AN ABSTRACT OF THE THESIS OF

Jianzheng Wu for the Master of Science

in Physical Sciences (Chemistry) presented on April 16, 2015

Title: The cloning, expression, and purification of CDC14 using *Escherichia coli*

Abstract approved:

Committee Chair: Dr. Kim Simons

Mitosis is a phase in the cell cycle when the replicated genetic information from a parent cell is separated and transmitted to its daughter cells. To ensure the genetic information is transmitted faithfully to the offspring, the cell monitors the progress in mitosis. In the late stage of mitosis, the activity of cyclin-dependent kinases (Cdks) is inhibited to complete and exit the mitosis. The phosphatase Cdc14p in budding yeast is a key component that counteracts the activity of Cdks and promotes mitotic exit. Cdc14p is regulated by the mitotic exit network (MEN) and the cell division cycle (CDC) fourteen early anaphase release (FEAR) network in budding yeast, and these two regulatory networks have been well studied at a genetic level. However, only a few studies explored the interactions of proteins in these two networks, and more investigations are necessary to understand these two regulatory networks at the protein level. We expressed the CDC14 gene in *E. coli* BL21 (DE3) strain, and purified the 6x histidine-tagged Cdc14p using a nickel metal

affinity column. We also co-expressed the CDC14 with the CDC15 or CDC5 gene in the *E. coli* BL21 (DE3) strain, but no proteins were produced. An active form of Cdc14p was isolated by varying conditions for its production and purification. We are now working on the expression and purification of a kinase that will be used to study the interaction between the kinase and the Cdc14p *in vitro*.

THE CLONING, EXPRESSION, AND PURIFICATION OF

CDC14 USING *ESCHERICHIA COLI*

A Thesis

Presented to

The Department of Physical Sciences

EMPORIA STATE UNIVERSITY

In Partial Fulfillment

of the Requirements for the Degree

Master of Science, Physical Sciences

Chemistry Concentration

By

Jianzheng Wu

April 2015

_____

Approved by the Department Chair

_____

Approved by the Dean of the Graduate School and Distance Education

_____

Approved by the Department Chair

_____

Committee Chair: Dr. Kim Simons

_____

Committee Member: Dr. Eric Trump

_____

Committee Member: Dr. Diane Nutbrown

_____

Committee Member: Dr. Melissa Bailey

_____

Dean of the Graduate School and Distance Education

ACKNOWLEDGEMENTS

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1 INTRODUCTION

Cell proliferation is an essential process in biological life, and it is vital for biological growth and development. The development of an embryo, the formation of organs, and the healing of tissues require the proliferation of cells. Cell proliferation is accomplished by replicating the genetic information and then dividing into two daughter cells during a period of time called the cell cycle. The period when the cell replicates the genetic information and prepares the materials for division is the interphase of the cell cycle; the period when the cell separates the replicated genetic information and divides into two daughter cells is the mitotic phase or M phase of the cell cycle. Because of these ordered series of stages, the cell cycle is a highly regulated process that ensures the fidelity of the genetic information transmission between the parent cell and its daughter cells.[1]

Cyclin-dependent kinases (Cdks) are involved in the progression of cell cycle, and cyclin is required for the activity of Cdks. In budding yeast, the cyclin-dependent kinase Cdc2p, when associated with cyclin B, promotes the entry to mitosis.[2] At the end of the M phase, the cell coordinately recruits some proteins to complete and exit the mitotic phase. To exit mitosis, the mitotic Cdks need to be inactivated, otherwise the cell would arrest in mitosis.[3] The protein Cdc14p in budding yeast promotes the mitotic exit by inhibiting the activity of Cdks.[4] A deficiency in mitosis causes chromosome instability, which is often found in some types of human cancer.[5]

The activity of important proteins including Cdc14p need to be regulated to ensure the precise process of mitosis. Extensive studies show that the activity of Cdc14p in late anaphase is mainly regulated by the mitotic exit network (MEN).[3] At the genetic level, many components in MEN are found to interact with CDC14. However, at the protein level, these interactions are not clearly understood and need further investigation. Thus, exploring the interactions between Cdc14p and other components in MEN is necessary to understand the network in the mitotic exit.

Understanding of the regulatory network at protein level would contribute to the research on mitosis, which may involve some diseases or human cancers. Our ultimate goal is to investigate the interactions of Cdc14p with other proteins that are involved in the mitotic exit. Are they interacting directly or indirectly with each other? How do they interact with each other and which part of the Cdc14p is involved in the interaction? As the first step of the project, we used an *E. coli* BL21 (DE3) strain to express CDC14 from the budding yeast *Saccharyomyces cerevisiae*. The histidine-tagged (His-tagged) Cdc14p was purified using a nickel affinity column. To isolate the active form of Cdc14p, the CDC14 was expressed at different temperatures and the Cdc14p was purified under different conditions.

CHAPTER 2 BACKGROUND

2.1 Brief overview of cell cycle

The cell cycle of eukaryotes is divided into four main phases. These four phases are the mitotic phase (M phase), the first gap (G1) phase, the DNA synthesis phase (S phase), and the second gap (G2) phase. During the G1 phase, the cell prepares to replicate its genetic information. In the S phase, the cell replicates its genetic information. During the G2 phase, the cell continues to grow and prepares conditions for the cell to proceed to the M phase. In the M phase, the cell divides into two daughter cells and the doubled chromosomes are partitioned equally between the two daughter cells.

The mitotic phase can be further divided into five stages: prophase, metaphase, anaphase, telophase, and cytokinesis. To ensure the genetic information is transmitted to the daughter cells without errors, the cell monitors the process. The cell cycle will not proceed to next phase until it is ready to progress. For example, in the G1 phase there is an important checkpoint called restriction or R point, and if cell damage occurs before this point, the cell cycle will stop until the damage is repaired.[6] Cyclin-dependent kinases (Cdks) are one of the major protein families that regulate the cell cycle, and Cdks regulate the progression of cell cycle by phosphorylating their target substrates.[7]

2.2 The discovery of the CDC14 gene

Leland Hartell's study about genetic control of cell division cycle (CDC) in budding yeast discovered CDC14.[8] In this series of studies, Hartell and his colleagues isolated temperature-sensitive mutants in the yeast *S. cerevisiae* to study the role of mutated gene in control of cell division cycle in the yeast. There are three main advantages to use yeast as a model organism. First, yeast, as a simple eukaryote, contains less genetic material compared to other eukaryotic cells, so it is relatively easy to study, and the findings in the study of yeast could be also applied to the research of human cells. Second, a yeast cell begins the cell cycle with the formation of a small bud, and the bud grows during the cell cycle, so the size of bud reliably indicates the different stages of the progression in the cell cycle. Last, both the haploid form and diploid form of yeast cells survive and grow, so these two forms of yeast cells are ideal for complementation study and recessive or dominant mutation study.

Temperature-sensitive mutants of the yeast *S. cerevisiae*, at a low temperature or "permissive temperature" (23 °C in this study), grow and reproduce, however, when incubated at a high temperature or "restrictive temperature" (36 °C in this study), the mutants will exhibit different defects in the cell function, such as abnormal morphology, loss of the ability to synthesize protein and RNA, and eventually, cell death.[9] Because the specific stages of cell division cycle are easily visualized by specific morphologies in *S. cerevisiae*, Hartwell, Culotti and Reid[10] were able to use time-lapse photomicroscopy

4

to detect the temperature-sensitive mutants of the yeast *S. cerevisiae* that have defects in cell division cycle. When incubated at restrictive temperature, the wild-type yeast cells continues to progress in the cell division cycle with normal morphologies, however, the temperature-sensitive mutants cells do not survive, and the morphologies of the dead yeast cells are often different when the mutants are caused by different genes. About one hundred and fifty mutants were isolated in the study, and based on the complementation study, these mutants were ordered into 32 groups, numbered from CDC1 through CDC32, and the CDC14 mutant was one of these groups.[11]

2.3 The function of Cdc14p

In 1991, Wan and his colleagues[12] mapped and sequenced the CDC14 gene. They found the complete deletion of CDC14 gene led to the death of budding yeast, and that the protein sequence encoded by the CDC14 gene contained a consensus sequence HCXAGXXR that is present in the active site of all tyrosine protein phosphatase. In 1997, Talyor, Liu, Baskerville, and Charbonneau found Cdc14p is actually a dual specificity phosphatase that can catalyze the dephosphorylation of a substrate containing a tyrosine or serine/threonine, and that the phosphatase activity of the Cdc14p is essential for progression of cell cycle in *S. cerevisiae*.[13] In 1998, Visintin *et al* demonstrated that Cdc14p promotes the exit from mitosis in *S. cerevisiae* by dephosphorylating, thus activating, Sic1p. Sic1p is an inhibitor of cyclin-dependent kinase-1 (Cdk1p). They also found that Cdc14p induces the degradation of mitotic cyclins in mitosis. Since cyclin is required for the activity of Cdks, the degradation of cyclin inactivates Cdks.

