000).

A)

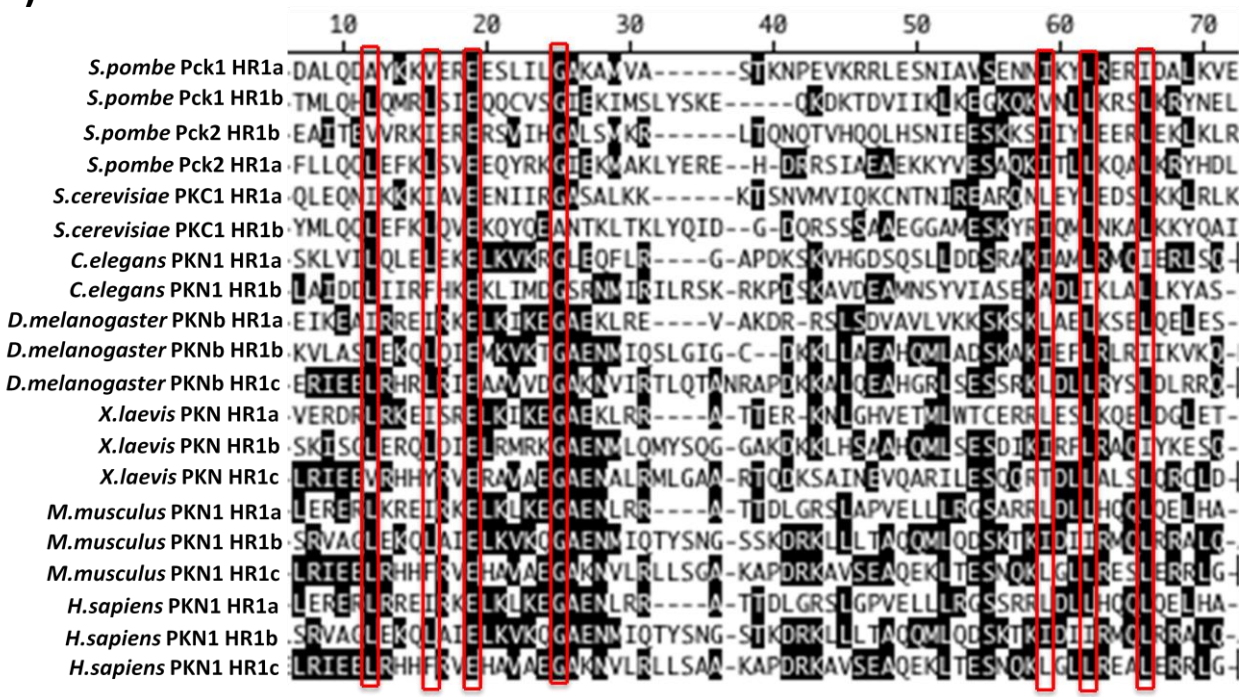


Figure 15 Alignments of HR1 domains all HR1 domains have two highly conserved motifs (highlighted in red box).

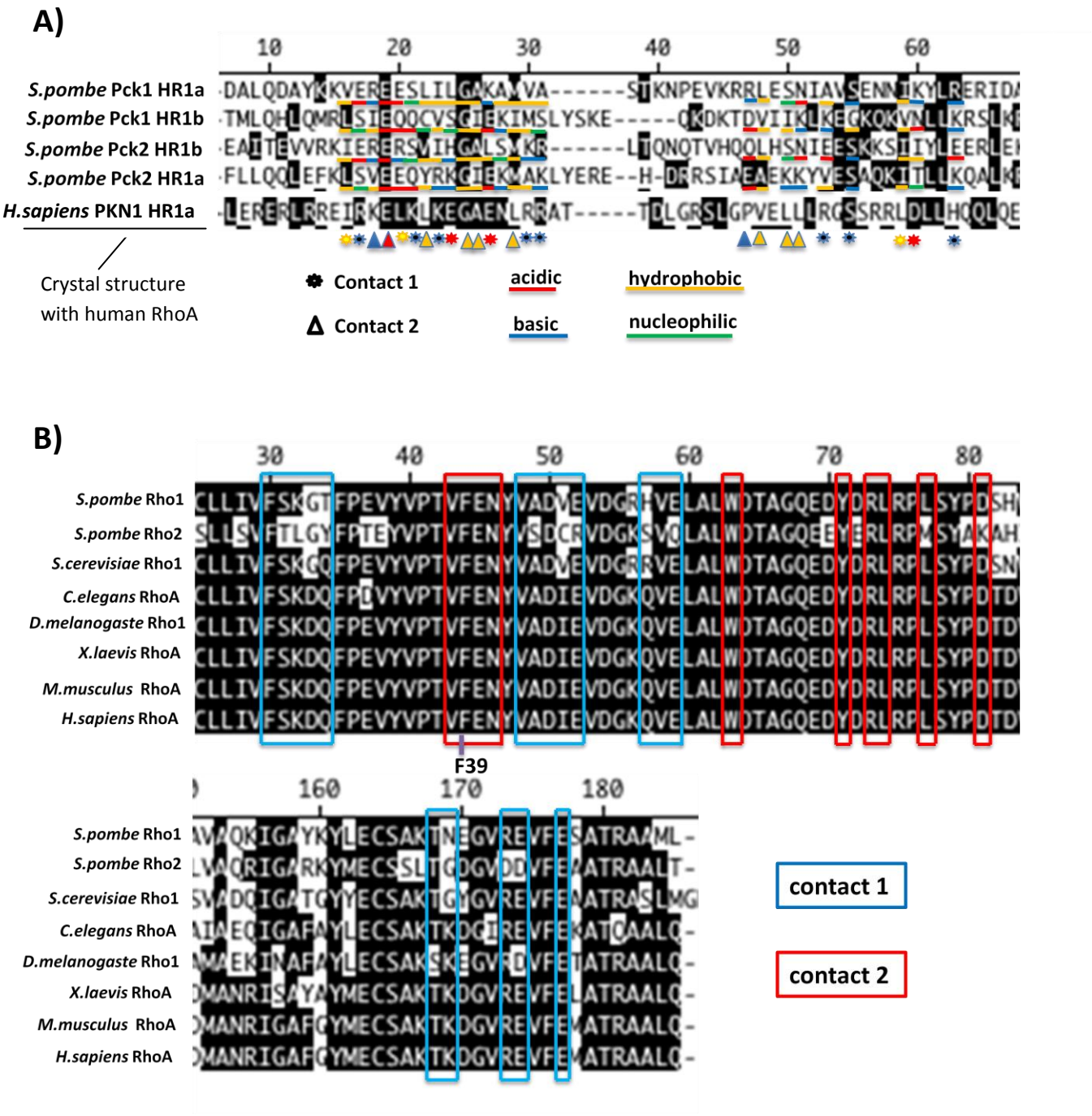


Figure 16 Alignments of HR1 domains and their binding sites on RhoA crystal structure showed human PKN1 HR1a domain can form two different types interaction with RhoA (Maesaki, Ihara et al. 1999). In contact 1, acidic and basic residues in PKN1 HR1a (marked with balls in **A**) form a network of hydrogen bonds with key residues in RhoA (highlighted in blue box in **B**). In contact 2, hydrophobic residues in PKN1 HR1a (marked with triangles in **A**) form hydrophobic interaction with key residues in RhoA (highlighted in red box in **B**).

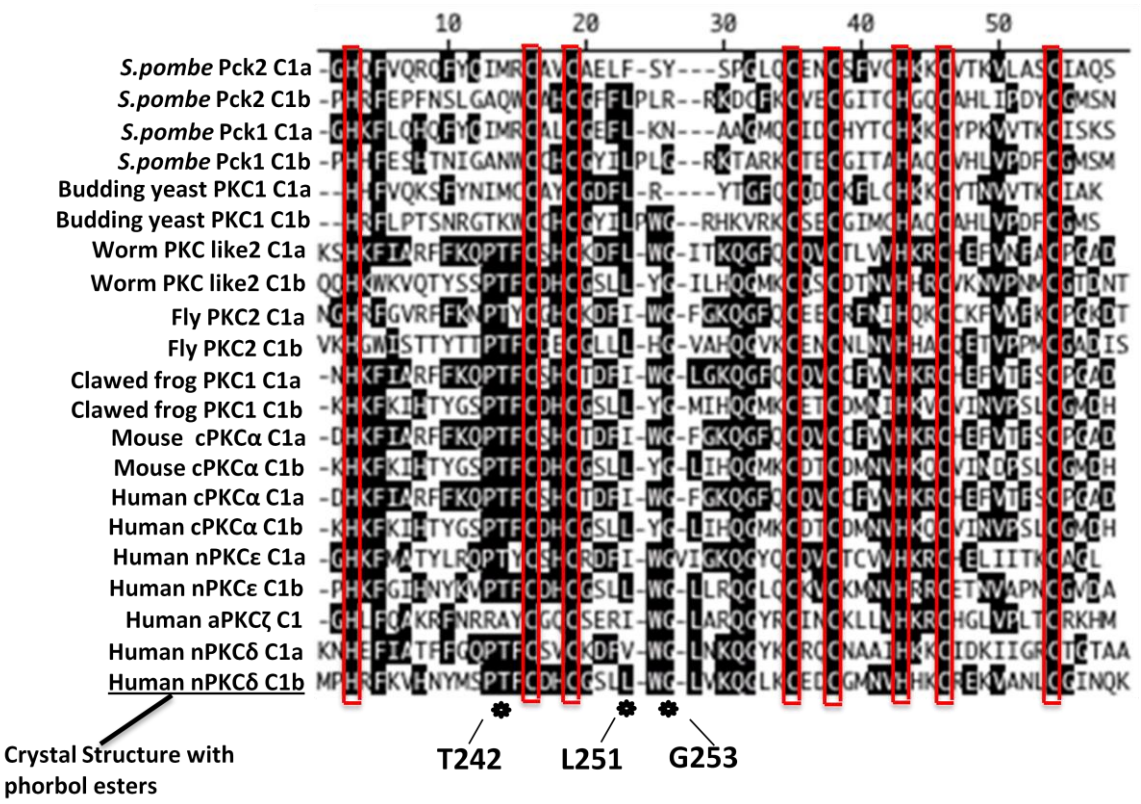


Figure 17 Alignments of C1 domains all the C1 domains have a cysteine-rich motif (highlighted in red box). Crystal structure showed the ligand forms a hydrogen network with main-chain carbonyl and amide of L251, G253, and T242 of human PKCδ (marked with balls) (Zhang, Kazanietz et al. 1995)

