The Assembly of CTP Synthase into the Cytoophidium in *Schizosaccharomyces pombe*

Jing Zhang

St Cross College

MRC Functional Genomics Unit

Department of Physiology, Anatomy and Genetics

University of Oxford



Supervisors: Professor Ji-Long Liu and Dr Andrew Bassett

A dissertation submitted for the degree of Doctor of Philosophy (DPhil)

Michaelmas Term 2018

Abstract

It is widely known that two factors can affect the activity of enzymes, the total molecular number in the cell or a subcellular compartment, or the activity of the molecule itself. Thus, spatial compartmentalization, such as sequestering enzymes in subcellular organelles, becomes an important method of regulating enzymes. Recently, several metabolic enzymes have been identified being able to form filamentous structures *in vivo*, indicating a new mode of enzymatic regulation. One of the representatives among these self-assembly enzymes is cytidine-5'triphosohate synthase (CTPS), a critical metabolic enzyme involved in *de novo* CTP synthesis. Although the catalytic function of CTPS has been well studied over decades, it is only recently observed that CTPS is able to self-assembly into filamentous structure, so called the cytoophidium (plural version as cytoophidia). The cytoophidium is evolutionarily conserved from bacteria (*Caulobactor crescentus* and *Escherichia coli*), yeast (*Saccharomyces cerevisiae*), fruit fly (*Drosophila melanogaster*) to rat (*Genus Rattus*) and human (*Homo sapiens*) cells.

In this thesis I describe using *Schizosaccharomyces pombe (shortened as S. pombe)* as a model to study the cytoophidium. Taking advantages of the *S. pombe* easily manipulated genome, I developed a set of basic protocols of manipulating fission yeast in our lab and established several libraries including hundreds of primers, plasmids and *S. pombe* strains, and therefore starts the yeast research system in our lab. Primary observation was taken in *S. pombe*, including

morphology observation, drug tests and live imaging, to have a general description for cytoophidia. The critical metabolic pathway, MAPK pathway and TOR pathway, were identified as the potential candidates involved in cytoophidia regulation. Several heat shock proteins were proved related with cytoophidia management. In addition, a novel phenomenon was observed that, unlike most other proteins, CTPS remain in filamentous form and are distributed asymmetrically during cytokinesis in *S. pombe*. All these experimental data can serve as a good primary exploration and provide a basic platform for future cytoophidia research in *S. pombe*.

Declaration

This work presented in this thesis has been conducted by the author Jing Zhang in the Department of Physiology, Anatomy and Genetics at the University of Oxford. Except acknowledgement is made, all the work presented in this thesis is all reserved by my own. I declare that this thesis has not been submitted either in the same or different form to this or other university for a degree.

Acknowledgments

This thesis includes my four years work and I received a huge amount of support from many people that I would like to acknowledge here.

First of all, I would like to thank my supervisor Dr Ji-Long Liu for giving me the opportunity to undertake this project. Without his support and help, this research and thesis will not be possible. I would say thanks for his support, patience and understanding during my difficult time and give me the freedom to approach the project.

I should also give a big thanks to my second supervisor Dr. Andrew Bassett who helped me a lot all through my research, providing ideas and help solving problems I faced with. Thanks to him sacrificing his time to discuss with me when I faced with troubles.

Another person I should also say thanks is Dr. Lydia Hulme who is very professional in fission yeast area and give me many suggestions in my project. Without her sharing experience with me, I would never be able to finish some of my experiments. I would like to express appreciation to Professor Stephen Kearsey and Dr. Israel Salguero for helping me establishing the *pombe* lab

during my first year. Samewise, I should also give a special thanks to Dr Luis Sanchez-Pulido who is very generous to spend time teaching me how to use the software PyMOL and the online database and therefore do the reconstruction of the protein 3D model. I also want to thanks our collaborator Lisa Folke who helped us with the CTP pool measurement and giving us *S. pombe* working notes and Li-lin Du who provide us *S. pombe* information that we need.

Furthermore, I wish to thanks all my lab members who are all generous with their time and knowledge, creating a friendly and positive research environment in the lab. My appreciation gives to all the students, post-docs and other members in Liu Lab including Gabriel Aughey, Jenna Schwarz, Qing-ji Shen, Ömür Yilmaz Tastan, Stuart Grice, Mayte Siswick, Yong Huang, Charlotte Tibbit, James Currie, Kemian Gou, Siân Davies and Ghows Azzam.

Aside from the academic aspect, I have also received huge support and encouragement from my friends and family outside of the lab. Thanks to my parents for funding my D.Phil research.

Thanks.

Contents

List of Figures
List of Tables
List of Videos
List of Abbreviations

1 - Introduction……..21
1.1 Metabolic regulation and enzymatic regulation……21
1.2 Self-assembly is a character of many enzymes……23
1.3 CTPS compartmentalisation is an example of enzyme self-assembly, and cytoophidia are evolutionarily conserved……27
1.4 CTPS properties and metabolic regulation……..…29
1.5 Cytoophidia can be repurposed for functions beyond catalytic functions ……34
1.6 Cytoophidia are potentially multi-component structures……36
1.7 CTPS and IMPDH related human diseases……37
1.8 *Schizosaccharomyces pombe* as a model organism……39
1.9 Aims and Outline……...……41

2 - Methods and Materials……43
2.1 Primers design and plasmids construction using overlap PCR, Infusion technique and site directed mutagenesis……43
2.2 *E. coli* transformation and plasmids purification……...……43
2.3 *S. pombe* transformation……44
2.4 Generating of CTPS-YFP expressing *S. pombe* strain……45
2.5 *S. pombe* cell culture and media making……45
2.6 DNA staining and microscopy……46
2.7 Live cell imaging……47
2.8 *S. pombe* mating and tetrad dissection……47
2.9 Random spore analysis……50
2.10 *S. pombe* whole genome DNA purification……...50
2.11 *S. pombe* total proteins purification……51
2.12 SDS-page and Western blot……52
2.13 Prediction of protein 3D structure using online database……53
2.14 Reconstruction of protein 3D model using PyMOL software……54
2.15 Establishment of basic *S. pombe* libraries, protocols and equipments…54

3 - CTPS compartmentalise into filaments (cytoophidia) in *S. pombe*……...55
3.1 Introduction……55
3.1.1 *S. pombe* CTP synthase……55
3.1.2 CTPS compartmentalisation is potentially regulated by different factors……57
3.1.3 The cell cycle and cell quiescence of *S. pombe*……59
3.1.4 Chapter aims……...……60
3.2 Results……61
3.2.1 Generation of CTPS-YFP expressing *S. pombe* strain……61
3.2.2 CTPS compartmentalisation happens in both cytoplasm and nucleus in *S. pombe*……64
3.2.3 CTPS compartmentalisation is regulated through the *S. pombe* growing stages……67
3.2.4 The stationary phase induced CTPS compartmentalisation is reversible in *S. pombe*……69
3.2.5 Cytoophidia are highly dynamic in *S. pombe*……71
3.2.6 Cytoophidia remain filaments during *S. pombe* cytokinesis……74
3.3 Discussion……77
3.3.1 Comparison of the cytoophidium between *S. cerevisiae* and *S. pombe* ……77
3.3.2 Cytoophidia regulation is related to the cell growing phase, and the stationary phase caused cytoophidia dispersal is reversible...……78
3.3.3 Cytoophidia remain filaments during cytokinesis……80
3.3.4 The nuclear cytoophidium……...81