The study of Cdc14p homologs from human showed that overexpression of the CDC14 human homolog, hCdc14A, disturbed the segregation of chromosomes and the mitotic spindle, and blocked cytokinesis in human bone osteosarcoma epithelial cells (U2OS).[14] The study of the Cdc14 orthologs in different organisms have revealed new functions.[15] For example, Cdc14p in budding yeast may be involved in DNA replication; hCdc14B in humans might play a role in the control of the DNA damage checkpoint at G2 phase, as without hCdc14B there is a deficiency in DNA repair.[16]

2.4 The regulation of Cdc14p

As an important part of the cell cycle, the mitotic process is precisely regulated to ensure a successful cell division. The Cdc14p, an essential protein in mitosis, is precisely coordinated during the process of cell cycle. In *S. cerevisiae*, Cdc14p associates with its inhibitor Cfi/Net1p, and the resulting complex is sequestered in the nucleolus from G1 phase until to early anaphase.[17] Some research[18,19] suggests that the replication fork block protein (Fob1p) also helps to sequester Cdc14p, since the inactivation of FOB1 results in the release of Cdc14p in the metaphase instead of anaphase.

The first release of the Cdc14p from nucleolus is achieved by the CDC fourteen early anaphase release (FEAR) network during early anaphase.[20] The components of the FEAR network, like CDC5, promote the phosphorylation of Net1p, leading to the dissociation of Cdc14p from Net1p.[21] The Cdc14p released through the FEAR network at the early anaphase is not enough to promote the mitotic exit, and in addition to FEAR network, there exists a more important network, the mitotic exit network (MEN), that releases more Cdc14p and sustains the activity of Cdc14p during the late stage of anaphase. The major components in MEN include Tem1p, Cdc15p, and Dbf2p-Mob1p complex. A genetic study shows that TEM1 is required for Cdc14p release[17] and works at upstream of CDC15.[22] The activation of Dbf2p requires Cdc15p, and Dbf2p-Mob1p promotes the release of Cdc14p by directly phosphorylating Cdc14p[23] (Figure 1).
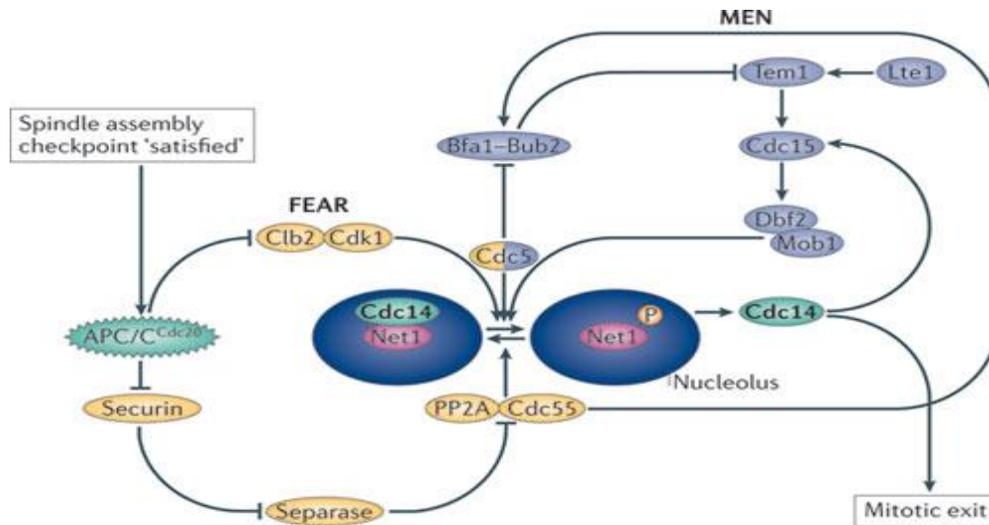
Nature Reviews | Molecular Cell Biology

Figure 1 Role of FEAR and MEN in regulating the release of Cdc14p.[24]

The components in the yellow ovals belong to FEAR network, and the components in light blue belong to MEN in budding yeast. Cdc5p participates in both the MEN and FEAR networks. The arrow represents the activation, and the bar-headed lines represent inhibition. In the FEAR network, the activation of the anaphase-promoting complex with Cdc20p (APC/C^{Cdc20}) degrades the securing (Pds1p) that inhibits the separase (Esp1p). The degradation of Pds1p releases the Esp1p that binds to and inhibits the PP2A-Cdc55p complex, leading to the release of Cdc14p from nucleolus. In the MEN, the release of Cdc14p depends on Tem1p and Cdc15p, and Tem1p works at upstream of kinase Cdc15p. The activation of Dbf2p requires Cdc15p, and Dbf2p-Mob1p promotes the release of Cdc14p from nucleolus by directly phosphorylating Net1p.

CHAPTER 3 METHODS

3.1 Cell transformation

50 ng of plasmid was added into 20 µL of competent *E. coli* cells in a pre-chilled microcentrifuge tube on ice. The tube that contains the mixture was incubated on ice for 10 minutes. Then, the tube was transferred to a 42 °C water bath for 45 seconds, followed immediately by returning the tube to ice for 5 minutes. 180 µL of LB media was added to the tube, and the tube was shaken with a speed of 200 rpm at 37 °C for 45 minutes. The mixture in the tube was spread on a LB agar plate containing the selective antibiotics, and the plate was then incubated overnight at 37 °C. The competent cells used for transformation were DH5α *E. coli* derived from a cell line purchased from New England Biolabs.

3.2 *E.coli* growth, and isolation of plasmid

To grow the *E. coli,* one colony from a plate was transferred to a 15 mL test tube containing 4 mL of LB media. The tube was shaken for 14 hours with a speed of 180 rpm at 37 °C. The cells were collected in a microcentrifuge tube by centrifugation at 8000 rpm for 5 minutes. The plasmids were isolated using QIAprep Spin Miniprep Kit from QIAGEN. The protocol included in the kit was followed to isolate the plasmids.

3.3 DNA digestion reaction

200 ng of DNA was mixed with 0.5 µL of restriction enzyme (2000 U/mL), 2 µL of reaction buffer, 2 µL of 10x bovine serum albumin (BSA) in a microcentrifuge tube, and nuclease-free water was added to a total volume of 20 µL. Then, the tube was incubated in the water bath at 37 °C for 2 hours. The restriction enzymes, the compatible buffers, and the BSA were all purchased from New England BioLabs.

3.4 Polymerase chain reaction (PCR)-based cloning of yeast CDC14 gene

The primers with an additional NdeI recognition sequence in forward primer and SalI site in reverse primer were designed based on the sequence of the CDC14 gene in yeast. PCR protocol for OneTaq DNA polymerase from New England BioLabs was followed to amplify the CDC14 gene, the size of which is 1656 base pairs (bp). The PCR product was doubly digested with NdeI and SalI restriction enzymes and was analyzed using DNA agarose gel electrophoresis. The DNA band around 1600 bp on an agarose gel was excised, and the DNA fragment was then purified from the agarose gel using QIAquick Gel Extraction Kit (Qiagen). This purified DNA fragment was inserted into the vector plasmid.

A pET-28 plasmid which contains a 6x histidine (His) tag was doubly digested with NdeI and SalI restriction enzymes. The size of the pET-28 plasmid is 5386 bp, and thus DNA fragment around 5300 bp was extracted using the QIAquick Gel Extraction Kit. The extracted pET-28 fragment was used as the vector for cloning.

The extracted CDC14 gene fragment and pET-28 vector were ligated at room temperature for 24 hours using the T4 DNA ligase from New England BioLabs. The ligation product was transformed into the competent cells, and the transformed cells were grown overnight on a LB plate with kanamycin. The colonies from the plate were then grown in LB media with kanamycin for 14 hours at 37 °C. The plasmids of the cells were isolated using a QIAprep Spin Miniprep Kit.

3.5 Gene expression using *E. coli* BL21 (DE3) strain

The plasmid construct which contains an N-terminal 6x His-tagged gene of interest was transformed into BL21 (DE3) competent cells, and the transformed cells were grown overnight on LB plate with selective antibiotics at 37 °C. One colony from the plate was grown in a 10 mL LB media with the selective antibiotics overnight at 37 °C. The 10 mL of overnight grown *E. coli* culture was transferred to a 500 mL LB media with antibiotics, and the *E. coli* culture was continuously incubated at 37 °C until the optical density at 600nm (OD_{600}) reached 0.7. Then, isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to 1 mM in the culture to induce expression, and the culture was incubated for an additional 5 hours at 37 °C, or the culture was incubated for 24 hours at room temperature. The cells were collected by centrifugation at 4000 xg for 25 minutes.