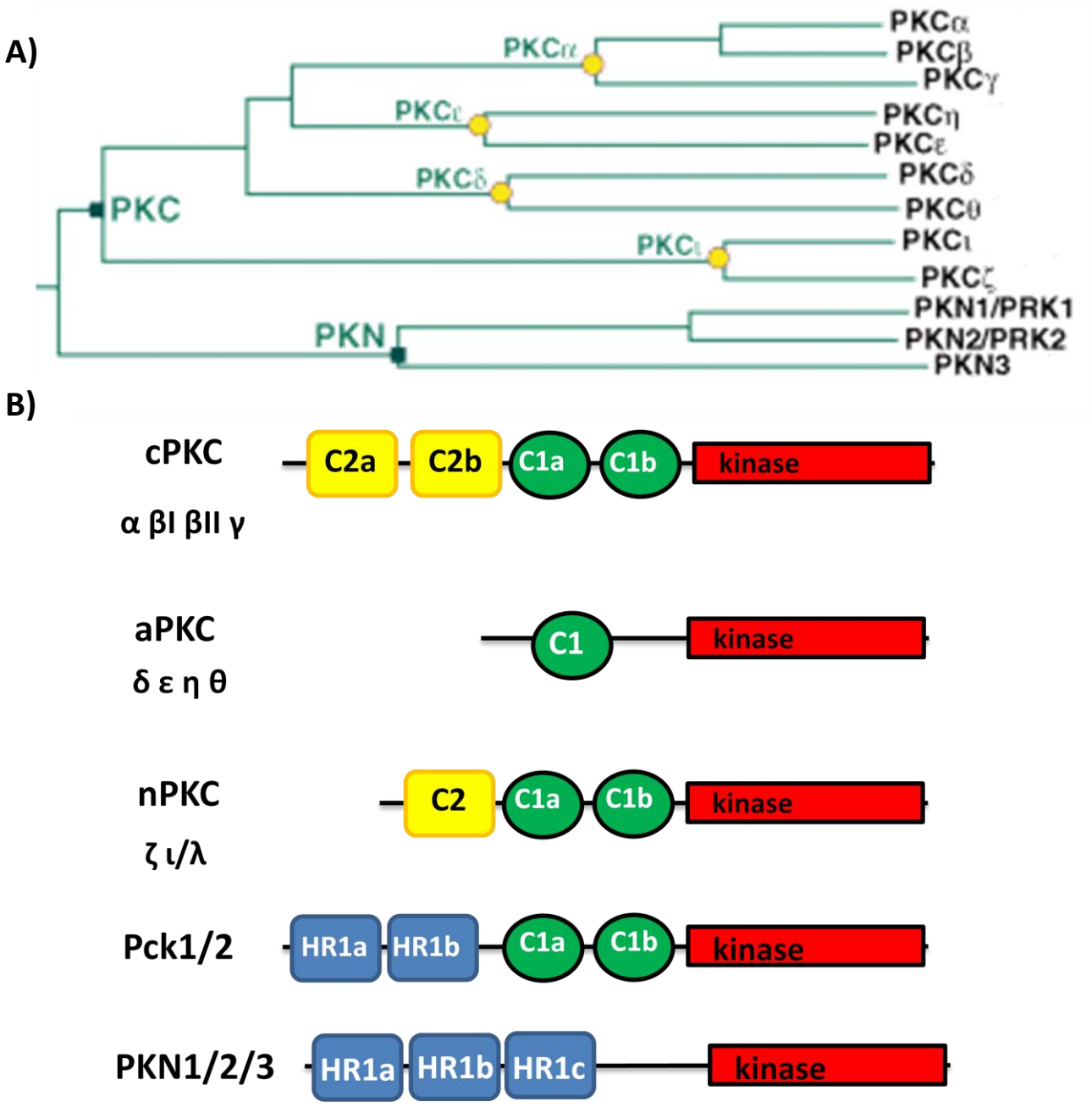


Figure 18 Pck1 and Pck2 are the homologues of protein kinase C-related kinase PRK. A) PKCs and PKN are descended from a common ancestor (Hanks and Hunter 1995). **B)** Like PKNs Both Pck1 and Pck2 have HR domains that bind to Rho GTPase. Pck1 and Pck2 also have two C1 domains with unknown function.

1 are well conserved in all the HR1a in PKNs, many are not conserved in *S.pombe*, whereas the hydrophobicity of many key residues around the ψ–x_3–ψ–x_2–E–x_5–G for contact 2 are conserved in PKN1/PKC (figure 16A). In RhoA/Rho1, these key residues for contact 2 are also better conserved than the key residues for contact 1 (figure 16B). Furthermore, RhoA mutants F39A and F39V impair the binding between RhoA and PKN1, and F39L (both Phe and Leu have a large hydrophobic side chains) has little effect on the binding (Sahai, Alberts et al. 1998), F39 is involved in contact 2 (figure 16B). Therefore, it is very likely that HR1a binds to RhoA/Rho1 through hydrophobic interactions. The Glu of the ψ–x_3–ψ–x_2–E–x_5–G motif is involved in both the interhelical interaction and contact 2, and it is the most conserved residue (98%) in HR1 domains in the Pfam database. It would be interesting to find out whether or not mutation of this residue can abolish the function of the HR1 domain in Pck1 and Pck2. Mammalian PKN1 kinase has three HR1 domains in its N-terminal regulatory region; they each have different affinities for the small GTPase RhoA: HR1a only binds to GTP- bound forms of RhoA whereas, HR1b binds both GTP- and GDP- bound forms of RhoA; HR1c does not bind to RhoA (Flynn, Mellor et al. 1998). It has also been demonstrated that the amino-terminal HR1 domains of Pck2 interact with the small GTPase Rho1 in *vivo* (Sayers, Katayama et al. 2000). Figure 15 shows that although both HR1a and HR1b in Pck1 and Pck2 contain the two highly conserved motifs described above, the sequences are not well conserved between HR1a and HR1b. this indicates different affinities for Rho1. Experiments are required to demonstrate whether or not the HR1 domains of Pck1 and Pck2 have a similar binding mechanism for Rho1 as the HR1 domains of PKN. It has been established for the binding of Rho1 by HR1 domains appears to have two functions 1) it controls the stability and kinase activity of Pck2. In Rho1-expressing (on pREP vector) cells, the highly conserved threonine in the activation loop (figure 14) of the kinase domain becomes phosphorylated (Sayers, Katayama et al. 2000). 2) It promotes co-localisation of Pck1 and Pck2 with Rho1 at the septum of dividing cells, where Rho1 is localised (Nakano, Arai et al. 1996)

45 out of 46 C1 domains in the Pfam database, Including Pck1 and Pck2, contain tandem repeats of a cysteine-rich motif that has the pattern: H-X_{12}-C-X_2-C-X_{11}-C-X_2-C-X_4-H-X_2-C-X_7-C, this suggest both the C1 domains in Pck1 and Pck2 can potentially bind to Zn^{2+} ions and fold into a similar 3D structure as the C1 domains found in other species (Hubbard, Bishop et al. 1991). In mammalian cells, it has been demonstrated that C1 domain of protein kinase C interacts with the secondary messenger DAG (diacylglycerol) or tumor-promoting phorbol esters (the interactions with those ligands are thought to be stereochemically equivalent (Rando and Kishi 1992), and binding of the ligand activates protein kinase C (Ono, Fujii et al. 1989)). The crystal structure of the C1 domain of PKCδ in a complex with

phorbol 13-acetate showed that the ligand forms a hydrogen bond network with the main-chain carbonyl and amide groups of L251, G253, and T242 (Zhang, Kazanietz et al. 1995). T242 is not conserved in yeasts, L251 is conserved and G is only conserved in Pck1 C1b (figure 17). However, the C1 domains uses its back bone for phorbol ester binding, so mutations that only alter the side chains of these residues might not affect the binding of the ligand. It is still unknown if C1 in Pck1 and Pck2 can bind to DAG. In several published papers (Toda, Shimanuki et al. 1993; Sayers, Katayama et al. 2000; Perez and Calonge 2002; Perez and Rincon 2010), Pck1 and Pck2 were thought to have only one C1 domain that resembles the non functional single C1 domain found in atypical PKC. However, here I show that Pck1 and Pck2 contain two C1 domains, which is reminiscent of both novel and conventional PKCs. The functions of the C1 domains in the *S.pombe* isoforms remain to be established. There are only three C1 containing proteins within *S.pombe* genome (GeneDB database) Pck1, Pck2 and Bzz1. Bzz1 is not a kinase and is uncharacterised protein, but its homologue in budding yeast regulates actin polymerization (Soulard, Lechler et al. 2002). Although there is no direct evidence that Pck1 and Pck2 is activated by DAG in *S.pombe*, and their budding yeast homologue PKC1 is insensitive to DAG *in vitro* (Antonsson, Montessuit et al. 1994), it has been proposed that Pck1 and Pck2 might be involved in DAG mediated lioapoptosis-a form of programmed cell death in yeast (Low, Shui et al. 2008). One possible way to look at the function of the C1 domains of Pck1 and Pck2 would be to mutate one of the residues described above that interacts with the ligand into a proline; because within an amino acid chain, proline has no hydrogen on its main-chain amide, and so cannot act as hydrogen donor. Alternatively, one or more of the Cys or His residues in the cysteine-rich motif near the functional residues could be mutated to abolish their Zn^{2+} binding ability so that the binding site for the ligand could be disrupted.

Neither Pck1 nor Pck2 have a C2 domain, and so are insensitive to Ca^{2+} ion themselves.

2. Inhibition of Pck2 delays mitotic commitment

One of the great advantages of using *S.pombe* to study cell division is that, under appropriate conditions, cells continuously grow via tip extension; and cell length increases significantly in G2 phase (Mitchison and Nurse 1985). Mutants such as *cdr2::kanMX6* that have delayed mitotic entry and stay longer in G2 are longer in length than wild type cells; whereas cells containing mutations such as *wee1::ura4*$^{+}$ that advance cells into mitosis are short in length. Therefore, cell length can be used as an indication of the duration of G2 phase.