4 – Cytoophidia respond to different environmental stresses in *S. pombe* …83
4.1 Introduction……83
4.1.1 DON is an unspecific CTPS inhibitor and induce cytoophidia in *Drosophila* and HeLa cells……83
4.1.2 Cytoophidia respond to environmental stresses in *S. cerevicae* and *Drosophila*……86
4.1.3 Heat shock proteins and temperature stress response……87
4.1.4 The MAPK pathway and stress response……91
4.1.5 The TOR pathway and nitrogen starvation……96
4.1.6 Chapter aims……99
4.2 Results……100
4.2.1 Cytoophidia respond to several drugs including DON……100
4.2.2 Cytoophidia respond to different environmental stresses……108

4.2.3 Both cold stress and heat stress disperse cytoophidia……115
4.2.4 Cytoophidia respond very quickly to both heat and cold stresses ……118
4.2.5 Cold-induced cytoophidia disassembly is reversible……121
4.2.6 Protein level of CTPS is not changed by cold stress……124
4.2.7 DON cannot prevent cold shock induced cytoophidia disassembly ……126
4.2.8 Heat shock proteins are related with cytoophidia assembly…129
4.2.9 Do MAPK pathway regulate cytoophidia? ……132
4.3 Discussion……135
4.3.1 Cytoophidia are promoted by DON and respond to several different drugs……135
4.3.2 Cytoophidia and stress response……139
4.3.3 Heat shock proteins and temperature stress response……140
4.3.4 Improvement of the cytoophidium analysis procedure……141
4.3.5 Cytoophidia assembly may be an energy consuming process…142
4.3.6 Potential functions of cytoophidia in *S. pombe*……143

5 - Asymmetric distribution of CTPS in *S. pombe* during cytokinesis….145
5.1 Introduction……145
5.1.1 *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*……145
5.1.2 Symmetric and asymmetric inheritance during cytokinesis…….146
5.1.4 Chapter aims……147
5.2 Results……149
5.2.1 Both cytoplasmic and nuclear cytoophidia are inherited asymmetrically during cell cytokinesis……149
5.2.2 Cytoophidia inheritance is a stochastic process……152
5.2.3 Both C-and N-cytoophidia are highly dynamic……155
5.2.4 Asymmetric inheritance of cytoophidia during cytokinesis leads to different CTPS level between the two daughter cells after cell division……159
5.3 Discussion……162
5.3.1 Cytoplasmic and nuclear cytoophidia……162
5.3.2 Dynamics of cytoophidia……163
5.3.3 Asymmetries of *S. pombe* cells……164

6 - Truncated deletions & Point mutations of CTPS……167
6.1 Introduction……167

6.1.1 CTPS N-terminal is conserved and critical for cytoophidia formation in *Drosophila*……167
6.1.2 CTPS structure models in *Escherichia coli, Thermus thermophiles* and *Sulfolobus solfataricus*……170
6.1.3 Chapter aims……172
6.2 Results……173
6.2.1 Prediction of CTPS 3D reconstruction in *S. pombe*……167
6.2.2 Truncated CTPS show different cluster patterns in *S. pombe*……177
6.3 Discussion……183
6.3.1 Truncated CTPS and the cytoophidium……183
6.3.2 Future work……184

7 – Discussion and conclusion……188
7.1 Overview……188
7.2 *S. pombe* as a tool for studying the cytoophidium……190
7.3 Cytoophidia and cytoskeletons……192
7.4 Possible advantages of filaments formation……193
7.5 CTPS and the cytoophidium in the study of disease ……196
7.6 Future work……...198
7.6.1 CTPS structure and point mutation design……198
7.6.2 The MAPK pathway related work……199
7.6.3 The TOR pathway and related work……199
7.7 Concluding remarks……200

References
Appendices

List of figures

Figures for Chapter 1

Figures	Page
Figure 1.1 Factors affecting the activity of enzymes.	22
Figure 1.2 Cytoophidia are highly conserved in different organisms.	28
Figure 1.3 The metabolic process of pyrimidine *de novo* synthesis.	31
Figure 1.4 The reaction of *de novo* synthesis of CTP catalyzed by CTPS.	32

Figures for Chapter 2

Figures	Page
Figure 2.1 Tetrad dissection matrix	49

Figures for Chapter 3

Figures	Page
Figure 3.1.1 CTPS is encoded at single locus *cts1* and has only one isoform.	56
Figure 3.1.2 Only CTPS isoform-A is able to compartmentalise into filaments in *Drosophila*.	58
Figure 3.1.3 Abbreviated diagram of *S. pombe* cell cycle.	60
Figure 3.2.1 Construction of the pSMUY2-*cts1* plasmid.	62
Figure 3.2.2 Generation of the CTPS-YFP expressing *S. pombe* strain.	63
Figure 3.2.3 The compartmentalisation of CTPS can be observed in both nucleus and cytooplasm in *S. pombe*.	65-66
Figure 3.2.4 Cells growth condition affects cytoophidia in *S. pombe*.	68
Figure 3.2.5 The stationary phase induced CTPS assembly is reversible in *S. pombe*.	70
Figure 3.2.6 Cytoophidia are highly dynamic in *S. pombe.*	72-73
Figure 3.2.7 Cytoophidia remain filaments during *S. pombe* cytokinesis.	75-76

Figures for Chapter 4

Figures	Page
Figure 4.1.1 The CTPS can form filaments in both cytoplasm and nucleus in HeLa cells.	85
Figure 4.1.2 Components and signalling model of the MAP kinase pathway in *S. pombe*.	94
Figure 4.1.3 Stress activation of the Sty1 MAP kinase pathway.	95
Figure 4.1.4 Schematic model of TORC1 and TORC2 in *S. pombe*.	97
Figure 4.1.5 A summary diagram of metabolic changes in *S. pombe* cells at the onset of nitrogen starvation.	99
Figure 4.2.1 DON treatment causes an increase of cytoophidia length in *S. pombe.*	103-104
Figure 4.2.2 DON treatment did not affect the CTPS protein level.	105
Figure 4.2.3 Cytoophidia respond differently with different drugs.	106-107
Figure 4.2.4 *S. pombe* cytoophidia respond to different environmental stresses.	111
Figure 4.2.5 H_2O treatment decrease the number of cytoophidia in *S. pombe*.	113-114
Figure 4.2.6 Both high and low temperature cause the decrease of cytoophidia in *S. pombe*.	116-117
Figure 4.2.7 Cytoophidia respond quickly to both heat and cold stresses.	119-120

Figure 4.2.8 Cold-induced cytoophidia dispersal is reversible and fast.	122-123
Figure 4.2.9 CTPS protein level remains unchanged under cold stress.	125
Figure 4.2.10 DON induced cytoophidia can be disassembled by cold stress.	127-128
Figure 4.2.11 Cytoophidia morphology in small heat shock protein deletion strains.	130-131
Figure 4.2.12 *atf1* and *pyp2* are potentioal regulators of cytoophidia	133-134