3.6 Purification of 6x His-tagged protein using nickel-nitrilotriacetic acid (Ni-NTA) agarose under native conditions and denaturing conditions

The cells collected from the expression procedure were resuspended in 6 mL of lysis buffer (20 mM Tris, pH 8, 300 mM NaCl, 1 mM PMSF, 10 mM imidazole). The resuspended cells were lysed by six times of 10 second-pulse of sonication using a Branson Sonifier at 14 outputs of display, and the cells were cooled on ice for 5 minutes between every sonication. The lysate was clarified by centrifuging at 10,000 xg for 25 minutes at 4 °C, and the supernatant was saved for purification. To purify the protein, the supernatant was loaded onto a column containing 4 mL of Ni-NTA agarose matrices, and the supernatant was allowed to flow through the column with a flow rate of one drop per 5 seconds. The column was then washed with 6 mL of wash buffer (20 mM imidazole, 20 mM Tris, pH 8, 300 mM NaCl). The His-tagged protein was eluted from the resin using 6 times of 1 mL of elution buffer (250 mM imidazole, 20 mM Tris, pH 8, 300 mM NaCl,), and the eluates were collected separately in six microcentrifuge tubes. 10 μL of sample from each step of the purification procedure was collected and mixed with 20 μL of 5x SDS loading dye (5% β-Mercaptoethanol, 0.02% Bromophenol blue, 30% Glycerol, 10% SDS, 250 mM Tris-Cl pH 6.8), which was used later for SDS-polyacrylamide gel electrophoresis (SDS-PAGE) analysis.

For purification under denaturing condition, all the procedures are the same with that of purification under native conditions except buffers. The buffers used in denaturing

condition are: lysis buffer (100 mM NaH$_2$PO$_4$, 10 mM Tris-Cl, 8 M urea, pH 8); wash

buffer (100 mM NaH$_2$PO$_4$, 10 mM Tris-Cl, 8 M urea, pH 6.3); elution buffer (100 mM

NaH$_2$PO$_4$, 10 mM Tris-Cl, 8 M urea, pH 5.9).

3.7 Phosphatase assay

Para-nitrophenyl phosphate (PNPP) is a non-specific substrate for phosphatases, and

when phosphatase is added to the PNPP solution that is colorless, the PNPP will be

converted into para-nitrophenol, which is a yellow color substance and absorbs light at

405 nm. To perform a phosphatase assay, 200 µL of 50 mM PNPP solution and 800 µL of

Tris buffer (20 mM Tris, 300 mM NaCl, pH 8) were first mixed in a 1.5 mL

microcentrifuge tube. Then the phosphatase of interest was added to test its activity by

observing the color change or measuring absorbance of the solution at 405 nm. The

amount of phosphatase added into the PNPP reaction buffer varies among different

phosphatases. 105 ng of the Cdc14p purified in our lab was able to make the PNPP

reaction buffer turn into bright yellow in 5 minutes. 1 µL of OPTIZYME™ Alkaline

Phosphatase (1 U/µL) purchased from Fisher BioReagents was used as a positive control.

CHAPTER 4 RESULTS

4.1 The CDC14 cloning construct was verified by DNA agarose gel electrophoresis and DNA sequence.

The CDC14 gene amplified by PCR was inserted into the pET-28 vector to make the CDC14 cloning construct as described in section 3.6 of the Methods chapter. A schematic map of the CDC14 cloning construct is shown in Figure 2. The resulting CDC14 cloning construct was checked using DNA digestion reaction followed by DNA agarose electrophoresis (Figure 3). In addition, the CDC14 cloning construct was sent for sequencing, and the sequence of the CDC14 cloning construct was analyzed by the Basic Local Alignment Search Tool (BLAST).[25]

The sequence of CDC14 (YFR028C) from *Saccharomyces* Genome Database (SGD) was used as the standard sequence for alignment. One example of the BLAST reports is shown in Figure 4. For nucleotides "N", not clearly identified, the chromatogram data from sequencing company (SeqWright Genomic Services) was used to analyze the identification of the nucleotides. Though there are some mutations in CDC14 cloning construct, all the mutations are silent. For example, in the BLAST report for positions 64-66, the sequence of subject is GCA, but the corresponding sequence of query is GCG. However, both GCA and GCG code for alanine. Based on the report from BLAST and chromatogram data, the open reading frame of CDC14 gene in the cloning construct was verified to be correct, and the CDC14 cloning construct was ready for expression.
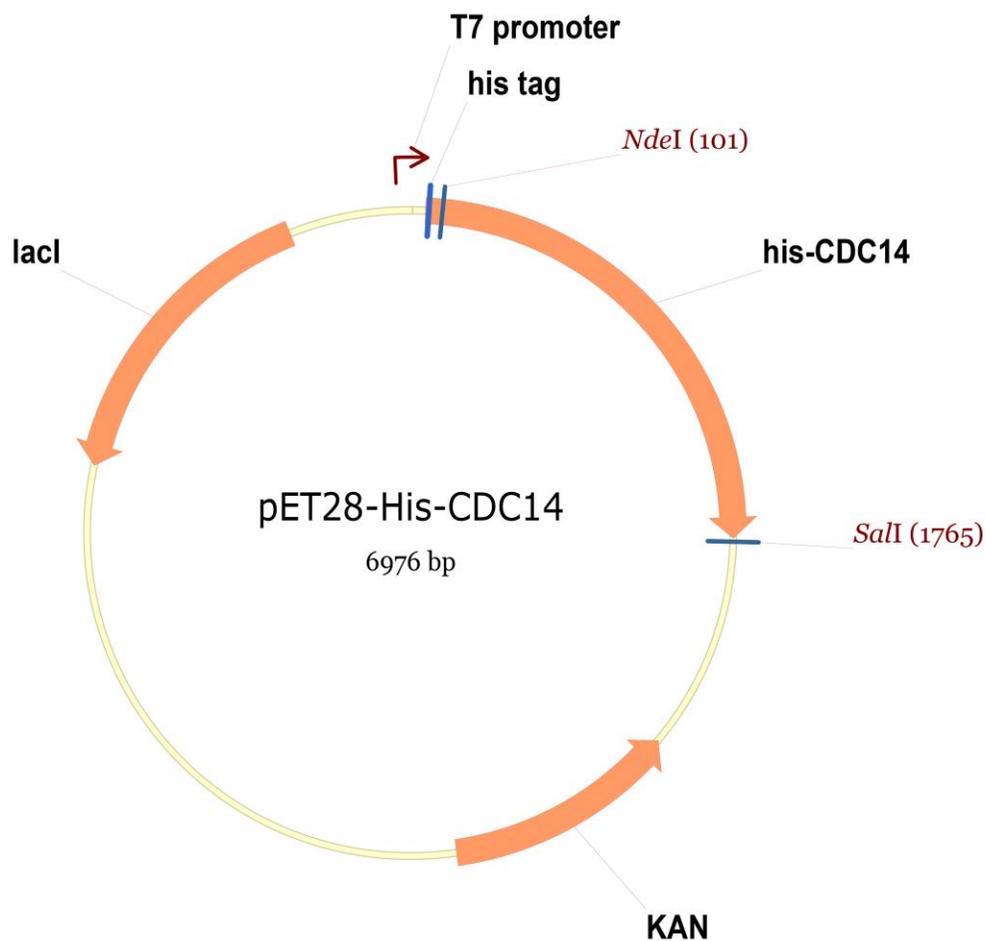
Figure 2 Map of the CDC14 cloning construct.