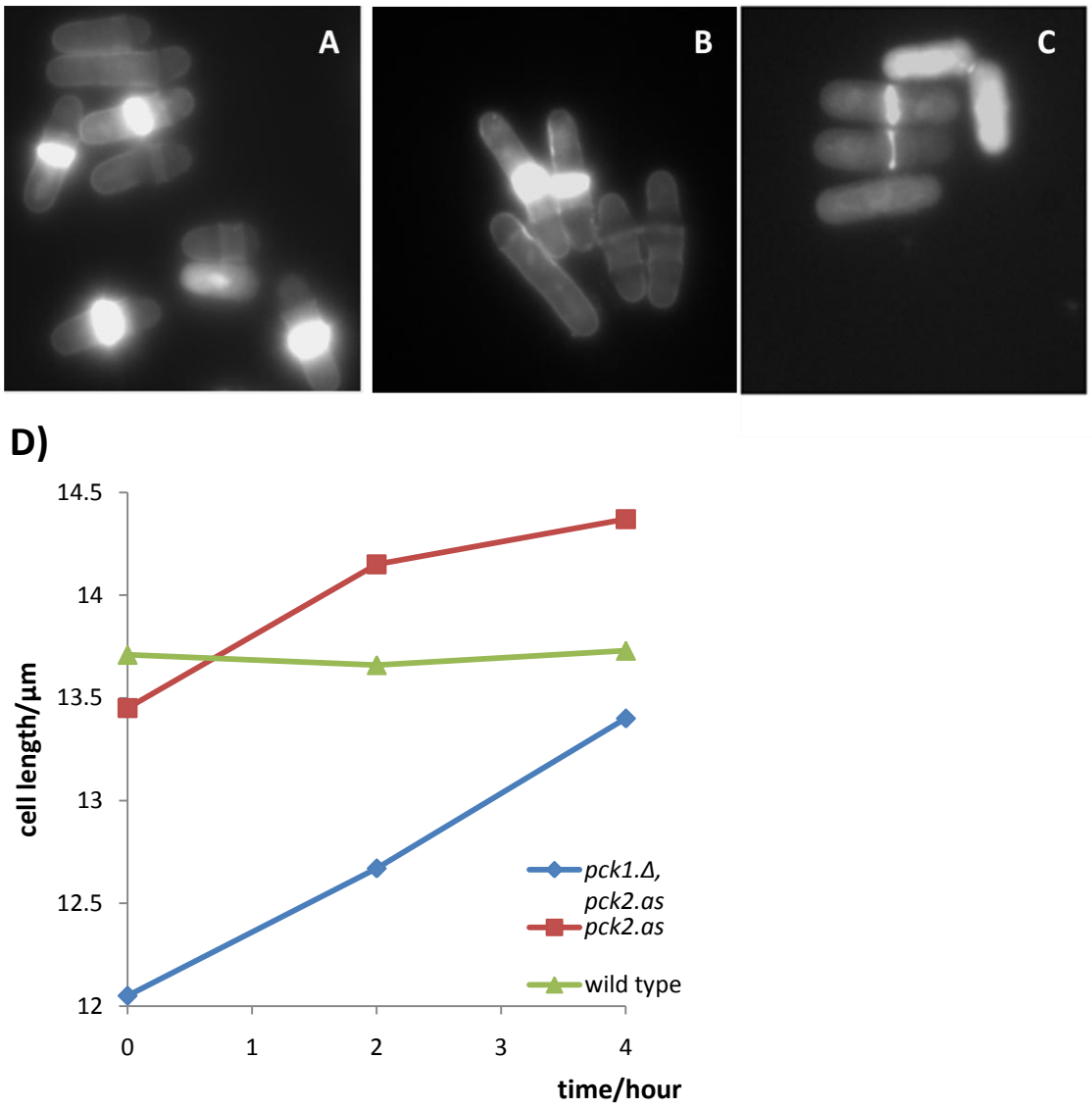


Figure 19 Calcofluor-Staining of early log phase cells in EMM2 liquid media at 25°C A) *pck1::ura4*$^{+}$ *pck2.as* without addition of the ATP analogue **B)** 4 hours after adding the ATP analogue to *pck1::ura4*$^{+}$ *pck2.as* cells, cells here are longer than the cells in B) and C) **C)** 4 hours after adding the ATP analogue to wild type cells. **D)** Quantitative cell length measurement. All cell lengths were measured via the condition and procedure described in Materials and Methods. Cells were grown in EMM2 liquid media for at least 18 hours to reach the early log phase, 50 cells were measured at each time point.

Pck2 might directly or indirectly signal MPF through protein phosphorylation. I asked whether or not inhibition of Pck2 kinase activity can delay in mitotic commitment. Early log phase *pck2.as* cells were treated with 20μM 3MBPP1. Cell lengths were measured at 0, 2 and 4 hours (figure 19) after analogue addition. Cell size at division increased in both *pck2.as* and *pck1::ura4*$^{+}$ *pck2.as* cells after adding the analogue to the culture; this indicates that inhibition of Pck2 kinase activity delays mitotic entry.

3. Pck2 activity promotes mitotic entry at 25^{o}C.

The Shokat washout approach (see Materials and Methods) was used to test whether the kinase activity of Pck2 promotes mitotic entry. In the presence of 3MBPP1, the kinase activity of Pck2.as is thought to be dramatically reduced (Gregan, Zhang et al. 2007), and the kinase activity of Pck2.as is thought to be restored by removal of 3MBPP1. At 25^{o}C, a burst of mitosis was observed 40 mins after removal of 3MBPP1 from *pck2.as* cells culture (figure 20); this indicates that Pck2 kinase activity is able to promote mitotic entry at 25^{o}C. The experiment was then repeated in a *pck1::ura4*$^{+}$ background. A stronger burst of mitosis was observed (figure 20A green), suggesting that Pck1 might also regulate mitotic entry, possibly in an additive manner with Pck2.

Interestingly, at 36^{o}C, the mitotic index first decreased for 40 mins after washing out the analogue, followed by a burst of mitosis at about 120 mins (figure 20A purple). This suggested that Pck2 kinase activity might inhibit mitotic entry at 36^{o}C.

4. Pck2 activity promotes mitotic entry via Wee1, not Cdc25.

The burst of mitosis in *pck2.as* mutants in the Shokat washout approach is specifically raised from the manipulation of Pck2 kinase activity that is able to promote mitotic entry. Having established this *pck2*-mitotic control phenotype (a burst of mitosis in Shokat washout approach), candidate genes can be tested for epstatic interactions with *pck2*$^{+}$: if a candidate gene is up- or down-stream of *pck2*$^{+}$ in its mitotic regulation pathway, deletion of this candidate gene will abolish the *pck2*-mitotic control phenotype as the pathway would have been blocked. On the other hand, if the candidate gene is not involved in the Pck2 mitotic regulation pathway, deletion of this candidate gene would have no impact

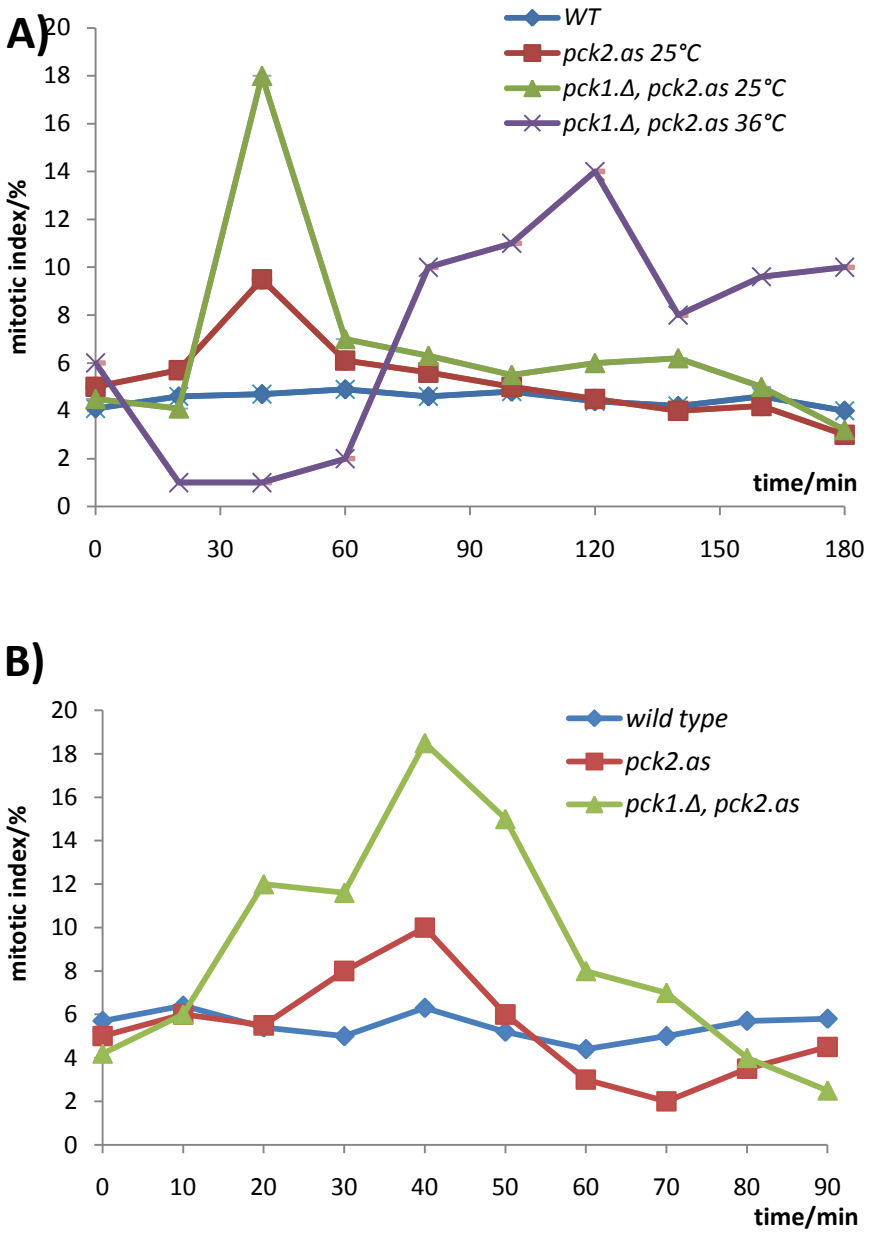


Figure 20 Pck2 kinase activity promotes mitotic entry at 25°C and inhibits mitotic entry at 36°C in EMM2. Early log phase cells were treated with 20µM 3MBPP1 for 5 hours, the analogue was then removed by filtration. The mitotic indexes (percentage of cells with mitotic microtubule) of the samples from each time points were measured. **A)** Samples were taken every 20mins **B)** At 25°C, Samples were taken every 10 mins to give more accurate measurement of the changes in mitotic indexes.