Figures for Chapter 5

Figures	Page
Figure 5.2.1 C-cytoophidia is inherited by only one daughter cell during *S. pombe* cytokinesis.	150
Figure 5.2.2 Differential inheritance of cytoophidia in *S. pombe*.	151
Figure 5.2.3 *S. pombe* cells are morphologically asymmetric and polarized.	154
Figure 5.2.4 Cytoophidia are dynamic and constrained in *S. pombe*.	157
Figure 5.2.5 *de novo* formation of cytoophidia in *S. pombe*.	158
Figure 5.2.6 Asymmetric distribution of CTPS between paired cells under cold stress.	160-161

Figures for Chapter 6

Figures	Page
Figure 6.1.1 The *Drosophila* CTPS gene locus encodes three isoforms.	168
Figure 6.1.2 BLAST result of CTPS among different species.	169
Figure 6.1.3 The sequence alignment of CTP synthases from *S. solfataricus, E. coli* and *T. thermophilus*	171
Figure 6.2.1 3D reconstruction of *S. pombe* CTPS	175
Figure 6.2.2 The 1-56 amino acids (coloured in red) are in the interior of the protein.	176
Figure 6.2.3 Design of truncate deletion strains.	178
Figure 6.2.4 Truncated CTPS-YFP proteins show different compartmentalisation patterns in *S. pombe*.	180
Figure 6.2.5 Analysis of CTPS truncated deletion strains in *S. pombe*: percentage of cells that contain CTPS foci or filaments.	181-182
Figure 6.3.1 High-throughput screen process and detail	187

List of Tables

Chapter	Tables	Page
1	Table 1.1 Enzymes being able to form into filamentous structures and their regulators.	26
4	Table 4.1.1 Enzymes inhibited by DON.	84
4	Table 4.1.2 Heat shock proteins and their functions in *S. pombe*	89
4	Table 4.1.3 Heat shock proteins and their orthologues.	90
4	Table 4.2.1 Effects and mechanisms of drugs tested in experiments.	101
4	Table 4.2.2 List of drugs and conditions that tested in experiments.	102
4	Table 4.2.3 List of stresses that tested	110
6	Table 6.3.1 Point mutation designs	186
appendices	Oligos used in experiments	225
appendices	Plasmids used in experiments	232
appendices	*S. pombe* strains used in experiments	236
appendices	Media used in experiments	243
appendices	CTPS related diseases	247

List of videos

Chapter	Videos
3	Video 1 Cytoophidia are highly dynamic in *S. pombe*.
3	Video 2 Cytoophidia remain filaments during *S. pombe* cytokinesis.
4	Video 3 Cold-induced cytoophidia disassembly is reversible in *S. pombe* and the recovery is fast.
5	Video 4 One cell shows the C-cytoophidium confined within the cytoplasm between the nucleus and cell tip, and N-cytoophidium confined within the nucleus.
5	Video 5 Several cells show the movement of C-cytoophidia and N-cytoophidia.
5	Video 6 G2 phase correlated with the differential inheritance of cytoophidia.
5	Video 7 Long-period time lapse live imaging shows the daughter cell generates *de novo* cytoophidia if not inherited cytoophidia from the mother cell.

List of Abbreviations

ACC	Acetyl-CoA carboxylase
Ack	Activated cdc42-associated kinase
Amp	Ampicillin
ATP	Adenosine triphosphate
BPB	Bromophenol Blue
Carb	Carbenicillin
C-cytoophidium	Cytoplasmic cytoophidium
Cdc2	Cell division control protein 2
Cdc42	Cell division control protein 42
CDK	Cyclin-dependent kinase
cDNA	Complementary DNA
CTP	Cytidine-5′-triphosphate
CTPS	CTP synthase (protein)
cts1	CTP synthase (gene)
C-terminus	Carboxyl-terminus
DNA	Deoxyribonucleic acid
DON	6-Diazo-5-oxo-L-norleucine
DTT	Dithiothreitol
EMM	Edinburgh minimal medium

EYFP	Enhanced yellow fluorescent protein
GFP	Green fluorescent protein
GTP	Guanosine triphosphate
HHpred	Homology detection & structure prediction by HMM-HMM comparison
HOG	High Osmolarity Glycerol
HSF	Heat shock factor
hsp	Heat shock protein (gene in *S. pombe*)
Hsp	Heat shock protein (protein in *S. pombe*)
HU	Hydroxyurea
IMP	Inosine-5′-monophosphate
IMPDH	Inosine-5′-monophosphate dehydrogenase
MAP	Mitogen-activated protein
MAPK	MAP kinase
MAPKK	MAP kinase kinase
MAPKKK	MAP kinase kinase kinase
MPA	Mycophenolic acid
N-cytoophidium	Nuclear cytoophidium
N-terminus	Amino-terminus
PBS	Phosphate buffered saline
PBST	PBS with Triton-X

PCR	Polymerase chain reaction
PFA	Paraformaldehyde
PMG	*S. pombe* glutamate medium
rpm	Revolutions per minute
RNA	Ribonucleic acid
RNR	Ribonucleotide reductase
SDS	Sodium Dodecyl Sulfate
SDS - PAGE	SDS - Polyacrylamide gel electrophoresis
SMN	Survivor Motor Neuron
SPAS	*S. pombe* Mating/Sporulation media
TBS	Tris-buffered saline
TBST	Tris-buffered saline, 0.1% Tween 20
TCA	Trichloroacetic Acid
TOR	The target of rapamycin
TORC	TOR complex
YES	Yeast extract with supplements
YFP	Yellow fluorescent protein
YPD	Yeast extract peptone dextrose

Chapter 1 Introduction

1.1 Metabolic regulation and enzymatic regulation

Metabolic regulation is a common central topic studied in biological research. It is important that the metabolic enzymes are tightly regulated and controlled so as to maintain the homeostasis of the cells and the whole organism. Cell metabolism is regulated by a variety of mechanisms, either by changing the number of enzyme molecules or by changing the existing enzyme's activity (**Figure 1.1**) (Lehninger, A. L., et al., 2013).

Protein degradation is one of the important methods of altering the number of enzyme molecules. Once synthesised, proteins have their specific half-lives depending on the conditions in the cell. These differs from one enzyme to another, ranging from minutes to several days. Enzymes with a short half-life have a rapid turnover (synthesis followed by degradation), which gives them a fast responsiveness but a high cost (Lehninger, A. L., et al., 2013).

Another strategy with lower cost to modulate an enzyme's effective activity is to sequester the enzyme and its substrate in different compartments. Enzymes can be compartmentalised into subcellular organelles to segregate them from their substrates and other enzyme systems. Once needed, the enzymes or their substrates will be transported to the target where the catalytic reaction takes

place. This strategy has the advantages of a lower consumption of energy and a more rapid response to environment changes (Lehninger, A. L., et al., 2013). For example, when enzymes are temporarily not in use, they can be stored in higher-order cytoplasmic structures for potential future use instead of being degraded, for instance filaments or other structures compounds. When cells are in specific conditions and the enzymes are needed, the compartmentalisation can be dispersed and depolymerised enzymes can take functions immediately instead of being resynthesised.