The size of the whole construct is 6976 base pairs (bp). LacI gene encodes *lac* repressor that is used to inhibit the expression of the gene on this construct. 6xHis tag gene is at the N-terminus of the CDC14 gene, and T7 promoter is located at upstream of the 6xHis tag gene. NdeI and SalI in red represent the recognition DNA sequence of NdeI and SalI restriction enzymes respectively, and these two recognition DNA sequences are unique in CDC14 cloning construct. The numbers in parenthesis indicate the site of the gene. KAN represents kanamycin resistant gene.
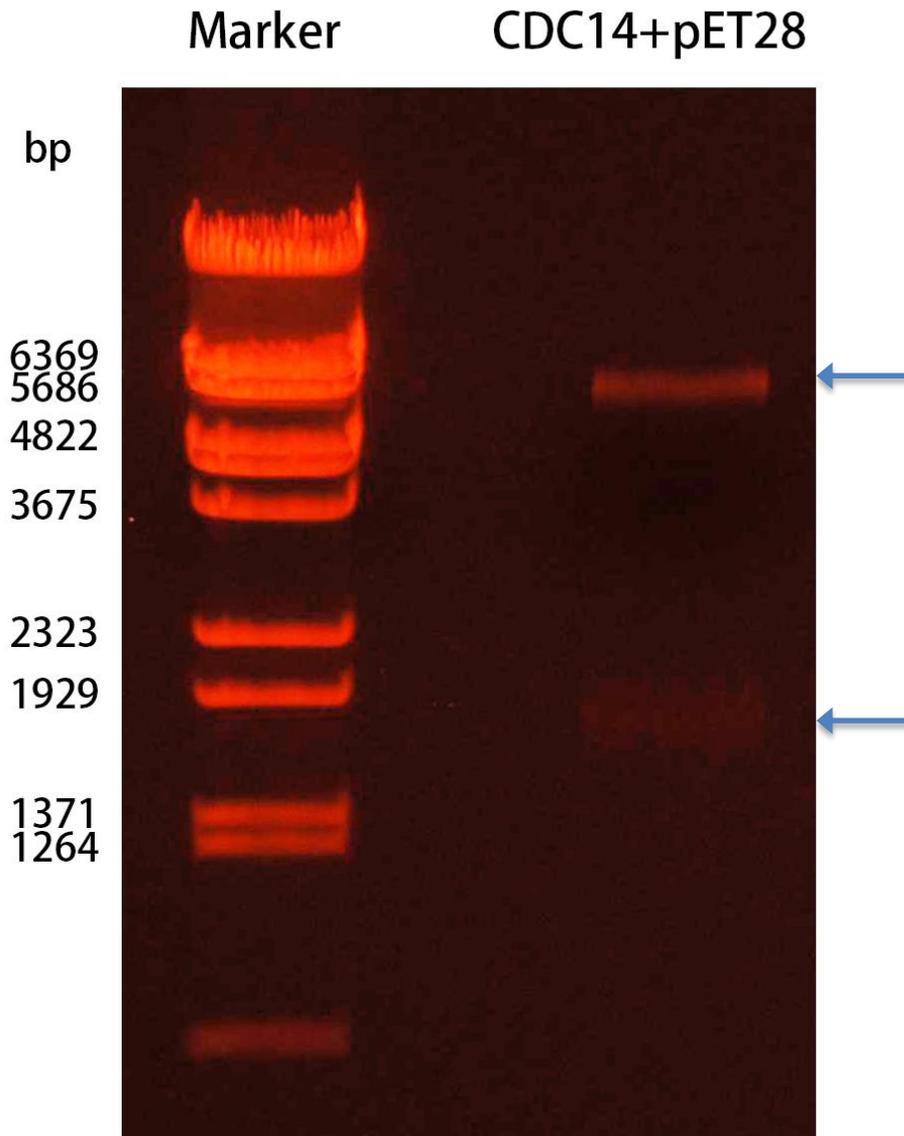
Figure 3 DNA agarose gel of CDC14 cloning construct digested by SalI and NdeI.

CDC14 cloning construct was digested with SalI and NdeI restriction enzyme. Left lane is DNA marker and the size of each band is displayed on the left. The right lane is the digested CDC14 cloning construct. The arrows indicate expected bands. The band around 5686 base pairs (bp) is the pET-28 vector, and the band near 1929 bp is the CDC14 gene.

Score	Expect	Identities	Gaps	Strand	Frame
1003 bits(543)	0.0()	564/582(97%)	1/582(0%)	Plus/Plus	

Features:

```
Query  126  ATGCGTAGGAGTGTATACCTCGACAACACGATCGAGTTCCTGCGAGGCAGAGTATATCTC
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1    ATGCGTAGGAGTGTATACCTCGACAACACGATCGAGTTCCTGCGAGGCAGAGTATATCTC

Query  186  GGTGCGTATGATTACACGCCGGAAGATACTGATGAGCTGGTGTTTTTTACCGTGGAGGAC
            |||||| |||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  61   GGTGCATATGATTACACGCCGGAAGATACTGATGAGCTGGTGTTTTTTACCGTGGAGGAC

Query  246  GCAATCTTTTACAACAGTTTCCATTTGGATTTCGGTCCCATGAACATTGGACATCTATAT
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  121  GCAATCTTTTACAACAGTTTCCATTTGGATTTCGGTCCCATGAACATTGGACATCTATAT

Query  306  CGTTTTGCCGTTATTTTCCATGAAATCTTGAATGACCCAGAAAATGCCAATAAAGCCGTT
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  181  CGTTTTGCCGTTATTTTCCATGAAATCTTGAATGACCCAGAAAATGCCAATAAAGCCGTT

Query  366  GTTTTCTATTCATCTGCATCGACCAGACAACGTGCTAACGCCGCATGTATGCTTTGCTGT
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  241  GTTTTCTATTCATCTGCATCGACCAGACAACGTGCTAACGCCGCATGTATGCTTTGCTGT

Query  426  TATATGATCCTCGTTCAGGCATGGACTCCGCATCAAGTGCTTCAGCCGTTGGCGCAANTA
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||| ||
Sbjct  301  TATATGATCCTCGTTCAGGCATGGACTCCGCATCAAGTGCTTCAGCCGTTGGCGCAAGTA
```

Figure 4 Report of sequence alignment from BLAST.

The query sequence is the sequence of the CDC14 cloning construct, and the subject sequence is the standard CDC14 sequence from SGD. "N" indicates that the nucleotide is not clearly identified by DNA sequencer, and the chromatogram data needs to be checked for nucleotide identification. The dash "-" means that the nucleotide is absent, and in this case the chromatogram data needs to be checked, also.

4.2 The expression of CDC14 and purification of Cdc14p was evaluated by SDS-PAGE.

The CDC14 gene was expressed by *E. coli* BL21 (DE3) strain at 37 °C for 4 hours as described in section 3.5, and the Cdc14p was purified using Ni-NTA agarose affinity column as described in section 3.6. The samples from the purification procedure were analyzed by SDS-PAGE as described in section 3.6. The expected size of Cdc14p is 62 kilo Daltons (kDa), and the SDS-PAGE result was shown in Figure 5. From the lane E2 to E5 there was only one band with expected size (62 kDa) in each lane, suggesting that the Cdc14p was purified successfully. Unfortunately, when 10 μL of sample from E3 was added to PNPP reaction solution to test the activity of Cdc14p as described in section 3.7, the solution did not turn yellow, indicating that the Cdc14p was inactive. The CDC14 gene was also expressed at room temperature as described in section 3.5, and the Cdc14p was purified as described in section 3.6. However, the purified Cdc14p was still inactive based on the result of phosphatase assay (5 μL of purified Cdc14p was used).
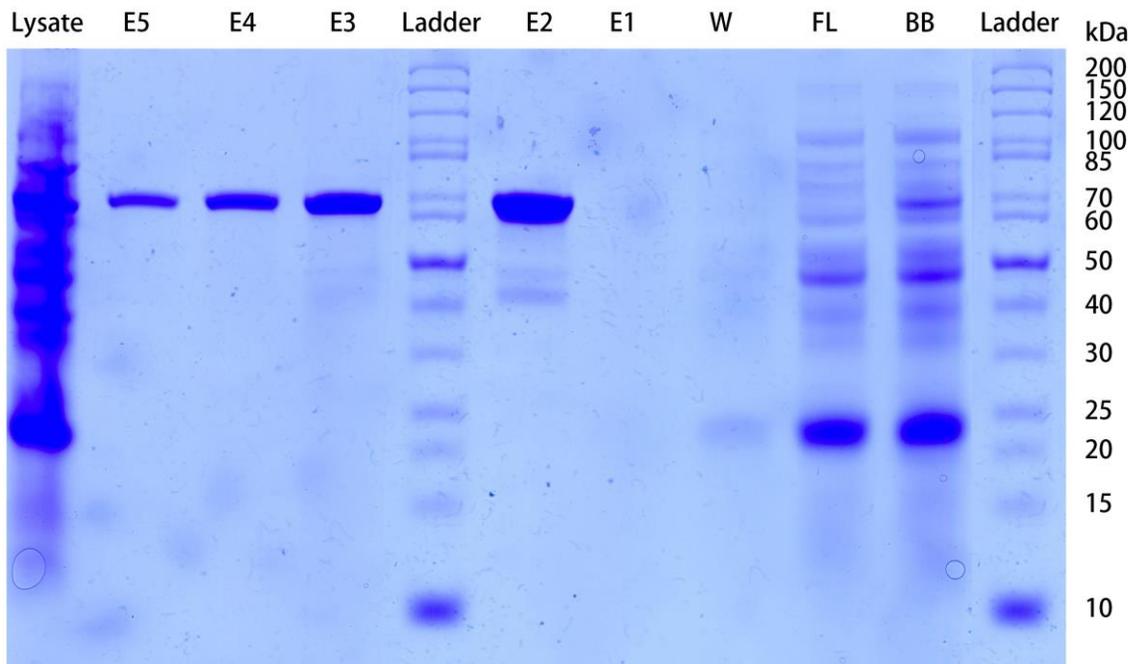
Figure 5 SDS-PAGE of Cdc14p purification under native conditions

The sizes of the ladder proteins are displayed on the right of this figure. Lysate is the sample from the whole crude lysate of the expression strain of *E. coli* after sonication; E refers to elution, and the number following represents fraction number. W is sample from collection of wash buffer passed through the column; FL is sample from collection of flow-through solution after the clarified lysate binding to the Ni-NTA column; BB is the sample collected before clarified lysate binding to the Ni-NTA affinity column.