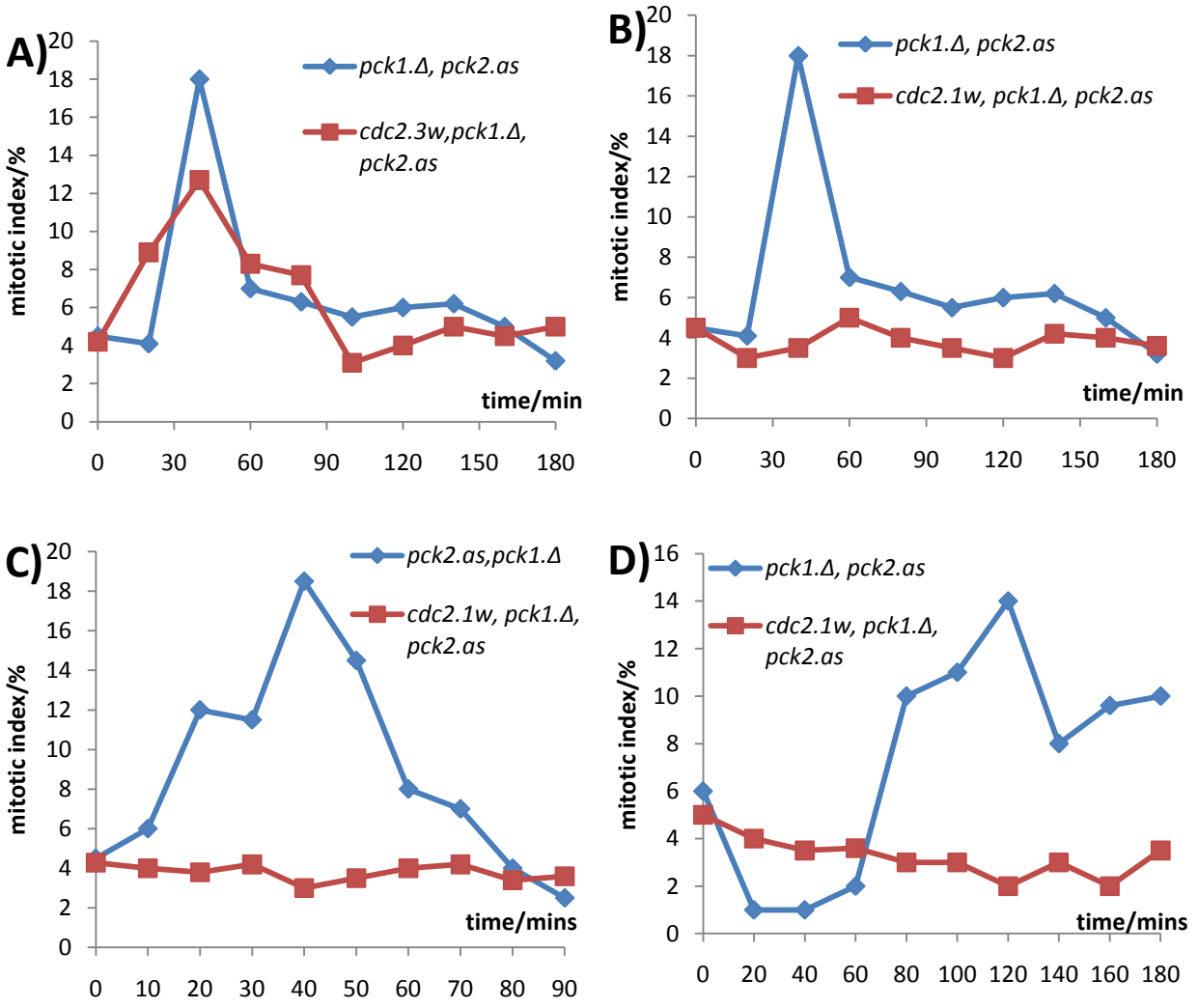


Figure 21 Pck2 regulates mitotic entry via Wee1 but not Cdc25. The indicated mutant cells were grown and manipulated by analogue addition and removal as described in figure 20. **A-C)** experiments were done at 25°C; while **D)** experiment was done at 36°C. **A, B and D)** Samples were taken every 20mins **C)** Samples were taken every 10mins.

on the *pck2*-mitotic control phenotype. Because Pck1 and Pck2 might have a redundant roles in mitotic commitment control and *pck1::ura4*$^{+}$ *pck2.as* double mutant has a stronger mitotic control phenotype than *pck2.as* single mutant in the Shokat washout approach as shown in (figure 20A). I decided to assess the impact of Pck2 on mitotic commitment, in *pck1::ura4*$^{+}$ background.

MPF is the master regulator of mitotic entry, the activity MPF is regulated by mitotic inhibitor Wee1 and mitotic inducer Cdc25 (Nurse 1990). Most pathways that regulate mitosis act via Wee1 or Cdc25 or both. If Pck2 is a mitotic inducer, it is most likely that the Pck2 mitotic pathway inhibits Wee1 or activates Cdc25 or does both. To test epstatic interactions with *wee1*$^{+}$, *cdc2.1w* was used. *cdc2.1w* is an allele specific *cdc2* mutation that makes Cdc2 largely insensitive to Wee1 (Russell and Nurse 1987). For epstatic interaction tests with *cdc25*, *cdc2.3w* was used. *cdc2.3w* is an allele specific *cdc2* mutation that makes Cdc2 largely insensitive to Cdc25 (Fantes 1979). Figure 21 shows that at 25^{o}C, rendering Cdc2 insensitive to Cdc25 function (*cdc2.3w* mutant) has no major impact on the *pck2*-mitotic control phenotype; whereas there was no induction of mitosis at 25^{o}C and no inhibition at 32^{o}C when Wee1 control of Cdc2 was blocked by the *cdc2.1w* mutation.

5. The Pmk1 pathway does not promote mitotic regulation downstream Pck2

Pck2 kinase activity might regulate mitotic entry through one of several characterised pathways such as the tip extension or the stress response pathways (see introduction). Alternatively, Pck2 might be part of a novel pathway that regulates mitotic entry. The epistatic interaction approaches described above were used to identify members of the Pck2 mitotic regulation pathway.

Pck2 is known to function upstream of the Pmk1 cell integrity pathway. The *pck2*-mitotic control phenotype might therefore be mediated by the Pmk1 stress pathway. However, this is not the case; Figure 22 shows that deletion of *pmk1*$^{+}$ has no major impact on the activity of Pck2 to induce mitosis at 25^{o}C at or delay mitosis at 36^{o}C. However, deletion of *pmk1*$^{+}$ delayed the timing of the burst of mitosis by 20 mins at 25^{o}C and accelerated it by 20 mins at 36^{o}C, suggesting that Pmk1 may influence the timing of Pck2 regulated mitotic entry at both temperatures.

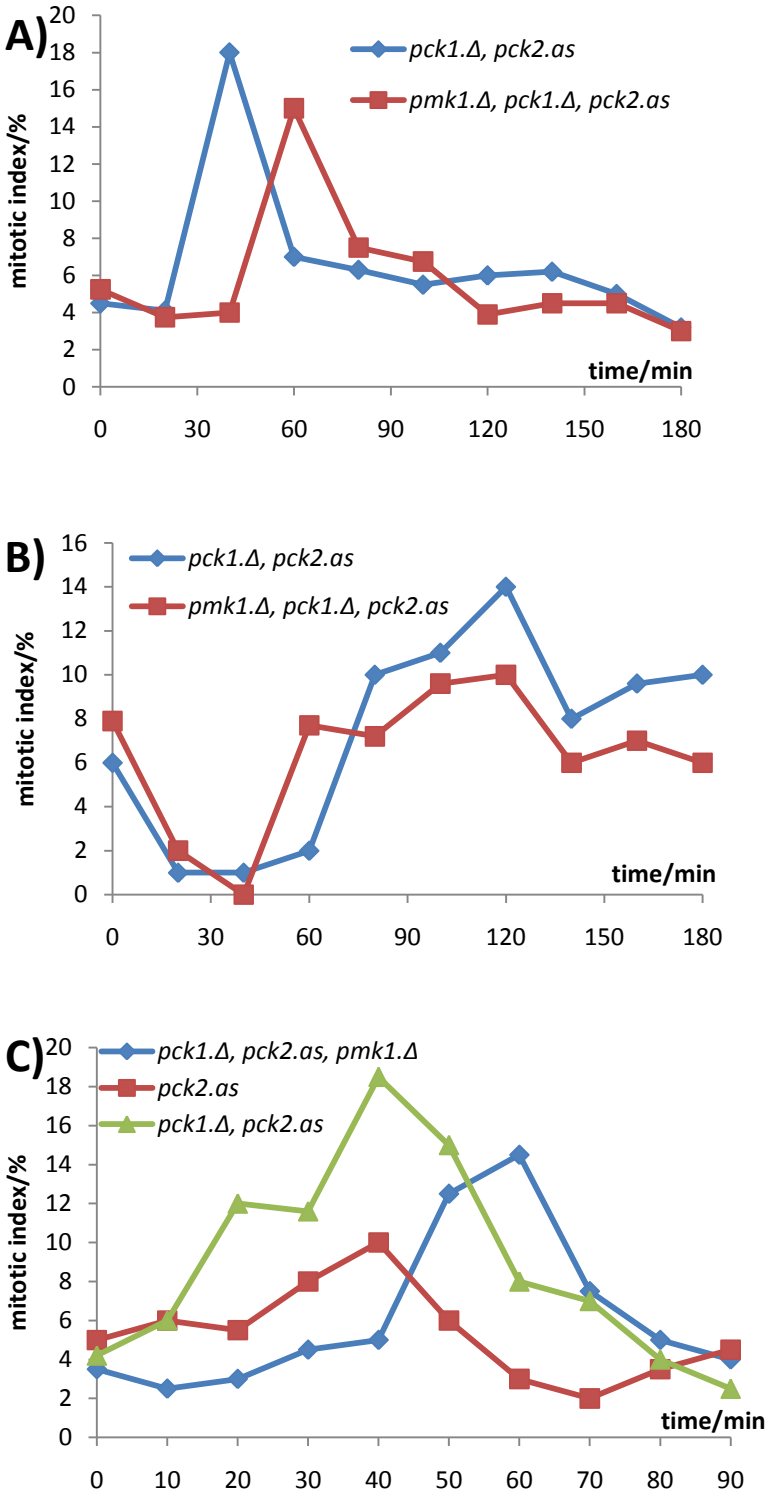
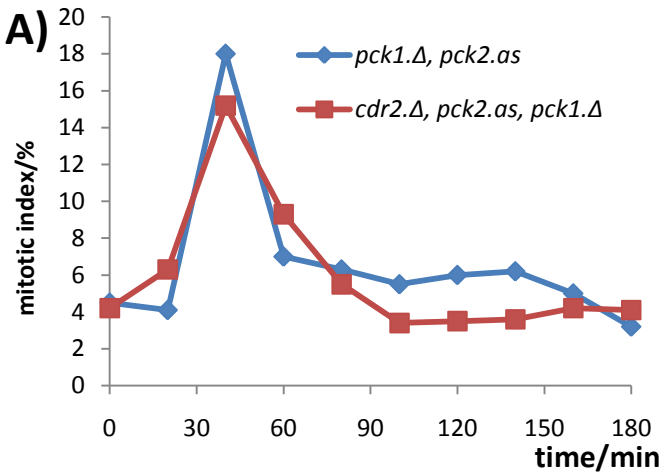
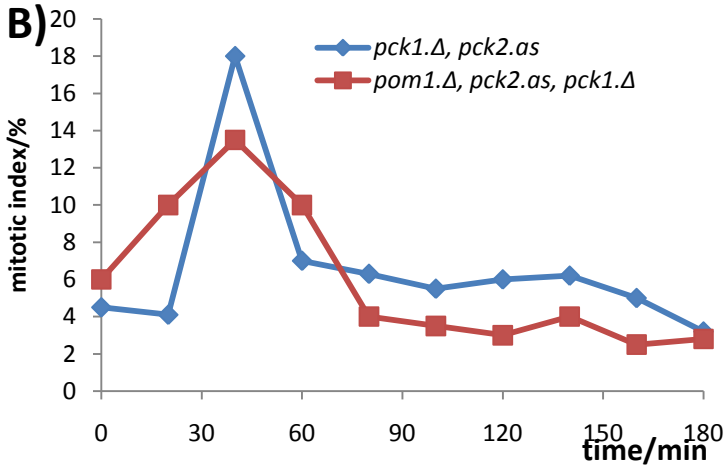