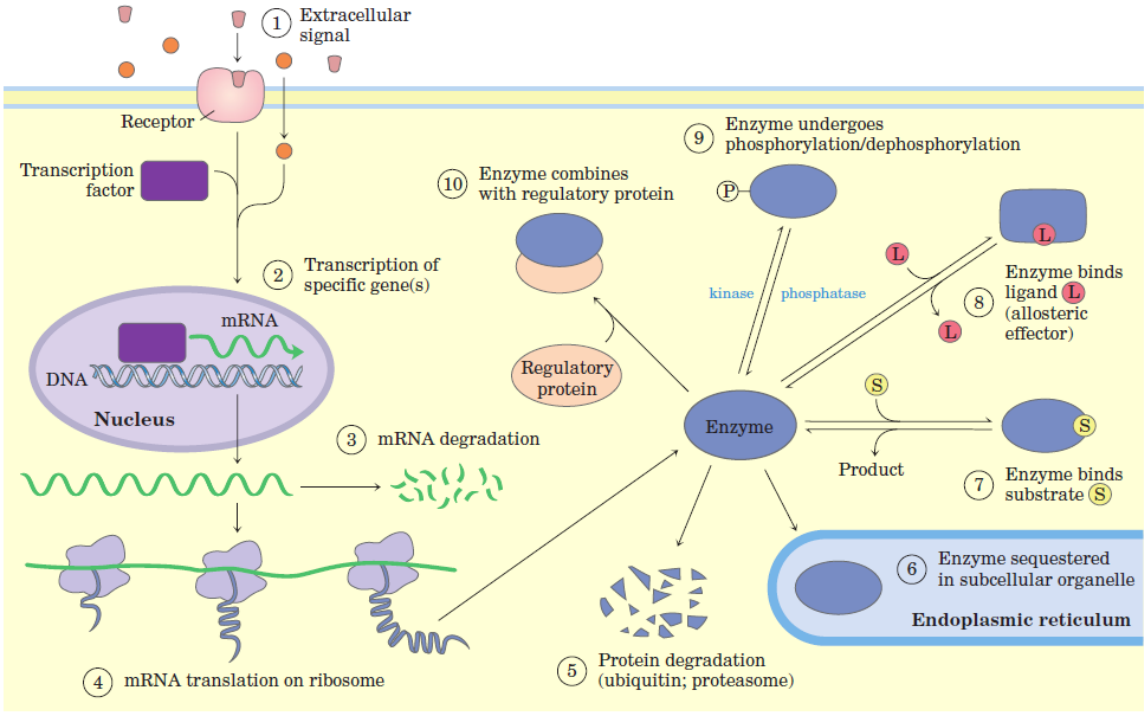


Figure 1.1 Factors affecting the activity of enzymes. The activity of an enzyme can be altered by the number of molecules in the cell, or its effective activity in a subcellular compartment (1 to 6), or by modulating the activity of existing molecules (7 to 10). An enzyme can be influenced by a combination of these factors. (Lehninger, A. L., et al., 2013)

1.2 Self-assembly is a character of many enzymes

Protein compartmentalisation is one of the commonly observed mechanisms in cells and is important for cell metabolic regulation (Ovadi, J. & Saks, V., 2004). There are hundreds of metabolic enzymes across diverse organisms that are capable of self-assembling into filaments (O'Connell, J. D., et al., 2012). The first discovered enzyme possessing the ability to self-compartmentalise was Acetyl-CoA carboxylase (ACC) (Gregolin, C., et al., 1966), an enzyme that is widely known to catalyse the irreversible step of acetyl-CoA carboxylation in fatty acid biosynthesis. The filamentous form of ACC shows an upregulated enzymatic activity (Gregolin, C., et al., 1966), and it is speculated that it takes the structural role in fatty acid synthesis, although no concrete evidence has been found to support this idea (Beaty, N. B. & Lane, M. D., 1983; Kim, C. W., et al., 2010).

Following the discovery of ACC filaments and their regulation mechanism through enzyme compartmentalisation, more and more metabolic enzymes have been observed forming filaments under different conditions (**Table 1.1**). For example, arginosuccinase (argininosuccinatelyase) and glutamate dehydrogenase are able to form filaments *in vitro* (Gregolin, C., et al., 1966; Kleinschmidt, A. K., et al., 1969; Haschemeyer, R. H. & De Harven, E., 1974). In budding yeast, CTPS, glutamate synthase, glutamine synthase and GDP-mannose pyrophosphorylase (Psa1p) are all able to form filaments (Noree, C., et al., 2010; Petrovska, I., et al., 2014). Interestingly, the filaments of budding yeast

CTPS, glutamine synthase and glutamate synthase can be promoted by starvation and low cellular pH and disassembled by high cellular pH (Petrovska, I., et al., 2014).

In 2006, the Yushida lab published the complete ORFeome cloning analysis of *S. pombe* protein localisation, showing that 953 proteins form dots and 19 proteins form filamentous structure in cells (Matsuyama, A., et al., 2006). One of our collaborators in Beijing, the Li-lin Du lab, who is working on *S. pombe* and did a genome-wide protein screening, providing a short supplemented list of proteins capable of compartmentalising into filaments. Among the 24 filament-forming proteins, 62.5% (15) are enzymes having catalytic functions (Matsuyama, A., et al., 2006), indicating that compartmentalisation of metabolic enzymes is far more widespread than previously thought.

Aside from the upregulated activity of ACC after compartmentalisation, other enzymes activity can be regulated through filament forming, including CTPS and IMPDH **(Table 1.1)**. Several mechanisms can be proposed and speculated about for enzyme regulation through compartmentalisation:

1. The polymerisation of enzymes can hide (or expose) the ubiquitin binding sites to prevent (or enhance) protein degradation;
2. The polymerisation of enzymes can hide (or expose) an enzyme's catalytic centre or substrate binding sites, and therefore inhibit (or

enhance) an enzyme's catalytic functions;

3. The compartmentalisation can hide (or expose) phosphorylation or other modulation sites to prevent (or enhance) an enzyme's post-translational process, and therefore prevent (or enhance) an enzyme's functions; and
4. The polymerisation can conformationally change an enzyme's structure, and consequently change (upregulate or downregulate) an enzyme's functions.

Enzyme name	Activity on assembly	Induced by	Dissociated by	References
Acetyl-CoA carboxylase (ACC)	Enhanced	Citrate; MIG12	Malonyl-CoA	Beaty, N. B. & Lane, M. D., 1983[b]; Kim, C. W., et al., 2010; Kleinschmidt, A. K., et al., 1969; Gregolin, C., et al., 1966.
Cytidine-5′-triphosphate synthase (CTPS)	Reduced	Glutamine analogues; Stationary phase; Low pH; Starvation; CTP	High pH	Ingerson-Mahar, M., et al., 2010; Liu, J. L., 2010; Noree, C., et al., 2010; Petrovska, I., et al., 2014.
Human inosine-5′-monophosphate dehydrogenase 2 (IMPDH2)	Enhanced	MPA, Ribavirin	Over-expression of IMPDH2	Chang, C. C., et al., 2015; Carcamo, W. C., et al., 2011
Glutamate synthase	Unknown	Starvation; Low pH; Stationary phase	High pH	Noree, C., et al., 2010; Petrovska, I., et al., 2014.
GDP-mannose pyrophosphorylase	Unknown	Unknown	Unknown	Noree, C., et al., 2010.
eIF2/2B	Unknown	Unknown	Unknown	Noree, C., et al., 2010.
Glutamine synthase	Reduced	Zinc; Low pH	High pH	Miller et al., 1974; Petrovska, I., et al., 2014.
Activated Cdc42 kinase (ACK)	Unknown	Unknown	Unknown	Strochlic, T. I., et al., 2014.
β-glucosidase	Enhanced	Unknown	Unknown	Kim, S. Y., et al., 2005; O'Connell, J. D., et al., 2012.
Glutamate dehydrogenase (GDH)	Controversial	ADP	GTP, NADH	Zeiri & Reisler, 1978; Haschemeyer, R. H. & De Harven, E., 1974.
Arginosuccinase	Unknown	Unknown	Unknown	Haschemeyer, R. H. & De Harven, E., 1974
IRE1	Enhanced	Stress response	Unknown	Korennykh et al., 2009; Li et al., 2010.
Asparagine synthetase	Unknown	Unknown	Unknown	Narayanaswamy, R., et al., 2009.
Mps1/Polo	Unknown	Hypoxia	Unknown	Gilliland et al., 2009.
Nitrilase	Unknown	Unknown	Unknown	Thuku et al., 2007.