4.3 Co-expression of CDC15 with CDC14, and Co-purification of Cdc15p with Cdc14p were analyzed by SDS-PAGE.

Since the Cdc14p produced previously was inactive, the CDC14 gene was co-expressed with CDC15 to test if Cdc15p helps make active Cdc14p. The CDC14 and CDC15 genes were both inserted into a pET-DUET plasmid using a similar strategy as described in section 3.4. In this construct the CDC15 gene was tagged by 6xHis, and the sequence of the construct was verified by DNA sequencing (Figure 6). Then, the CDC14-CDC15 cloning construct was co-expressed using *E. coli* BL21 (DE3) strain for 24 hours at room temperature and for 4 hours at 37 °C respectively. After expression, the Cdc15p and Cdc14p were co-purified under native conditions as described in section 3.6. Unfortunately, the expected proteins of Cdc15 (110 kDa) or proteins of Cdc14p-Cdc15p complex were not visible on the protein gel (Figure 7). The Cdc14p and Cdc15p were also co-purified under denaturing conditions as described in section 3.6. Again, no bands with expected sizes were evident in the SDS-PAGE (Figure 8).
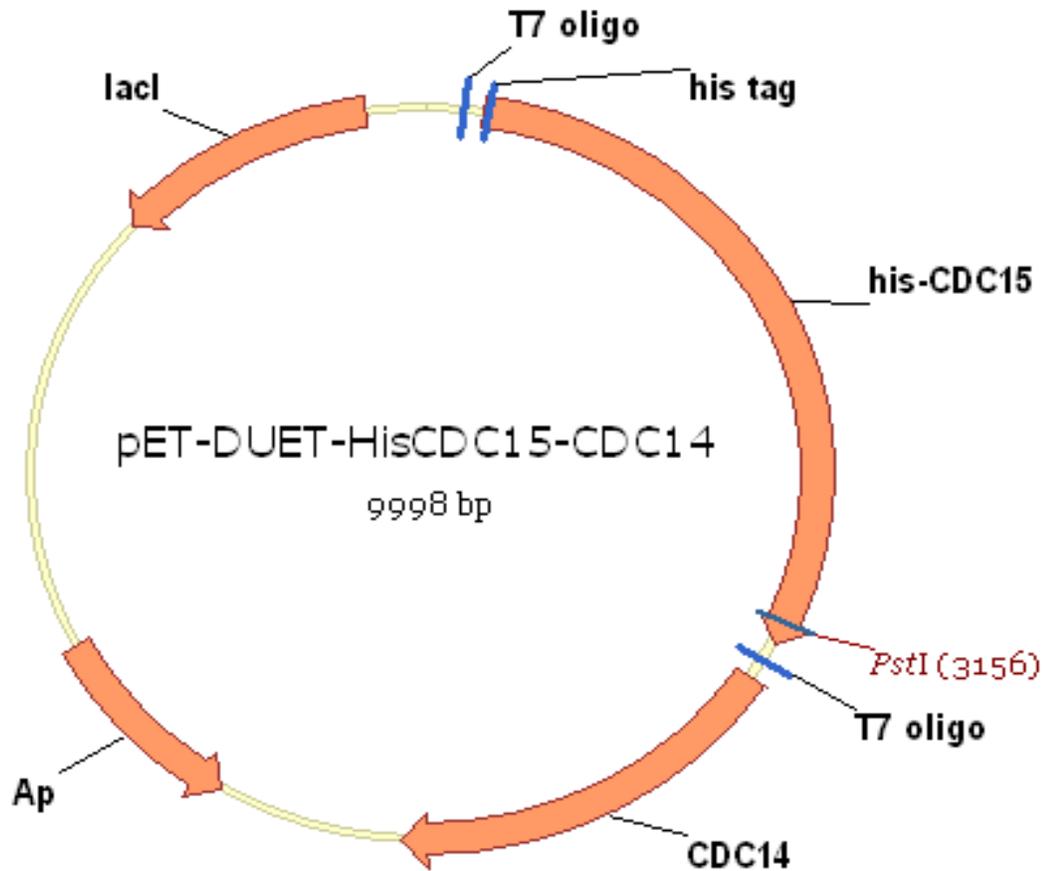
Figure 6 Map of the CDC15-CDC14 co-expressing cloning construct.

The size of the whole construct is 9998 base pairs (bp). LacI gene encodes *lac* repressor that is used to inhibit the expression of the gene on this construct. 6xHis tag gene is at the N-terminus of the CDC14 gene, and T7 oligo is the promoter located at upstream of the 6xHis tag gene. PstI in red represents the recognition DNA site of the PstI restriction enzyme. The number in parenthesis indicates the site of the gene. Ap represents ampicillin resistant gene.
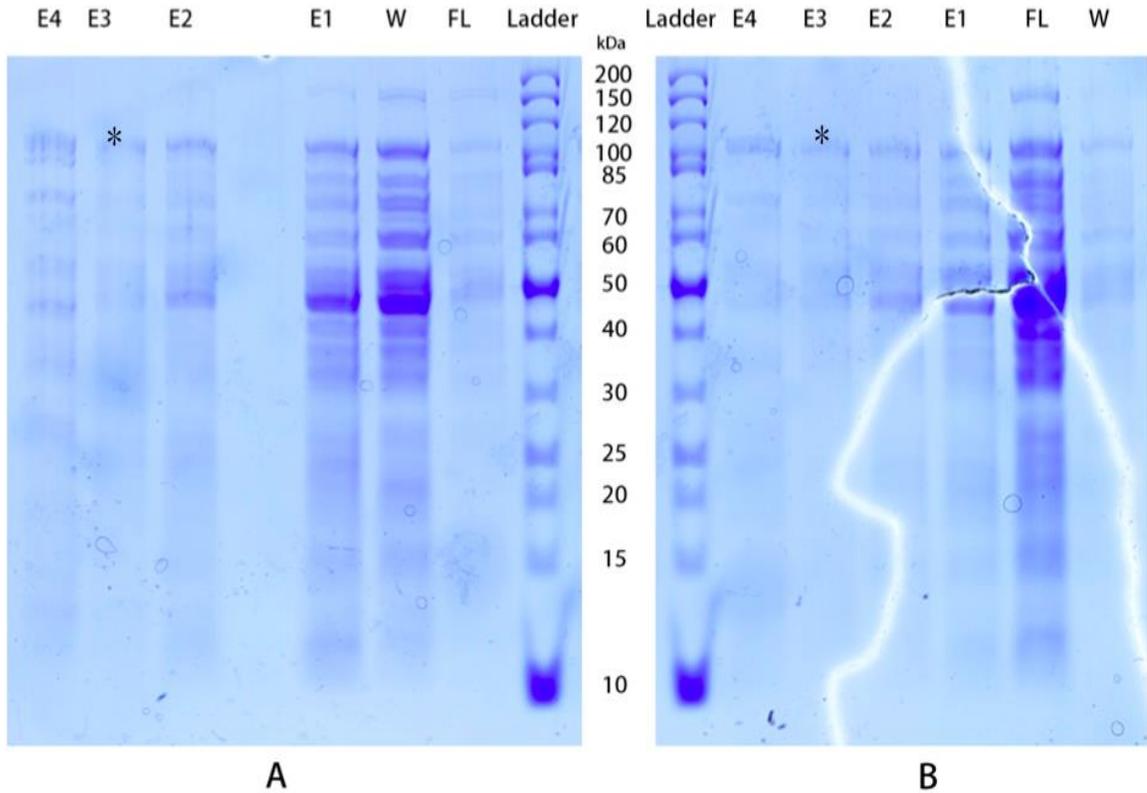
Figure 7 Co-purification of Cdc15p and Cdc14p under native conditions