Figure 22 Pmk1 pathway is not directly involved in mitotic regulation of Pck2. The indicated mutant cells were grown and manipulated by analogue addition and removal as described in figure 20. **A and C)** were done at 25°C. **B)** were done at 36°C. **C)** Samples were taken every 10 mins.





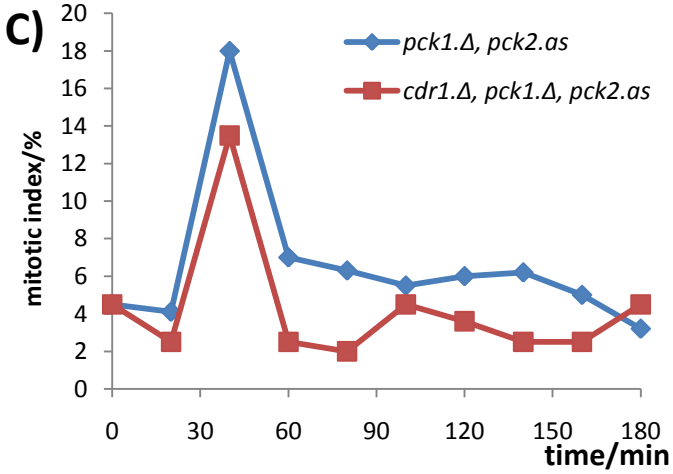


Figure 23 The tip extension pathway is not directly involved in mitotic regulation of Pck2 The indicated mutant cells were grown and manipulated by analogue addition and removal as described in figure 20. **A, B and C)** were done at 25°C.

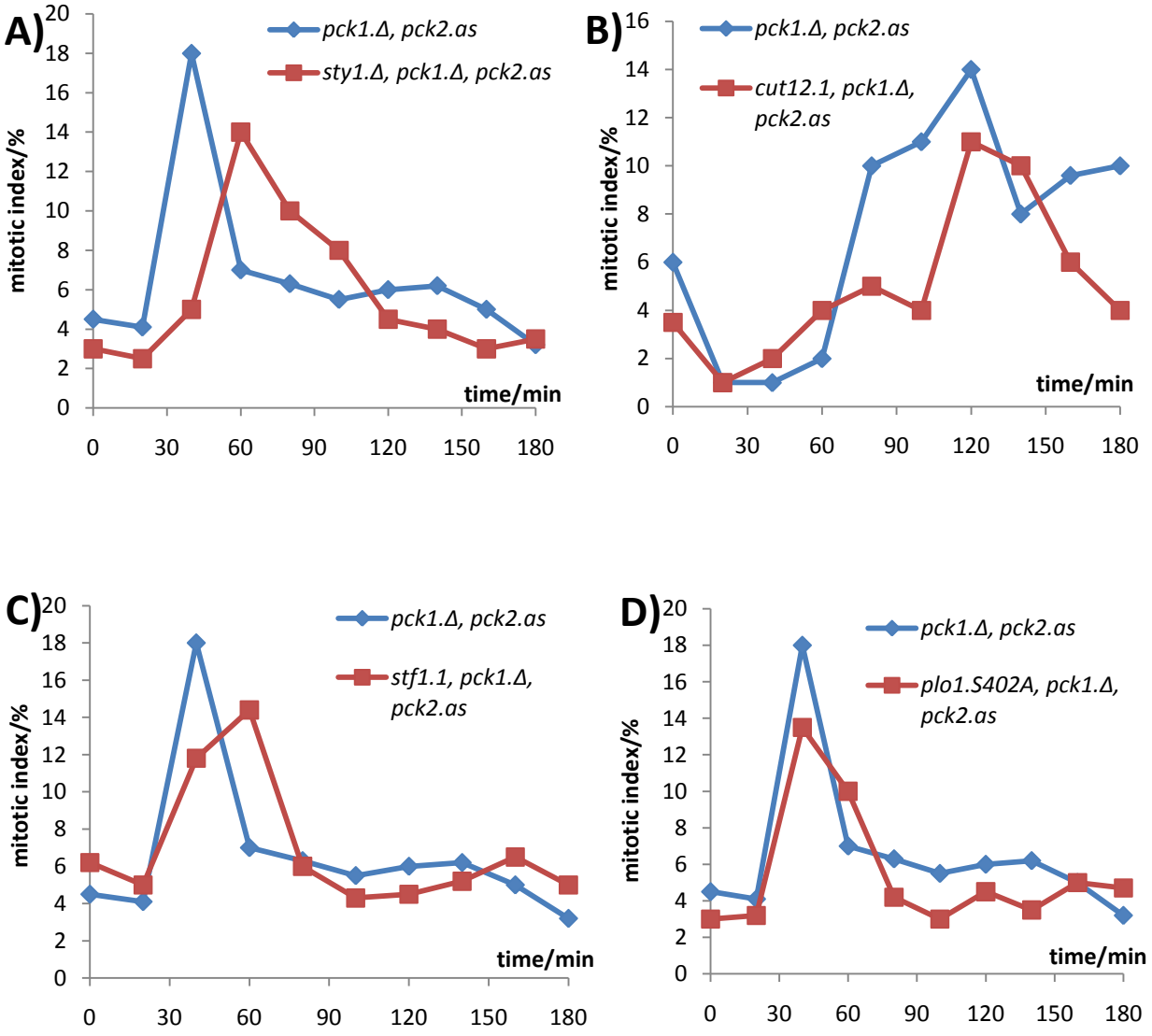


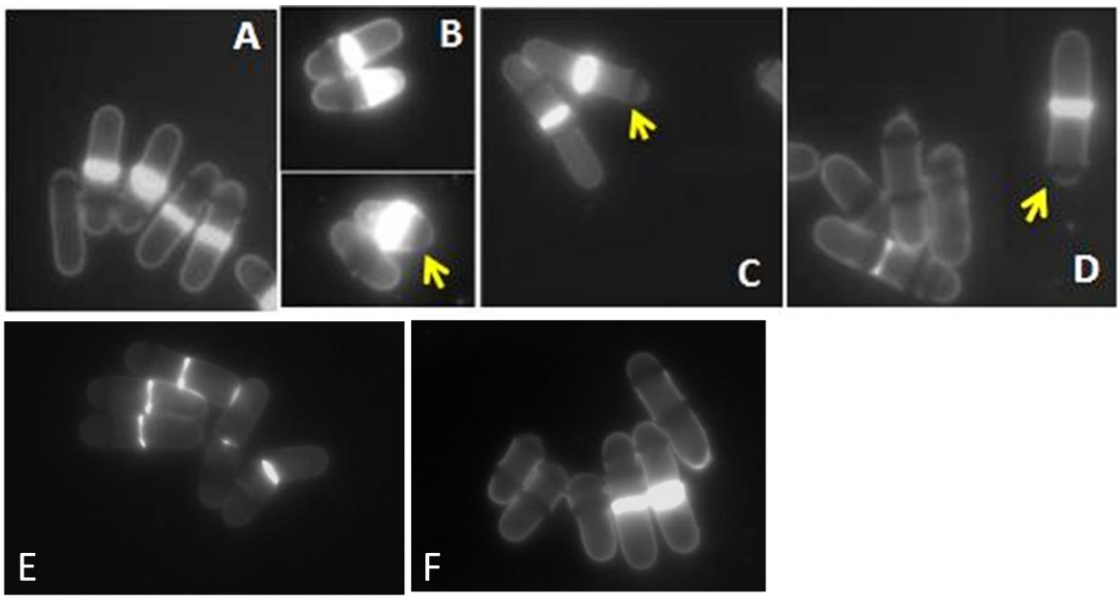
Figure 24 The Sty1- stress pathway is not directly involved in mitotic regulation of Pck2. The indicated mutant cells were grown and manipulated by analogue addition and removal as described in figure 20. **A, C and D)** were done at 25°C. **B)** was done at 36°C.

6. Mitotic regulation of Pck2 at 25^{o}C is independent of the tip extension and stress response pathways.

In *S.pombe* cells, the metabolic rates and the activities of enzymes can be very different at 25^{o}C from 36^{o}C, hence the mitotic regulation is very different at two different temperatures (Mitchison 2003). I decided to focus on the Pck2 mitotic regulation at 25^{o}C for my project.