Table 1.1 Enzymes being able to form into filamentous structures and their regulators.

1.3 CTPS compartmentalisation is an example of enzyme self-assembly, and cytoophidia are evolutionarily conserved

As early as 1987, a novel filamentous structure was observed in cultured cells using a monoclonal antibody (Willingham, M., 1987). Morphologically similar cellular filaments have since been observed several times (Berman, J., 1989; Carcamo, W.C., et al., 2011). However, the constituent proteins of these filaments has never been identified. In 2010, an important metabolic enzyme, Cytidine-5'triphosohate synthase (CTPS/CTP synthase), was identified by several groups of researchers independently as the major constituent of a novel filamentous structure in *Drosophila melanogaster* **(Figure 1.2 B)** (Liu, J.L., 2010), bacteria *C. crescentus* **(Figure 1.2 E and F)** (Ingerson-Mahar, M., et al., 2010), budding yeast *S. cerevisiae* **(Figure 1.2 C and D)** and rat hippocampal neurons (Noree, C., et al., 2010). These newly discovered CTPS filaments were morphologically similar to each other and have been termed "the cytoophidium" (plural version as cytoophidia), due to their snake-like morphology (*cyto* means cell in Greek, and *ophidia* means serpent in Latin) (Liu, J.L., 2010). Later on, the CTPS filaments are also observed in human cell line HeLa cells (**Figure 1.2 A**) (Chen, K., et al., 2011), indicating that self-assembly is a widely conserved property of CTPS. The lineages for bacteria and humans separated more than 3 billion years ago, yet the cytoophidia in both organisms are highly conserved, suggesting that cytoophidia might represent a novel type of organelle.

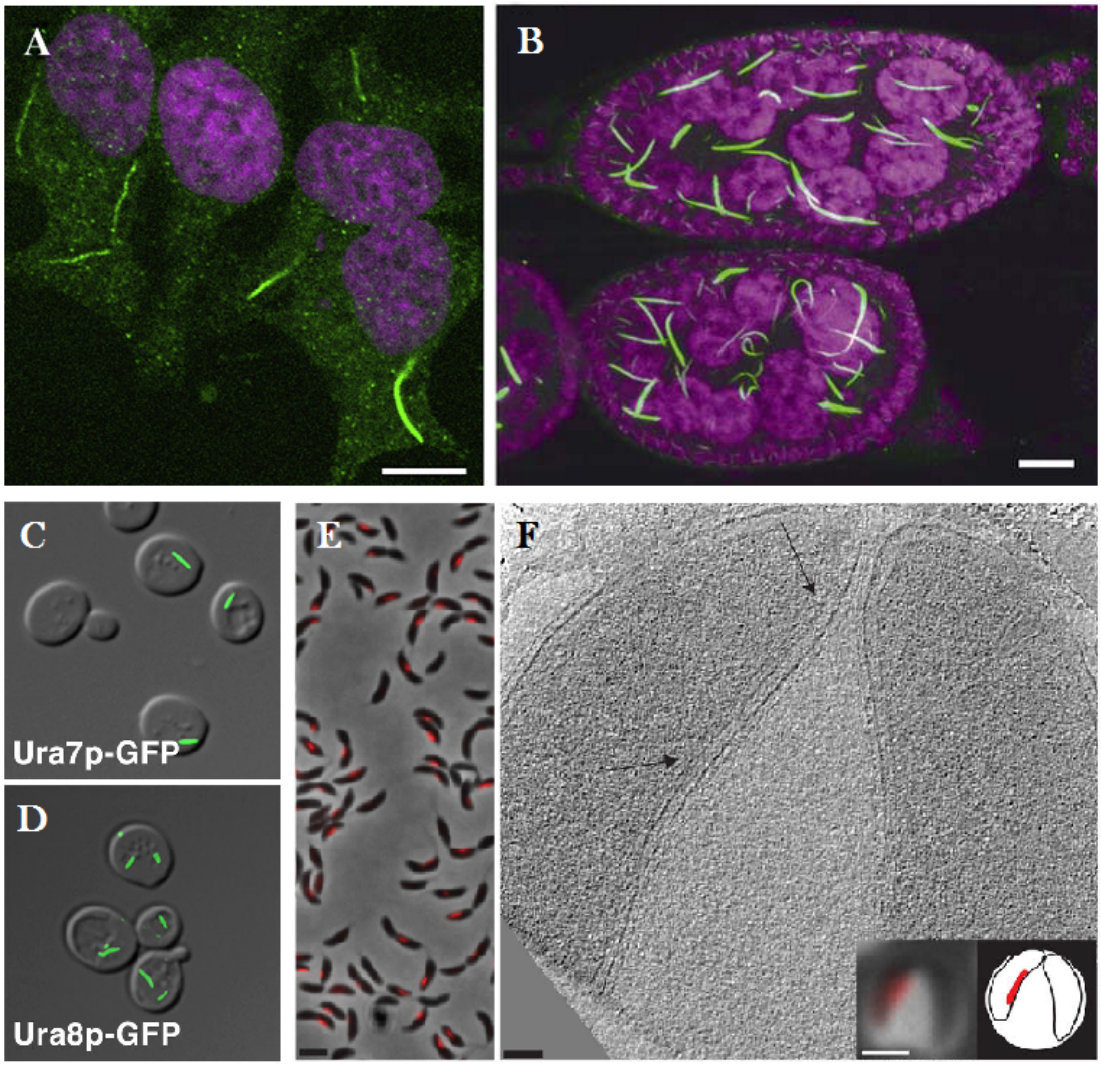


Figure 1.2 Cytoophidia are highly conserved in different organisms. (A) Cytoophidia in Hela cells. Scale bar: 10μm (Chen, K., et al., 2011) (B) Cytoophidia in a *Drosophila* egg chamber. Scale bar 10μm (Liu, J. L., 2010) (C-D) Both isoforms of CTPS Ura7p and Ura8p can compartmentalise into cytoophidia in budding yeast. (Noree, C., et al., 2010) (E) Cytoophidia in *Caulobacter crescentus*. Scale bar: 2μm (Ingerson-Mahar, M., et al., 2010) (F) electron cryotomography (ECT) image of cytoophidia in *Caulobacter crescentus*. Scale bars: 100nm for ECT image and 1μm for the inset light microscope images (Ingerson-Mahar, M., et al., 2010)

In higher-level multicellular organisms, cytoophidia show tissue-specific expression. In fruit flies (*D. melanogaster*), cytoophidia can be observed only in some specific cell types under normal conditions, including female ovaries, the optic proliferation centre of the larval CNS, the larval lymph gland, and the testis (Liu, J. L., 2010). The cytoophidia can also be observed in the male accessory gland, gut, trachea, and salivary gland (Liu, J. L., 2010). In rats (*Rattus norvegicus*), cytoophidia are restricted to the axons of hippocampal neurons (Noree, C., et al., 2010). The widespread conservation of CTPS compartmentalisation among diverse species suggests that the formation of cytoophidia might have some potential biological functions other than a basic CTPS role in catalysis (Liu, J. L., 2011).