Panel A is the co-expression of CDC15 and CDC14 at room temperature for 24 hours, and panel B is the co-expression at 37 °C for 4 hours. The sizes of the ladder proteins are displayed in the middle of this figure. Lysate is the sample from the whole crude lysate of the expression strain of *E. coli* after sonication; E refers to elution, and the number following represents fraction number. W is sample from collection of wash buffer passed through the column; FL is sample from collection of flow through after the clarified lysate binding to the Ni-NTA column; BB is the sample collected before clarified lysate binding to the Ni-NTA affinity column. An asterisk shows the expected site (110 kDa) for Cdc15p.
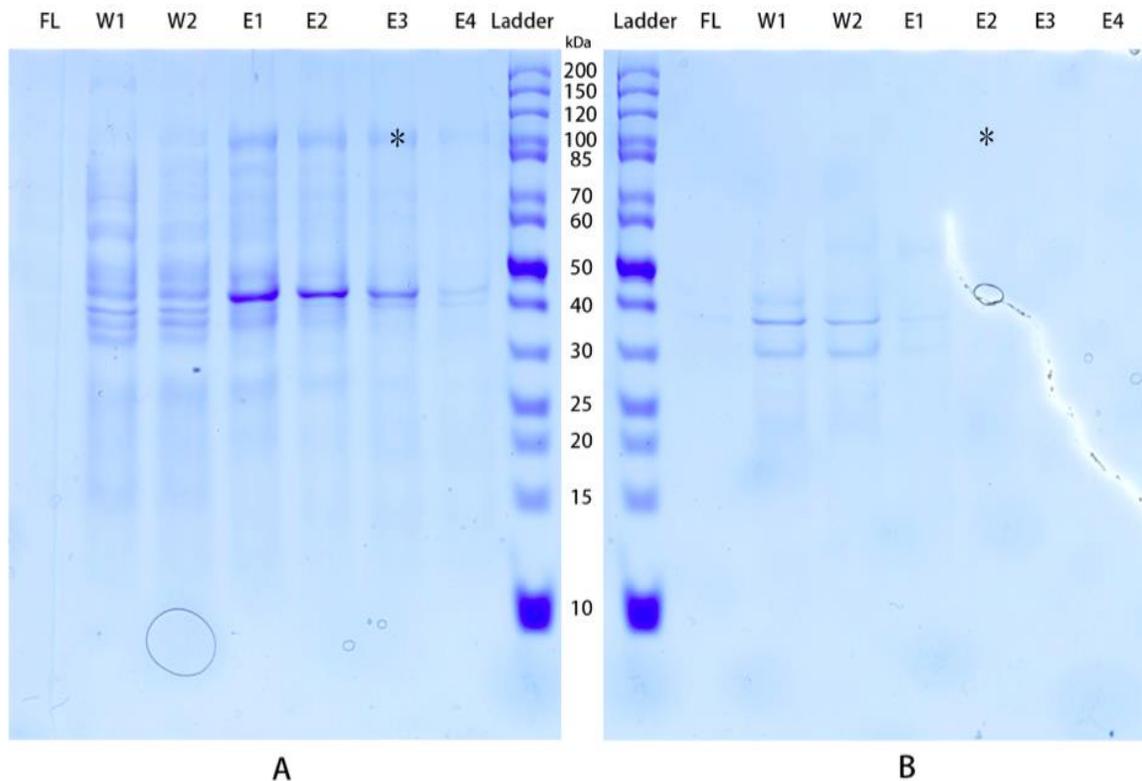
Figure 8 Co-purification of Cdc15p and Cdc14p under denaturing conditions

Panel A (left) is the co-expression of CDC15 and CDC14 at 37 °C for 4 hours, and panel B (right) is the co-expression at room temperature for 24 hours. The sizes of the ladder proteins are displayed in the middle of this figure. Lysate is the sample from the whole crude lysate of the expression strain of *E. coli* after sonication; W is sample from collection of wash buffer passed through the column; E refers to elution, and the number following represents fraction number. FL is sample from collection of flow-through solution after the clarified lysate binding to the Ni-NTA column; BB is the sample collected before clarified lysate binding to the Ni-NTA affinity column. An asterisk shows the expected site (110 kDa) for Cdc15p.

4.4 The expression of the phosphatase part of CDC14, and purification of phosphatase part of Cdc14p were evaluated by SDS-PAGE.

To test if the phosphatase part of CDC14 gene is active, the CDC14 gene was truncated to retain only the phosphatase part of the CDC14 gene using Q5 Site-Directed Mutagenesis Kit purchased from NEB. The phosphatase part of CDC14 was expressed and purified as described in section 3.5 and 3.6. The single band of expected size (42 kDa) showed in the E4, E5, and E6 lane (Figure 9). However, when 20 µL of the E4 was added to the PNPP reaction buffer, the solution did not turn into yellow, suggesting that the phosphatase part of Cdc14 is inactive.
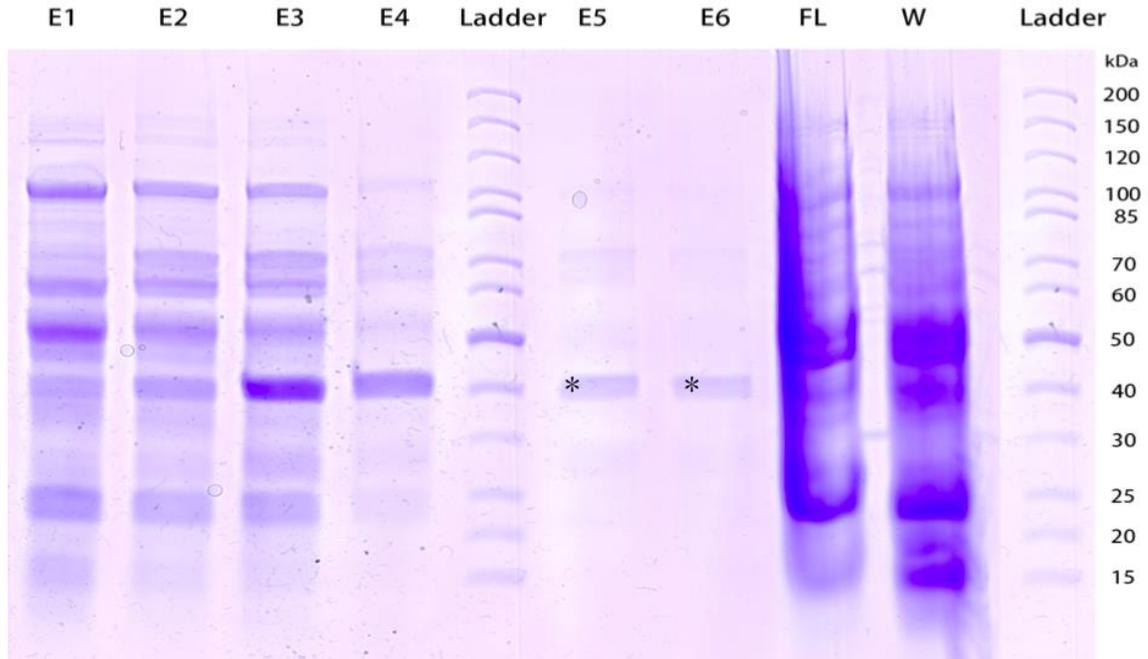
Figure 9 Purification of phosphatase part of Cdc14p under native conditions

The sizes of the ladder proteins are displayed on the right of this figure. Lysate is the

sample from the whole crude lysate of the expression strain of *E. coli* after sonication; E

refers to elution, and the number following represents fraction number. W is sample from

collection of wash buffer passed through the column; FL is sample from collection of

flow-through solution after the clarified lysate binding to the Ni-NTA column; BB is the

sample collected before clarified lysate binding to the Ni-NTA affinity column. The

bands with size of 42 kDa in E4, E5, and E6 lanes are phosphatase part of Cdc14p. The

asterisk shows the expected site (42 kDa) for phosphatase part of Cdc14p.