Figures 19 shows that Pck2 inhibition cause the cells to become longer than the wild type, and Pck2 regulates mitotic commitment in a Wee1 dependent manner. Pom1 also regulates mitotic commitment via Wee1, suggesting that Pck2 may promote mitotic entry through the tip extension pathway. Figure 23 however shows that deletion of the members of the tip extension pathway *cdr1^{+}*, *cdr2^{+}* and *pom1^{+}* have no major impact on *pck2*-mitotic control phenotype. Furthermore, Shokat washout approaches showed that *fin1^{+}* interacts with *pom1^{+}* but not with *pck2^{+}* at the top of the tip extension pathway (Dr. Agnes Grallert unpublished data). Therefore the tip extension pathway is unlikely to be involved in mitotic regulation of Pck2. It has been demonstrated that there is crosstalk between different stress pathways in higher eukaryotes (Tian and Karin 1999). In *S.pombe*, it has been shown that the Pmk1 pathway also promotes phosphorylation of the Sty1 pathway downstream factor Aft1, however it is not known if both phosphorylations are on the same site (Takada, Nishimura et al. 2007). During the Sty1 stress response, phosphorylation Plo1 at S402 is dependent on Sty1 activity. This phosphorylation event promotes recruitment of Plo1 to SPB via Cut12, to promote mitotic entry (Petersen and Hagan 2005). I therefore asked if Pck2 induces mitotic entry through the Sty1 stress pathway. *plo1.S402A* cells was used, in which the Serine at residue 402 is substituted by a unphosphorylatable Alanine, this mutation largely reduces recruitment of Plo1 to SPB (Petersen and Hagan 2005). The *stf1.1* mutant was also used to address whether Pck2 regulated mitotic commitment by controlling the affinity of Plo1 for the spindle pole body, *stf1.1* is gain of function mutation *cut12^{+}* which leads to Plo1 premature association with the SPB, and this inappropriate polar recruitment of Plo1 promotes the activity of Plo1 on the SPB and overrides the requirement for Cdc2 activation by Cdc25 (Mulvihill, Petersen et al. 1999)

Figure 24 shows that neither blocking the Sty1 pathway nor mis-regulating Plo1 SPB recruitment had major effect on the *pck2*-mitotic control phenotype. However, in *stf1.1 pck2.as pck1::ura4^{+}*and *sty1::kanMX6 pck2.as pck1::ura4^{+}* cells, the bursts of mitosis were delayed by 20 mins in comparison with *pck2.as pck1::ura4^{+}* cells, which is similar to the results reported in the experiment in figure 22. Therefore the mechanism by which Pck2 regulates mitotic entry might not go directly through the Sty1–Plo1 pathway, but Sty1 and Cut12 may exert some influence on mitotic regulation by Pck2.



Cell length at 25°C	***day1***	***day2*** in μm
wild type IH5974	13.4±0.9	13.8±1.1
pck1:: ura4⁺.dis from Toda T	14.4±1.2	14.9±1.4
pck2::LEU2⁺.dis from Toda T	12.8±1.7	12.3±2.0
pck2::ura4⁺.del from Bimbo A	15.0±1.3	15.5±1.4
pck2::kanMX6.del	14.2±0.8	14.4±1.1
pck2.as	13.5±1.0	13.7±1.2
pck2.as pck1:: ura4⁺dis	12.2±1.1	12.7±1.4

Figure 25 Calcofluor-Staining of early log phase cells for cell length measurement in EMM2 liquid media at 25°C A) wild type cells **B)** *pck2::LEU2⁺.dis* from Dr. Toda **C)** *pck2::ura4⁺.del* from Dr. Bimbo **D)** *pck2::kanMX6.del.* **E)** *pck2.as* **F)** *pck2.as pck1::ura4⁺ dis.* The yellow arrows point to the non-growing cell tips of monopolar cells (described in text). **The table** in lower panel shows quantitative cell length measurement, cell lengths were measured in the first day, and the cultures were then diluted to give early log phase for repeating cell length measure in the second day. All cell lengths were measured via standard conditions (1×10^6 cells/ml, in EMM2 at 25*°C*) using standard procedure (describe in Materials and Methods).

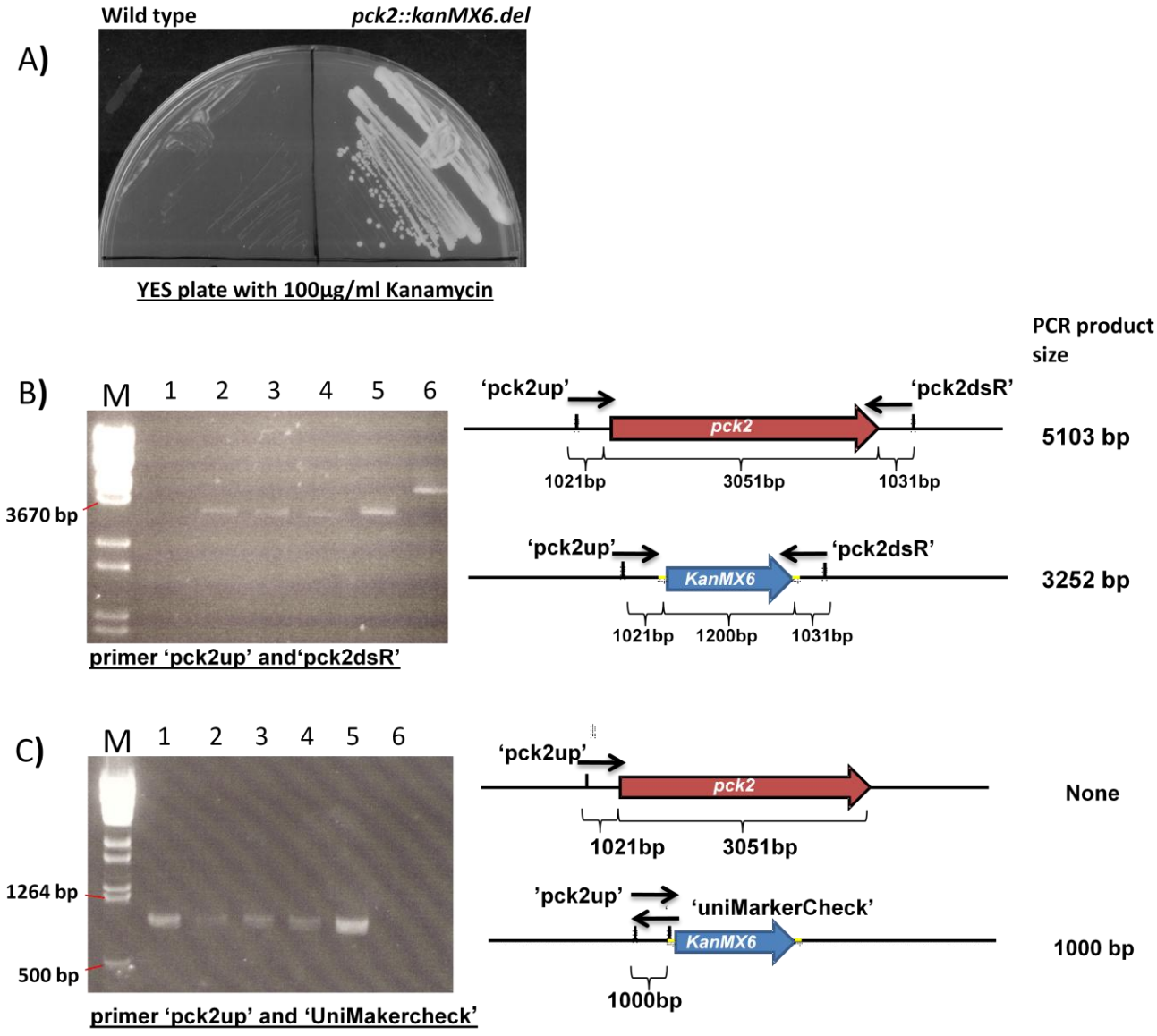


Figure 26. Validation of *pck2::kanMX6.del*. A) Cells have their *pck2*$^{+}$ displaced by *kanMX6* are able to grow on YES plate with kanamycin. Colony PCRs were preformed with two different primer sets (figure11). **B)** primers flank ORF of *pck2*$^{+}$ with colony DNA from *pck2::kanMX6.del* cells (lane 5) and wild type cells (lane 6). **C)** primers against the promoter region of *kanMX6* with colony DNA from *pck2::kanMX6.del* cells (lane 1-5) and wild type cells (lane 6). The short arrows indicate the direction of the primers. The sizes of the genes and distance between the primer binding sites and the ORF of the genes are labeled.

7. Complete deletion of *pck2*$^{+}$

In the original analysis of *pck2*$^{+}$, the gene was disrupted by insertion of the *LEU2*$^{+}$ gene into the ORF (*pck2::LEU2*$^{+}$*.dis*)(Toda, Shimanuki et al. 1993). These cells are shorter than wild type cells at 25^{o}C (figure 25B). Cells with a complete deletion of *pck2*$^{+}$ (*pck2::ura4*$^{+}$*.del*), however, are longer than wild type cells at 25^{o}C (figure 25C). I then deleted *pck2*$^{+}$ by completely replacing the open reading frame with the *kanMX6* maker (described in Materials and Methods). PCR analysis with two sets of primers shown figure 26 was used to confirm correct targeting. The advantage of using drug resistant makers over autotrophy markers such as *ura4*$^{+}$ for gene deletions in *S.pombe* is that the autotrophy markers might cause physiological changes of the cell, because the mutant has to express the autotrophy genes (e.g. *ura4*$^{+}$) for metabolism at different loci and promoter that might affect the regulations of gene expression, drug resistant genes on the other hand are not required for general metabolism. *pck2::kanMX6.del* cells are longer than wild type cells at 25˚C (figure 25D) in minimal medium suggesting that Pck2 might act as a mitotic inducer that positively regulates mitotic commitment. *pck1::ura4*$^{+}$*.dis* cells are also slightly longer than wild type cells, suggesting that Pck1 might also regulate mitotic commitment. In the 25˚C in minimal medium *pck2.as* cell has cell size that is similar as wild type (figure 25E), however *pck2.as pck1::ura4*$^{+}$*.dis* cells are significantly shorter than wild type cells; and they have no morphological defect in Calcofluor-staining (figure 25F). One possible explanation for this phenotype is that the point mutation in the ATP binding site of Pck2.as might alter the kinase activity of Pck2 (Gregan, Zhang et al. 2007); and only in the background of Pck1 disruption, Pck2.as can induce early mitotic commitment.