1.4 CTPS properties and metabolic regulation

CTPS is an important metabolic enzyme which catalyses the final step of pyrimidine nucleotides *de novo* synthesis **(Figure 1.3)**. It contains two major domains, the CTP synthase domain and the glutamine amidotransferase (GATase) domain, which are responsible respectively for the synthase and aminotransferase activity. The reaction of CTP synthesis involves all four nucleotides **(Figure 1.4)**, especially the substrate uridine-5'-triphosphate (UTP) and the product cytosine-5'-triphosphate (CTP), which are essential building blocks of RNA, as well as the structural components of a series of enzyme cofactors and metabolic intermediates (Lehninger, A. L., et al., 2013). During the reaction, the glutamine is first hydrolysed in the GATase domain, generating an amino group, before being transferred to the synthase domain, where

the ATP-dependent phosphorylation of UTP takes place and reacts with the generated amino group, resulting in the synthesis of CTP (Endrizzi, J. A., et al., 2004) **(Figure 1.4).**

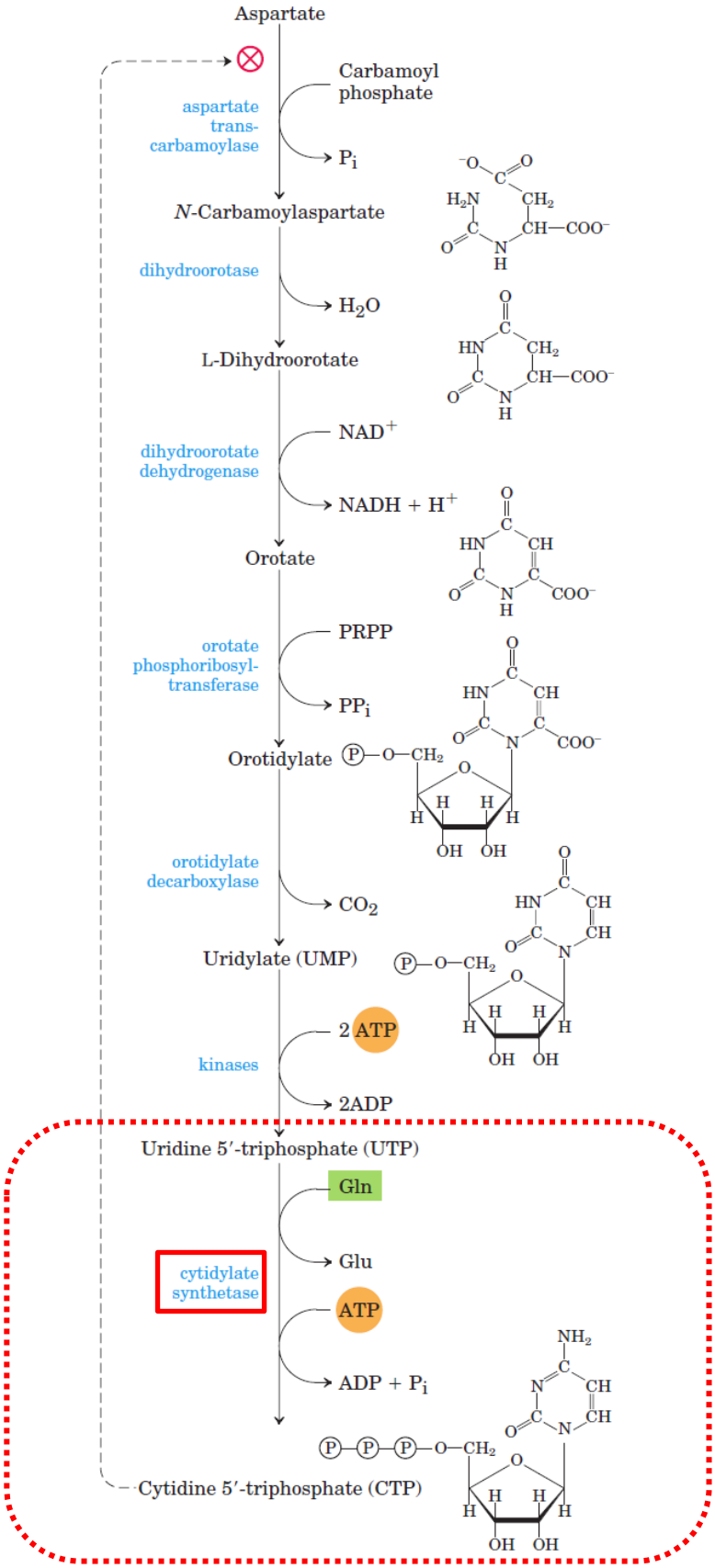


Figure 1.3 The metabolic process of pyrimidine *de novo* synthesis. CTPS catalyses the final step of CTP synthesis (in the red frame). Figure adapted from Lehninger, *Principles of Biochemistry*.

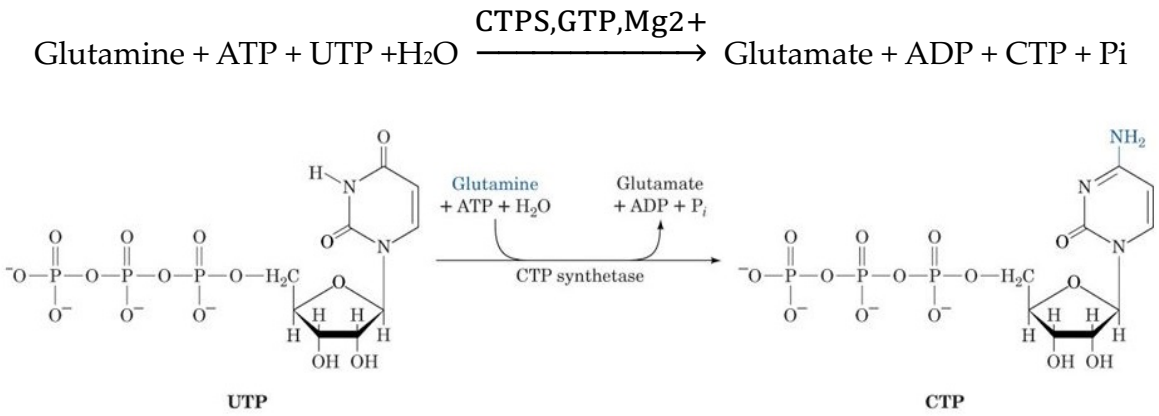


Figure 1.4 The reaction of *de novo* synthesis of CTP catalysed by CTPS. Figure adapted from Lehninger, *Principles of Biochemistry*.