4.4 Increased concentration of purified Cdc14p shows activity.

During one test of the activity of Cdc14p, the assay remained on the bench. Surprisingly, two weeks later the PNPP reaction solution in the tube turned slightly yellow, indicating that the Cdc14p might be active. Since the yellow color was not obvious, the concentration of the Cdc14p was increased to see if a higher amount of the Cdc14p show a more obvious yellow color. One liter of transformed *E. coli* BL21 (DE3) strain by the CDC14 construct was used to express the CDC14 at room temperature for 24 hours, and then the Cdc14p was purified under native conditions. This time a more concentrated Cdc14p was obtained (Figure 10), and when 50 µL of E2, E3, and E4 was added respectively into the PNPP reaction solution as described in section 3.7, the solution turned into bright yellow in 5 minutes (Figure 11), suggesting that the Cdc14p is active.

To identify if different buffers affect the activity of the Cdc14p, the phosphatase assay was performed in two different buffers. The activity of the purified Cdc14p was tested by measuring absorbance of the PNPP reaction solution at 409 nm, and the result indicated that the buffer did have an effect on the activity of Cdc14p (Table 1). However, which component of buffer affects the activity of Cdc14p still need more experiments.
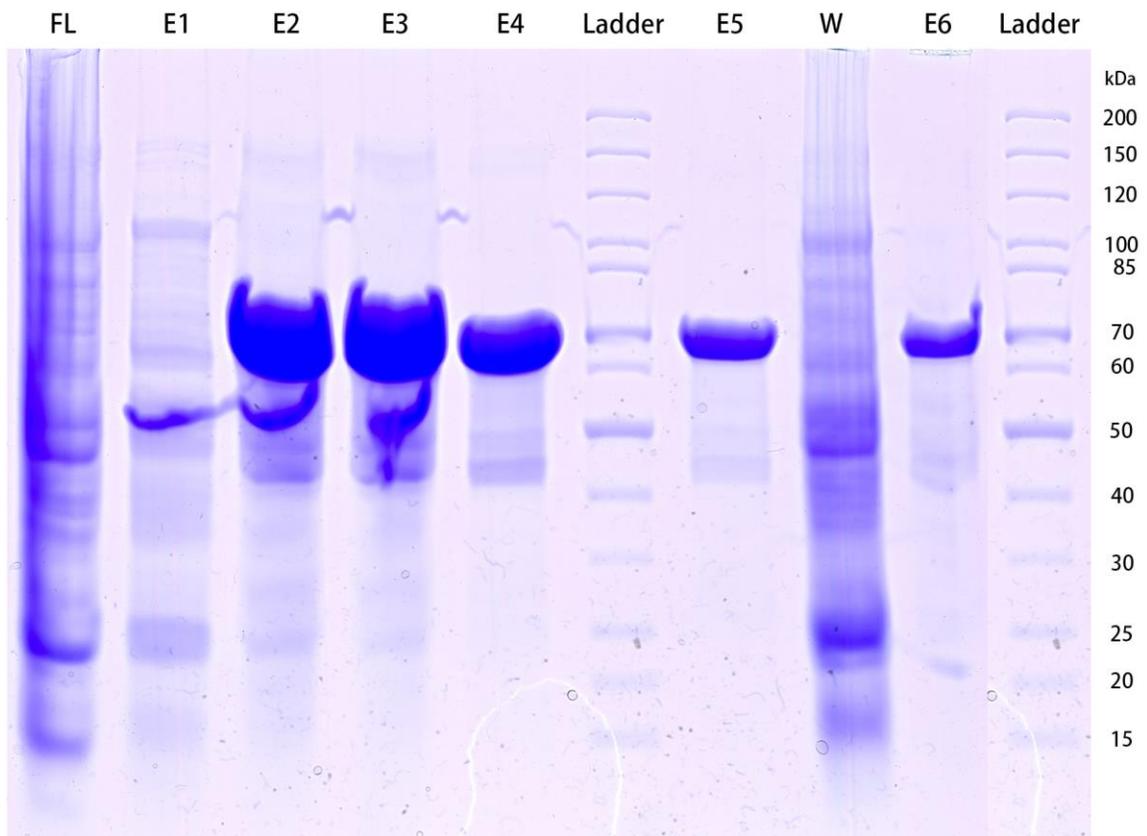
Figure 10 SDS-PAGE of Cdc14p purification under native conditions

The CDC14 was expressed at room temperature for 24 hours and the Cdc14p was purified under native conditions. The bands with size of 62 kDa in E2, E3, E4, E5, and E6 lanes are Cdc14p.
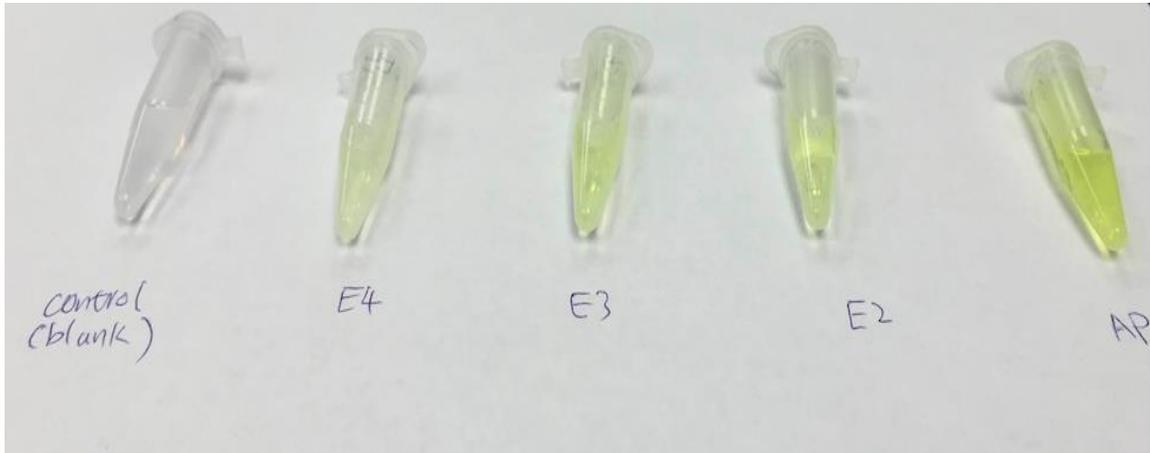
Figure 11 Result of phosphatase assay at room temperature

The blank control, the tube with colorless solution, is composed of 200 μL of 5 mM

PNPP, and 800 μL of buffer (20 mM tris, 300 mM NaCl, pH 8). AP is the alkaline

phosphatase and acts as a positive control, and 1 μL of AP was added. E2, E3, E4 are

purified Cdc14p, and 50 μL of each was added. All the solutions except blank control

turn into yellow, suggesting that the purified Cdc14p is active.

Table 1 Result of PNPP assay in two different buffers

The amount of Cdc14p	Volume of 5mM PNPP	Type of buffer (770 μL)	Absorbance at 409 nm
105 μg	200 μL	20mM Tris, 300mM NaCl, pH 8	1.066
105 μg	200 μL	50mM imidazole, 1mM DDT, 1mM EDTA, pH 6.9	0.183

CHAPTER 5 DISCUSSION

Brian Dorsey and Ethan Deckert were working on the CDC14 as part of an undergraduate research project, and they obtained large amounts of purified Cdc14p, but the Cdc14p produced by *E. coli* previously was inactive (Brian Dorsey and Ethan Deckert, unpublished results). It is possible that the Cdc14p might need to be activated by posttranslational modifications, because Cdc14p is the protein from yeast, a eukaryote, however, the CDC14 was expressed in the prokaryotic cells, where the posttranslational modification would not happen. Phosphorylation is one type of the posttranslational modification and there are some kinases, like Cdc5p and Cdc15p, which show important roles in the regulation of Cdc14p. Thus, if CDC14 is co-expressed with either CDC5 or CDC15, would the kinase Cdc5p or Cdc15p phosphorylate the Cdc14p and activate Cdc14p? To test this idea, CDC14 was co-expressed with CDC15, but the expected proteins were not produced, suggesting that the co-expression did not work.

The failure to get the Cdc15p protein or Cdc14p-Cdc15p protein complex could be due to three main reasons: 1) the CDC15 gene that contains the 6xHis tag gene was not expressed or expressed incorrectly; 2) the protein was degraded by intracellular bacterial proteases; 3) the Cdc14p and Cdc15p interaction buried the 6x His tag, making the 6x His tag not accessible to the Ni-NTA affinity column. Since no errors were found in the sequence of the cloning construct, the possibility for incorrect expression is low. A protease inhibitor PMSF had also been added during the purification procedure. To test if

the failure was caused by the hiding of the 6x His tag, the protein was purified under denaturing condition as described in section 3.6. Under denaturing condition, the protein-protein interaction and the tertiary structure of the protein would be disrupted, making the 6xHis tag be exposed to the nickel column. However, this method did not work. Next, CDC14 was co-expressed with CDC5 just like in co-expressing the CDC14 with CDC15. Again, nothing was obtained during purification based on the result of SDS-PAGE (data not shown).