In the original analysis of *pck2*$^{+}$, *pck2::LEU2*$^{+}$*.dis* was thought to have Calcofluor-staining defects; those cells appeared as white chalk sticks under fluorescence microscope after being treated with Calcofluor (Toda, Shimanuki et al. 1993). Here, I used a much lower concentration of Calcofluor and only added Calcofluor just prior to observation (see Methods and Materials). By using our protocol, *pck2::LEU2*$^{+}$*.dis* and *pck2::kanMX6.del* cells give the characteristic appearance of Calcofluor-staining (figure 25). An interesting observation from the Calcofluor-Staining of some *pck2.del* and *pck2.dis* cells is that the birth scar (the site of previous round of division) is very close to one of the cell tip in many septating cells i.e. there was no growth at the new end after previous round of division as growth should extend the distance between the new end and the birth scar. Thus Pck2 appears to have impact upon new end take off (NETO), i.e. these cells are monopolar, and they only grow at one cell tip rather than at both cell tips as in the wild type cell.

8. *pck2*$^{+}$ mutants have defect in microtubule dynamics

Immunostaining of tubulin allows us to look at the microtubule distribution at different stages of the cell cycle. In wild type cells, the rate of polymerization of interphase microtubules slows after they establish end-on associations with cortex at cell tips (Tran, Marsh et al. 2001). Mutations in genes such as *peg1*$^{+}$ (CLIP170-associated protein) that regulates microtubule dynamics reduces the degree to which contact with the tip slows down microtubule polymerization; such that cells with *peg1*$^{+}$ depletion have very long interphase microtubule bundles that bend near cell tips (Grallert, Beuter et al. 2006). In both *pck2::kanMX6* cells (figure 25 A and B) and *pck1::ura4*$^{+}$ *pck2.as* cells with 5 hours analogue inhibition (figure 25 C and D), some of the interphase cells also have longer microtubule bundles that bend near cell tips. This phenotype suggests that *pck2::kanMX6* and *pck2.as* cells have defects in regulation of microtubule dynamics at cell tips.

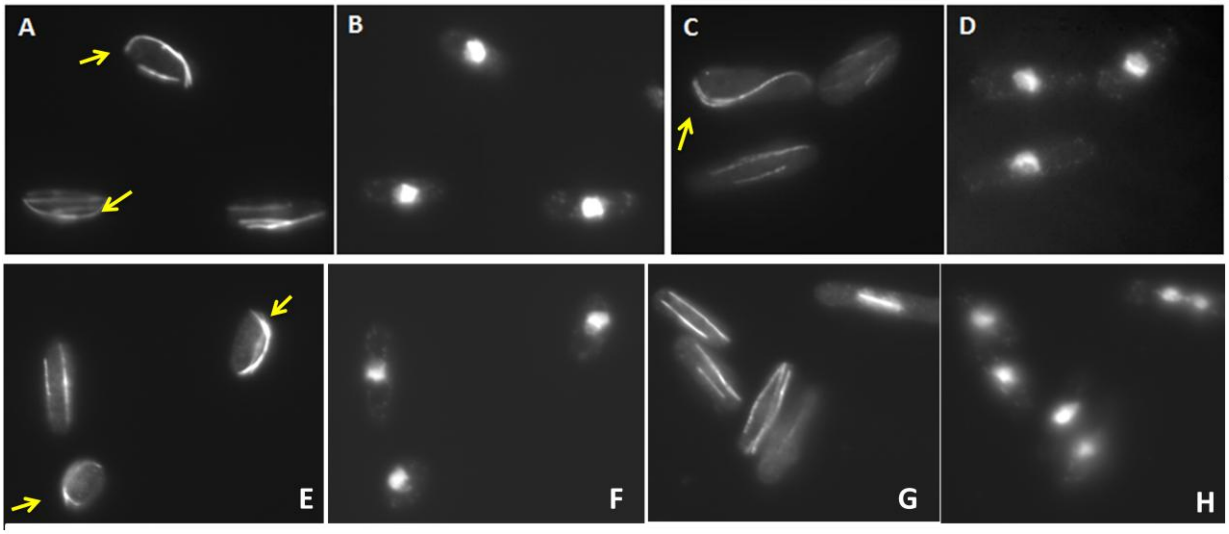


Figure 27 *pck2*$^{+}$ mutants have defect in microtubule dynamics. in the standard condition, at 25^{o}C, early log phase cells of *pck2::kanMX6.del* **A and B)**, *pck2::LEU2*$^{+}$*.dis* **E and F)**, wild type cells **G and H)** and *pck1::ura4*$^{+}$ *pck2.as* after treatment of 20µM 3MBPP1 for 4hours in EMM2 liquid media **C and D)**. Cells were fixed for immunostaining of tubulin by TAT1 antibody **A, C, E and G)**, and DAPI staining of DNA B**, D, F and H)**. The yellow arrows point to the microtubule bundles bend near cell tip.

Discussion and Future Direction

1. Pck1 and Pck2 are the homologues of protein kinase C-related kinase PRK, and the two isotypes in S.pombe have different functions.

By analyzing the primary sequences of Pck1 and Pck2, I tried to address the question:"Which protein kinase C isoforms in other species do Pck1 and Pck2 most resemble?", and identify the domains and the functional and structural motifs within these domains for future molecular analysis.

All members of the protein kinase C family are activated through one or more allosteric inputs, which can be lipids, proteins or both (Roffey, Rosse et al. 2009). Both Pck1 and Pck2 contain two HR1 tandem repeats that bind to the small GTPase Rho1 in other systems. Protein kinase C related kinase PRK (also known as protein kinase N or PKN) in human cells also contains two N-terminal HR1 domains, in this case, these domains bind to RhoA (homologue of Rho1) in an association that stimulates the activity of the kinase (Flynn, Mellor et al. 1998). Therefore, Pck1 and Pck2 are considered to be homologues of PRK1/PRK2 (figure 18). Pck1 and Pck2 also contains two C1 tandem repeats; although there is no direct evidence as to whether Pck1 and Pck2 bind to and are activated by DAG, the structural residues within the C1 domain are highly conserved across different yeast species, suggesting that their significant biological functions may be well conserved. In *S.pombe, plc1*$^{+}$ is thought to encode a putative phosphoinositide phospholipase C that can produce DAG as secondary messenger from inositol lipid (Andoh, Yokoo et al. 1995). Furthermore, in human cells, novel and conventional PKCs have two C1 tandem repeats, and they are activated by DAG (figure 18B) (Newton 2003). Pck1 and Pck2 might also be able to bind to DAG through their C1 tandem repeats; and if so, they could be classified as novel protein kinase C isoforms that are activated by DAG but not Ca^{2+}. However, the biological functions of DAG signaling through C1 domain of Pck1 and Pck2 remain to be explored. Further studies such as mutating the functional residues identified in figure 17 are required to functionally assess the roles of the C1 domains of Pck1 and Pck2. It has been shown that PRK2-depleted Hela S3 cells are delayed in G2/M progression (Schmidt, Durgan et al. 2007). A recent quantitative phosphoproteomics of the kinome across the cell cycle of human cells showed that the phosphorylation of PRK1/PRK2 and PKCε (a novel PKC) by Cdc2 is significantly increased during mitosis (Daub, Olsen et al. 2008), suggesting that these kinases play roles during mitosis. It would be interesting to determine if these phosphorylation controls are also conserved in *S.pombe* and define the function of these phosphorylation events during cell division. PKCs and PKN are descended from a common ancestor (figure 18B) (Hanks and Hunter

1995). This common ancestor has evolved into different isotypes of PKCs and PKNs, which gained different regulatory domains that enable each kinase isoform to bind to different combinations of regulatory factors a such as Ca^{2+} ions. This extends the variety of functions executed by protein kinase C in more complex higher organisms. In *S.pombe*, the common ancestor appears to have evolved in different ways to produce only two isoforms for this branch of protein kinases.

Although Pck1 and Pck2 have a similar domain arrangement, they are unlikely to have entirely redundant functions for a number of reasons: 1) the N-terminal regulatory region of Pck1 and Pck2 exhibit a low percentage of homology (figure 14), so they might be regulated in different ways. Perhaps the affinity for ligands differs. 2) *pck2::ura4$^+$.del* is moderately thermosensitive, whereas *pck1::ura4$^+$* has no temperature sensitivity (Arellano, Valdivieso et al. 1999). 3) *pck2$^+$* overexpression is lethal and cells have very thick cell walls, whereas *pck1$^+$* overexpression has no apparent phenotype (Arellano, Valdivieso et al. 1999). 4) Genetic evidence showed that Pck2 regulates NETO (new end talk off, see introduction) at both 25 and 36°C, whereas Pck1 only regulates NETO at 36°C but not 25°C, indicating that Pck1 and Pck2 regulate NETO by different mechanisms (Koyano, Kume et al. 2010).

The bioinformatics results can greatly help us to study the functions of Pcks in mitotic control. For example the key residues in figure 15 and 17 in the HR1 and C1 domains can be mutated, and then the phenotypes such as cell length, microtubule dynamics and the *pck2*-mitotic control phenotype in the Shokat washout approaches could be investigated to find out the contribution of each domain to Pck2 function. To avoid the complication of studying two functionally different genes together, we decided to focus on *pck2$^+$* first, then look at the function of *pck1$^+$* in mitosis following the generation of a analogue-inhibitable *pck1.as* mutant in the lab in the future.

2. Pck2 is involved in mitotic commitment

Since protein kinase C was first characterised in *S.pombe*, the researches on this kinase have mainly focused on its roles in cell wall synthesis and the cell integrity pathway. However, *pck1.dis pck2.dis* double disruption is lethal (Toda, Shimanuki et al. 1993), whereas deletion of any members in Pmk1 pathway is not lethal; clearly indicating that Pck1 and Pck2 must play essential roles other than in the cell integrity pathway. The first goal of my project is to find out whether or not Pck2 is involved in the control of mitotic commitment.