CTPS is the rate-limiting enzyme for both *de novo* and the uridine salvage pathway of cytosine nucleotide synthesis. Active CTPS presents in the homotetrameric form. When CTPS concentration is low and ATP and UTP are absent, CTPS exists as an inactive monomer. As the CTPS concentration gets higher, it forms first into dimers, then (in the presence of ATP and UTP) it polymerises into tetramers and becomes active (Lauritsen, I., et al., 2011).

As the rate-limiting enzyme for one of the most important anabolic reactions, CTPS is tightly regulated through several different mechanisms, including phosphorylation (Yang, W. L. & Carman, G. M., 1995; Yang, W. L. & Carman, G. M., 1996; Choi, M. G., et al., 2003; Park, T. S., et al., 2003; Kassel, K. M., et al., 2010), feedback inhibition (McLaren, J. D. & Chu, E. H., 1984; Endrizzi, J. A., et al., 2005; Oberti, D., et al., 2015), competitive and allosteric interactions (Ovejera, A. A., et al., 1979; Bearne, S. L., et al., 2001) and transcriptional control (Meng, Q., et al., 2004). CTPS activity can be directly regulated by all four nucleotides. ATP and UTP can positively regulate CTPS activity as the substrates binding (Long, C. W. & Pardee, A. B., 1967; Robertson, J. G., 1995). CTP can bind the CTPS with negative cooperativity as a feedback inhibition mechanism (McLaren, J. D. & Chu, E. H., 1984; Endrizzi, J. A., et al., 2005). Notably, GTP is a positive allosteric effector and promotes glutamine hydrolysis at low concentration. In contrast, it becomes the negative allosteric regulator when the concentration exceeds 0.15mM (MacDonnell, J. E., et al., 2004).

A newly established regulation mechanism of CTPS has recently been demonstrated. Several published papers have revealed that compartmentalisation of CTPS can downregulate its activity (Barry, R. M., et al., 2014; Noree, C., et al., 2014; Aughey, G. N., et al., 2014). CTP strongly induces compartmentalisation of CTPS filaments *in vitro,* and a downregulated activity of filamentous CTPS was detected by simultaneously monitoring CTPS assembly and enzymatic activity (Barry, R. M., et al., 2014). This result is supported by the experiment in budding yeast, which showed that a mutation at the CTP feedback inhibition binding site (E160K) lead to the dispersal of CTPS filaments (Noree, C., et al., 2014). A similar experiment with *Drosophila* showed that the inhibition or over-expression of CTPS can highly promote cytoophidia formation (Aughey, G. N., et al., 2014). These data have promoted the idea of a new mechanism of enzyme regulation, in which the oligomerisation of CTPS can downregulate the CTPS catalytic activity.

1.5 Cytoophidia can be repurposed for functions beyond catalytic functions

The compartmentalisation of CTPS has shown a downregulation of CTPS catalytic activity, indicating the cytoophidium playing a role of enzyme activity regulator (Barry, R. M., et al., 2014; Noree, C., et al., 2014; Aughey, G. N., et al., 2014). Aside from the catalytic regulator, cytoophidia can be repurposed for other functions.

The structural function is one of the potential roles cytoophidia may serve. Several reviews have discussed the evolution of cytoskeletal proteins, speculating that they

may have evolved from a common ancestor. It has also been proposed that dual-role filaments formed by self-assembly metabolic enzymes may be the origin of cytoskeletal proteins (Barry, R. M. & Gitai, Z., 2011; Ingerson-Mahar, M. & Gitai, Z., 2012; Ingerson-Mahar, M., et al., 2010). In bacteria *C. crescentus*, CTPS filaments participate in the regulation of cell shape independently of its enzymatic activity, and regulate cell curvature through crescentin (a curvature regulator in *C. crescentus*). CTPS and crescentin functionally interact with each other and colocalise to the inner curvature of *Caulobacter* cell. In addition, *E. coli* CTPS compartmentalisation happens both *in vivo* and *in vitro*, resembling many cytoskeletal proteins. *E. coli* CTPS can complement both the metabolic and cytoskeletal functions of *C. caulobacter* CTPS (Ingerson- Mahar, M., et al., 2010). These data lead to the speculation that CTPS acquired the ability of filament formation first, then evolved the structural function and served as the dual-role protein in *C. caulobacter*.

An enzyme "depot" is another potential function for cytoophidia that has been proposed. The purified *E. coli* CTPS can form filaments *in vitro*, and was sequestered in a tetramer state with the active sites being readily accessed but conformationally changed, so that the CTPS could be rapidly reactivated and depolymerised (Barry, R. M., et al., 2014). This strategy can store enzymes in a conformationally restricted form and offer a quick response when CTPS is required, giving cells the advantage of maintaining metabolic homeostasis more easily and more quickly. Whether other enzymes aside from CTPS also employ filament-mediated regulatory strategies might

be an interesting topic for future research.

1.6 Cytoophidia are potentially multi-component structures

Cytoophidia were first described as a filamentous structure consisting of CTPS and potentially other constituents (Liu, J. L., 2010; Noree, C., et al., 2010; Ingerson-Mahar, M., et al., 2010). Later on, a critical enzyme involved in purine *de novo* synthesis, inosine-5′-monophophate dehydrogenase (IMPDH), was identified of colocalisation with CTPS (Carcamo, W. C., et al., 2011; Calise, S.J., et al., 2014), indicating it may be another constituent of cytoophidia. IMPDH catalyses the rate-limiting and reversible step of GTP *de novo* biosynthesis, converting inosine monophosphate (IMP) to xanthosine monophosphate (XMP) (Hedstrom, L., 2009). Although the colocalisation of CTPS and IMPDH has been observed, there is no direct evidence showing whether these two enzymes bind directly, but cross-talk between the two enzymes has been speculated due to their closely related functions (pyrimidine and purine *de novo* biosynthesis) (Aughey, G. N., Liu, J. L., 2015). A more recently published paper, however, revealed that CTPS and IMPDH are only partially colocalised and form two independent but closely related filaments (Keppeke, G. D., et al., 2015).

In *Drosophila*, the activated CDC42 kinase (Ack), also known as DAck or p145, has also been observed to colocalise with CTPS (Strochlic, T. I., et al., 2014). *Drosophila* Ack is conserved with mammalian ACK1 (TNK2), which explains why ACK1 also colocalises with CTPS in the human MCF7 cell line, indicating that the colocalisation of CTPS and

Ack is evolutionarily conserved (Strochlic, T. I., et al., 2014). Mutation of Ack, however, only partially inhibits cytoophidia assembly (Strochlic, T. I., et al., 2014). The previously published study in budding yeast has demonstrated the insignificant influence that phosphorylation has on CTPS assembly (Noree, C., et al., 2014), suggesting that Ack may act as an indirect regulator of CTPS compartmentalisation.

No other component of cytoophidia has yet been reported. The confocal microscopy scanning result in *Drosophila* with anti-CTPS antibody staining has demonstrated the observation of 'gaps' in cytoophidia, suggesting there may be other constituents aside from CTPS in cytoophidia (Liu, J. L. 2010).