As an alternative, the conditions were optimized that might affect the activity of the Cdc14p when expressing and purifying the Cdc14p. First, the CDC14 was expressed at room temperature for 24 hours instead of at 37 °C for 4 hours. However, the phosphatase assay showed that the Cdc14p was still inactive. Next, other buffers were tried when performing the purification, and the buffers tried were PBS (Phosphate-buffered saline) buffer and HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid] buffer with pH of 8. However, none of these buffers led to a positive result of phosphatase assay (data not shown).

A literature [26] showed that only part of the Cdc14p is needed for the phosphatase activity. The auto-inhibition of some phosphatases[27] gave us a hint that the phosphatase activity of the Cdc14p might be self-inhibited by the regulatory part of the protein that is not responsible for the phosphatase activity. The phosphatase part of CDC14 gene was expressed and purified. Though the phosphatase part of Cdc14p was successfully purified

(Figure 10), the phosphatase assay showed that it was still inactive.

During one test of the activity of Cdc14p, the assay remained on the bench. Surprisingly, two weeks later the solutions in the reaction tube turned slightly yellow, indicating that the Cdc14p might be active. To confirm the Cdc14p was active, the phosphatase assay was repeated. This time, one reaction tube was incubated in 37 °C water bath (in order to speed up the reaction) and another reaction tube was left on the bench at room temperature. The solution of the reaction tube at 37 °C turned slightly yellow after 7 days, but the solution in the other tube at room temperature still remained colorless. Though this result indicated that Cdc14p was active, the intensity of the yellow color was not convincing. The positive control showed an obviously bright yellow color, but the purified Cdc14p only produced a pale yellow color. This suggested that increasing the concentration of the Cdc14p could show a more convincing result. Instead of 10 μL, 50 μL of the purified Cdc14p was added to the phosphatase assay, and the bright yellow color showed up in 5 minutes.

One literature[13] suggests another buffer for Cdc14p to perform phosphatase assay. To identify if different buffers affect the activity of Cdc14p, phosphatase assay was tested in two different buffers (Table 1), and the result indicated that the buffer did have an effect on the activity of Cdc14p. However, which component of the buffer affected the activity of Cdc14p still need more experiments. In addition to buffer, will different substrates affect the activity of the Cdc14p? If we add a kinase to the Cdc14p, will this

change the activity of Cdc14p? Next, the activity of Cdc14p under different conditions will be tested. The kinase, Cdc15p or Cdc5p, will be produced, and the interactions between Cdc14p and Cdc15p or Cdc5p will be studied *in vitro*. The interactions between Cdc14p and other components in MEN will be studied, also. Exploring the interactions between Cdc14p and other components in MEN is necessary to understand the network in the mitotic exit. Understanding of the regulatory network at protein level would contribute to the research on mitosis. The research on mitosis will promote the progress in discovering the new therapies for treatment of some diseases or cancers that are caused by the deficiency in mitosis.

LITERATURE CITED

1. Lodish H, Berk A, Zipursky SL, *et al*. (2000). Molecular Cell Biology. 4th edition. New York: W. H. Freeman; Section 13.1, "Overview of the Cell Cycle and Its Control."

2. Morgan, David O. (1997). "Cyclin-dependent kinases: Engines, clocks, and microprocessors." *Annual review of cell and developmental biology*.

3. Frank Stegmeier and Angelika Amon. (2004). "Closing mitosis: The functions of the Cdc14 phosphatase and its regulation." *Annu. Rev. Genet*.

4. Visintin, Rosella, *et al*. (1998). "The phosphatase Cdc14 triggers mitotic exit by reversal of Cdk-dependent phosphorylation." *Molecular cell*.

5. Lengauer, Christoph, Kenneth W. Kinzler, and Bert Vogelstein. (1998). "Genetic instabilities in human cancers." *Nature*.

6. Pardee, Arthur B. (1974). "A restriction point for control of normal animal cell proliferation." *Proceedings of the National Academy of Sciences*.

7. Malumbres, Marcos, and Mariano Barbacid. (2009). "Cell cycle, CDKs and cancer: a changing paradigm." *Nature Reviews Cancer*.

8. Culotti, J., and Hartwell Leland H. (1971). "Genetic control of the cell division cycle in yeast: III. Seven genes controlling nuclear division." *Experimental cell research*.

9. Hartwell Leland H. (1967). "Macromolecule synthesis in temperature-sensitive

mutants of yeast." *Journal of bacteriology*.

10. Hartwell, Leland H., Joseph Culotti, and Brian Reid. (1970). "Genetic control of the cell-division cycle in yeast, I. Detection of mutants." *Proceedings of the National Academy of Sciences*.

11. Hartwell, *et al*. (1973). "Genetic control of the cell division cycle in yeast: V. Genetic analysis of cdc mutants." *Genetics*.

12. Wan, Jackson, Haixin Xu, and Michael Grunstein. (1992). "CDC14 of Saccharomyces cerevisiae. Cloning, sequence analysis, and transcription during the cell cycle." *Journal of Biological Chemistry*.

13. Taylor, Gregory S., *et al*. (1997). "The activity of Cdc14p, an oligomeric dual specificity protein phosphatase from Saccharomyces cerevisiae, is required for cell cycle progression." *Journal of Biological Chemistry*.

14. Kaiser, Brett K., *et al*. (2002). "Disruption of centrosome structure, chromosome segregation, and cytokinesis by misexpression of human Cdc14A phosphatase." *Molecular biology of the cell*.

15. Mocciaro, Annamaria, and Elmar Schiebel. (2010). "Cdc14: A highly conserved family of phosphatases with non-conserved functions?" *Journal of cell science*.

16. Mocciaro, Annamaria, *et al*. (2010). "Vertebrate cells genetically deficient for Cdc14A or Cdc14B retain DNA damage checkpoint proficiency but are impaired in DNA repair." *The Journal of cell biology*.

17. Shou, Wenying, *et al*. (1999). "Exit from mitosis is triggered by Tem1-dependent release of the protein phosphatase Cdc14 from nucleolar RENT complex." *Cell*.

18. Visintin, Rosella, Ellen S. Hwang, and Angelika Amon. (1999). "Cfi1 prevents premature exit from mitosis by anchoring Cdc14 phosphatase in the nucleolus." *Nature*.

19. Stegmeier, Frank, *et al*. (2004). "The replication fork block protein Fob1 functions as a negative regulator of the FEAR network." *Current biology*.

20. D'Amours, Damien, and Angelika Amon." (2004). "At the interface between signaling and executing anaphase—Cdc14 and the FEAR network." *Genes & development*.

21. Azzam, Ramzi, *et al*. (2004). "Phosphorylation by cyclin B-Cdk underlies release of mitotic exit activator Cdc14 from the nucleolus." *Science*.

22. Visintin, Rosella, and Angelika Amon. (2001). "Regulation of the mitotic exit protein kinases Cdc15 and Dbf2." *Molecular biology of the cell*.

23. Mohl, Dane A., *et al*. (2009). "Dbf2–Mob1 drives relocalization of protein phosphatase Cdc14 to the cytoplasm during exit from mitosis." *The Journal of cell biology*.

24. Wurzenberger, Claudia, and Daniel W. Gerlich. (2011). "Phosphatases: Providing safe passage through mitotic exit." *Nature reviews Molecular cell biology*.

25. "Basic Local Alignment Search Tool (BLAST)." Retrieved from

http://blast.ncbi.nlm.nih.gov/Blast.cgi

26. Gray, Christopher H., *et al*. (2003). "The structure of the cell cycle protein Cdc14 reveals a proline-directed protein phosphatase." *The EMBO journal*.

27. Krauss, Gerhard. Biochemistry of signal transduction and regulation. (2006). John Wiley & Sons. Chapter 9, "Ser/Thr-Specific Protein Kinases and Protein Phosphatases."

I, <u>   Jianzheng Wu   </u>, hereby submit this thesis to Emporia State University as partial fulfillment of the requirements for an advanced degree. I agree that the Library of the University may make it available to use in accordance with its regulations governing materials of this type. I further agree that quoting, photocopying, digitizing or other reproduction of this document is allowed for private study, scholarship (including teaching) and research purposes of a nonprofit nature. No copying which involves potential financial gain will be allowed without written permission of the author. I also agree to permit the Graduate School at Emporia State University to digitize and place this thesis in the ESU institutional repository.

_____

Signature of Author

_____

Date

<u> The cloning, expression, and purification of CDC14 using *Escherichia coli*</u>

Title of Thesis

_____

Signature of Graduate School Staff

_____

Date Received