Cell length at division has been used as an accurate measurement of the rate of G2 phase progression (Mitchison and Nurse 1985), because, cells keep on growing via elongation at the cell tips during G2 phase. In normal condition (25^{o}C in EMM2 liquid medium), soon after the cell reaches the critical size (about 13.4µm in wild type cells), it enters M-phase. Both *pck2::ura4^{+}.del* from Dr Bimbo and *pck2::kanMX6.del* are longer than wild type cells at 25°C (figure 25), which is an indication of a delay in the timing of mitotic commitment. In contrast, *pck2::LEU2^{+}.dis* from Dr Toda, is shorter than wild type cells. The explanations for this difference in phenotype are that firstly, in Toda, Shimanuki et al. 1993 two EcoRI sites (at 1412 and 2150) within the *pck2^{+}* open reading frame were used for one step gene disruption (Rothstein 1983). This approach leaves a gene fragment which would be sufficient to produce a 470 amino acid long peptide from the N-terminus of the sequence of *pck2^{+}* ORF in the genome. This fragment contains the HR1a, HR1b and C1a domains. It produced these domains might still be able to bind to Pck2 partners such as Rho1 and alter their functions. Cell morphology appears to be affected, as cells assumed a ball-shaped morphology. *pck1*::*ura4^{+}* from (Toda, Shimanuki et al. 1993) was also disrupted by the same method, also leaving a fragment containing the HR1a, HR1b and C1a domains. *pck1*::*ura4^{+}* however, has a cell size similar to that of wild type cells (figure 25) and it has no observed defects in cell morphology or microtubule dynamics. A possible explanation for these observations is that those two peptides have different binding partners, or significantly different binding affinity for the same partner(s). The functions and regulations of Pck1 remain largely unknown (figure 10). Secondly, *LEU2^{+}* is a gene from budding yeast; if a heterologous gene is introduced into *S.pombe*, changes in physiology of some mutants can be unpredictable. Although *pck2::kanMX6.del* is only slightly longer than wild type cells, cells were immediately getting longer within one generation of the inhibition of Pck2.as kinase activity. This indicates that blockage of Pck2 kinase activity must be required somewhere in G2 and G2/M for cell cycle to progress.

The Shokat washout approach is specifically designed to study kinases that regulate mitotic commitment. In *S.pombe*, as soon as cells enter M-phase (right after activation of MPF) microtubules undergo the characteristic re-distribution from cytoplasmic interphase array into mitotic spindle (Hagan and Hyams 1988). The mitotic spindle is therefore a definitive mitotic marker that can be scored to monitor the frequency of cells in mitosis within a population (Hagan 1998). Even a small increase in the numbers of cells entering mitosis can be detected during a time course. The Shokat washout approach clearly demonstrated that restoration of Pck2 kinase activity to cells that have been depleted of this activity for an entire generation can induce mitotic entry at 25^{o}C. Interestingly, Pck2 kinase activity might inhibit

mitotic entry at 36^{o}C, an interesting behaviour for a kinase that has never been recorded before in *S.pombe*.

Pck1 may also regulate mitotic entry. In *pck1*::*ura4*$^{+}$ background *pck2.as* cells gave stronger mitotic control phenotype in Shokat washout approach (figure 20) this indicates that Pck1 promotes mitotic entry in manner that is additive with Pck2. In the beginning of my project, we thought that Pck1 and Pck2 were likely to regulate mitotic entry via the same pathway; therefore all the Shokat washout approaches were done in *pck1*::*ura4*$^{+}$ background. However, data from a recent publication (Koyano, Kume et al. 2010) and results from my bioinformatics analysis all highlight differences between Pck1 and Pck2, and it is still unclear whether Pck1 and Pck2 regulate mitotic entry via the same or different pathways. Therefore, some Shokat washout experiments have to be repeated with *pck2.as pck1*$^{+}$. It would also be informative to generate a Pck1 analogue-sensitive strain to study the role played by Pck1 in the control of mitotic commitment.

3. Pck2 promotes mitotic entry at 25^{o}C via Wee1.

Activation of MPF depends on the relative activity of Wee1 and Cdc25, and the pathways that control mitotic commitment all regulate into MPF via Wee1, Cdc25 or both regulators. During G2/M-phase Wee1 becomes hyperphosphorylated and inactivated in a Cdc2 dependent manner (Hoffmann, Clarke et al. 1993) as well as a number of mitotic inducers such as Cdr2 (Kanoh and Russell 1998) and Blt1 (Martin and Berthelot-Grosjean 2009). The experiments in figure 21 demonstrated that Pck2 is a mitotic inducer at 25^{o}C that acts through Wee1, but not Cdc25. It is unlikely that Pck2 regulates Wee1 through the tip extension pathway, because Pck2 can still promote mitotic entry even when the tip extension pathway is blocked at different levels (figure 23). It is also unlikely that Pck2 regulates Wee1 directly through the Pmk1 cell integrity pathway (figure 22). However, in the absence of Pmk1, the *pck2.as* Shokat washout mitotic control phenotype was delayed by about 20 mins. One possible explanation for this is that, removal of Pmk1 pathway might reduce Pck2 activity, because there might be a positive feedback loop between Pck2 and Pmk1 as feedback loops have been reported in the Sty1 stress pathway (Wilkinson, Samuels et al. 1996). This delay is also observed in *sty1::kanMX6* or *cut12.s11* background (figure 24), thus the Sty1-Plo1 pathways might also influence Pck2 mitotic regulation. To further investigate the impact of *pmk1*$^{+}$*, sty1*$^{+}$ *and cut12*$^{+}$ on the *pck2*-mitotic control phenotype , the Shokat washout could be repeated in background mutated to generate constitutively activated Pmk1 or Sty1 signaling, for

example via the *wis1.DD* mutation that constitutively activates the MAPK kinase Wis1 that acts in the Sty1 pathway (Shiozaki and Russell 1995) (see introduction 2.2.1).

To look at the interaction between Pck2 and Wee1 in the future, *in vitro* kinase assay and co-immunoprecipitation assay could be used to see whether Pck2 is capable of phosphorylating Wee1 directly. The Pck2 phospho site on Wee1 *in vivo* could then be identified by comparing the phosphorylations on Wee1 (via Mass spectrometry) before and after analogue inhibition of Pck2.as. Alternatively Pck2 might indirectly regulate Wee1 through a novel pathway. A combination of analogue inhibitable Pck2.as mutants and quantitative mass spectrometry could be used for comprehensive identification of sites of Pck2 phosphorylation on large numbers of substrates *in vivo* (Holt, Tuch et al. 2009) to identify the candidate genes that are involved in the Pck2 regulated mitotic pathway.

I cloned the region of Pck2 spanning amino acid 527 to 676, which is unique region in Pck2 (data are not shown). This protein has been purified and is ready to send for antibody generation. The antibodies generated against recombinant protein could be used for molecular analysis of Pck2 regulated mitotic entry.

4. Pck2 positively regulate NETO

NETO takes place in G2 phase (details are described in Introduction 2.1), after which cell growth switches from monopolar to bipolar at 25°C in minimal medium. It requires that 1) cell growth must reach a critical length of 9.0-9.5 µm. 2) cells must have transited at least the first one third of one cell cycle (Mitchison and Nurse 1985). The signaling pathways regulating NETO remain largely uncharacterised, but a number molecules have been identified and classified into four types 1) protein kinases such as Pom1 (Bahler and Nurse 2001) and Kin1 (Drewes and Nurse 2003). 2) microtubule related proteins such as Tea1 (Mata and Nurse 1997). 3) actin binding proteins such as For3 (Nakano, Imai et al. 2002) 4) Others. Recently, Pck1 and Pck2 have also been shown to positively regulate NETO (Koyano, Kume et al. 2010).

In (Koyano, Kume et al. 2010) deletion of *pck2*$^{+}$ or *pmk1*$^{+}$ but not *pck1*$^{+}$ at 25^{o}C increased the percentage of monopolar cells in asynchronious culture; deletion of *pck2*$^{+}$, *pmk1*$^{+}$ or *pck1*$^{+}$ at 36^{o}C increased the percentage of monopolar cells. These results suggest that A) Pck1 and Pck2 positively regulate NETO in

different pathways. B) Pck2 might regulate NETO via the Pmk1 pathway; C) Pck1 but not Pck2 regulation of NETO is temperature dependent. Here, I also observed this phenotype in *pck2::kanMX6* cells (figure 25). In the Shokat washout approach used here at 25°C *pck1*::*ura4*$^{+}$ *pck2.as* and *pck2.as* mutant cells display a burst of mitosis around 40 mins after the ATP analogue is washed out, in *pck1*::*ura4*$^{+}$ *pck2.as pmk1::ura4*$^{+}$ mutant however, the burst of mitosis appears about 60 mins after the ATP analogue is washed out (figure 22); at 36°C *pck1*::*ura4*$^{+}$ *pck2.as* mutant have a burst of mitosis around 120 mins after the ATP analogue is washed out. It seems that there is a correlation between NETO and the timing of *pck2*-mitotic control phenotype. It would be interesting to establish whether there is a correlation between NETO and mitotic entry regulation of Pck2, that is, NETO might be a part of the mechanism by which Pck2 regulates mitotic commitment, or Pck2 might regulate mitotic entry and NETO independently. To answer this question the Shokat washout approach could be used, test for epistatic interactions between Pck2 and genes that regulate NETO such as *pom1*$^{+}$, *kin1*$^{+}$, *tea1*$^{+}$ and *for3*$^{+}$. Additionally, it would be informative to Inhibit Pck2.as just before or after NETO takes place in synchronised culture, and measure the mitotic index to find out whether or not Pck2 kinase activity is still required for mitotic entry.

Pck2 might positively regulate interphase microtubule stability. However, there are only small number of cells with the microtubules phenotype (figure 27), suggesting that it is unlikely that Pck2 directly regulates microtubule dynamics itself. This phenotype might arise as a consequence of misregulation of the proteins that regulate microtubule dynamics such as MAPs (microtubule associated proteins). It would be interesting to know whether or not the misregulation of NETO is a consequence of the bent microtubule phenotype in *pck2*::*kanMX6* cells or vice versa. The bent microtubule phenotype of *pck2::kanMX6* can be used to test epistatic interactions between *pck2*$^{+}$ and candidate genes that regulate NETO.

As described in introduction, Rho GTPases are highly conserved from yeast to mammalian cells; they are the key regulators of cell polarity. Pck1 and Pck2 are immediate downstream kinases of Rho1 and Rho2 in *S.pombe*. We speculate that Pck1 and Pck2 might function above NETO in a novel pathway that regulates mitotic commitment through Wee1 to coordinate the two cellular processes, cell division and morphogenesis.

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