1.7 CTPS and IMPDH-related human diseases

Nucleotide synthesis is one of the most important metabolic processes in all organisms. The failure in maintenance of the nucleotide homeostatic level is often the cause of human diseases, and the enzymes responsible for nucleotide biosynthesis are often the clinical and therapeutic targets for cancer and disease treatment. Nucleotide analogues and their precursor analogues are often used as chemotherapeutic agents to treat cancers and other diseases. CTPS and IMPDH are both critical enzymes involved in nucleotide *de novo* synthesis (CTPS catalyses the last step of CTP *de novo* synthesis, and IMPDH is responsible for the rate-limiting step of GTP *de novo* synthesis). In the Chinese hamster ovary cell model of leukaemia, mutations of CTPS-specific conserved sites induced the resistance of chemotherapeutic drugs such as

cytosine arabinoside (ara-C) and the increase of the intracellular CTP level (Whelan, J., et al., 1994). Upregulation of CTPS activity has also been widely observed in human and rodent tumours (Williams, J. C., et al., 1978; Kizaki, H., et al., 1980) and is inhibited by an anticancer agent called cyclopentenyl cytosine (van den Berg, A. A., et al., 1995; Verschuur, A. C., et al., 2000); this is reasonable because the biological activities and cellular metabolism are normally maintained at a much higher level in tumours compared with normal condition cells, and the increased CTPS activity can support the large demand of nucleotides and lipid biosynthesis required by the cancer cell for proliferation. The increased activity of CTP synthase may confer selective advantages on proliferating cells, suggesting that CTPS is an important target in chemotherapy design.

CTPS is also related to severe immunodeficiency disease. The loss-of-function mutation of human CTPS gene CTPS1 leads to the expression of the abnormal transcriptional product and results in a complete loss of CTPS expression. The lack of CTPS can cause the defects in T and B cells responding to antigen-mediated activation, and consequently appears as a life-threatening immunodeficiency disease (Martin, E., et al., 2014). Other CTPS related diseases and the detailed information about them are listed in **Appendix 5**. The human IMPDH1 is related to the hereditary disease Retinitis pigmentosa (RP) and Leber congenital amaurosis (LCA) (Bowne, S. J., et al., 2006). The mutation in IMPDH1 results in a gradual loss of rod photoreceptors and cone photoreceptors, as well as causing the pigment deposits on the fundus, consequently causing the patients to lose their vision (Bowne, S. J., et al., 2006).

In *Drosophila*, the CTPS expression level is consistent with the expression level of the proto-oncogene Myc during different developmental progress (Aughey, G. N., 2016). The knocking down of Myc leads to cytoophidia disassembly while Myc overexpression promotes cytoophidia length growing. In human cells, Myc-bingding sites have been identified in the CTPS gene locus, suggesting the potentially direct role Myc takes in transcriptional regulation of CTPS (Wu, C. H., et al., 2008; Liu, Y. C., et al., 2008). This is supported by the experiment in *Drosophila,* which showed that the CTPS mRNA level was increased by the overexpression of Myc and decreased when Myc was knocked down (Aughey, G. N., et al., 2016). Though there is no clear evidence showing direct regulation of Myc upon cytoophidia, the correlation of cytoophidia with Myc makes it a potentially marker for monitoring the Myc expression level.

1.8 *Schizosaccharomyces pombe* as a model organism

Schizosaccharomyces pombe, so-called fission yeast, is a traditional organism model often used in molecular and cell biology research. It was first isolated in 1893 by Paul Lindner from East Africa millet beer, and used as an experimental tool in 1950s by Urs Leupold for studying genetics and by Murdoch Mitchison for studying the cell cycle. In 2001, Paul Nurse won the Nobel Prize in Physiology or Medicine for his work in fission yeast genetics and cell cycle research (Fantes, P. A. & Hoffman, C. S., 2016). The following year, the whole genome sequence of *S. pombe* was published, making it the sixth model eukaryotic organism to have its genome fully sequenced (Wood, V., et al.,

2002). In 2006, using green fluorescent protein tags, almost all the protein's sub-cellular localisation in *S. pombe* was confirmed and published (Matsuyama, A., et al., 2006).

S. pombe is a unicellular eukaryote with a genome of approximately 14.1 million base pairs, containing around 4970 protein-coding genes and at least 450 non-coding RNAs. Among all these genes, only 5% have duplicated genes, making it easier for researchers to approach functional studies, and a large number (43%) of the *S. pombe* genes contain introns among studied 4739 genes. Compared with *S. cerevisae*, which has 16 short chromosomes, *S. pombe* has only 3 chromosomes, 100-300 times longer than those of *S. cerevisae* chromosomes. In addition, a 97% conserved 1780 bp region has been discovered in the DGS region of the centromere in *S.* pombe (Wood, V., et al., 2002). *S. pombe* does cell fission and splits cells into two identical daughter cells during cytokinesis, which is very similar to human cell cytokinesis. The similitude between *S. pombe* cells and human cells, and the simplicity of the *S. pombe* genome, make fission yeast a good model system to apply in the study of higher eukaryotes for their functions and mechanisms.

The budding yeast *Saccharomyces cerevisiae* and the fission yeast *Schizosaccharomyces pombe* are the most studied yeast species, and perhaps the most studied unicellular eukaryotes (Forsburg, S. L., 2005). Both yeast species provide excellent models for fundamental biological research. They have been used for the study of various

biological processes including cell growth, cell cycle, intracellular trafficking, metabolism, organelles, and cell division (Mitchison, J. M., 1957; Mitchison, J. M., 1958). *S. pombe* is also widely used in studying DNA damage response and DNA replication processes. Notably, *S. pombe* has been revealed of 50 genes or mutants linked to human diseases, nearly half (23) of which are cancer-related, making it a good model for studying human diseases (Wood, V., et al., 2002).

1.9 Aims and outline

The property of protein to compartmentalise into filaments has been observed among more and more enzymes. Notably, one of the central cell metabolic enzymes, CTPS, is evolutionarily conserved to form filamentous structures among cells from bacteria, yeast, fruit flies, rats and humans. The CTPS filaments, termed cytoophidia, represent a group of enzymes sequestrating into cytoplasmic filaments and are a good model for studying protein compartmentalisation mechanisms. Research has been carried out into several organisms, but never in *S. pombe*. The aims of this thesis is to establish the basic platform for cytoophidia research to *S. pombe,* and to make up for the lack of functional studies in the field of cytoophidia.

The data in this thesis demonstrate that cytoophidia are highly dynamic in cells, and that the assembly of cytoophidia is reversible. We also show that cytoophidia can be observed in both nucleus and cytoplasm. Interestingly, cytoophidia remain filamentous form and the CTPS showed asymmetric distribution during cell

cytokinesis, which is firstly observed in *S. pombe*. Cytoophidia in *S. pombe* have been observed to respond to different drugs as well as to several environmental stresses, including oxidative stress, osmotic stress, temperature stress (cold shock and heat shock) and nutrient deprivation, promoting the speculation that the MAPK pathway and the TOR pathway may be involved in the regulation of cytoophidia, and providing a better understanding of cytoophidia functions in *S. pombe*. Future experiments related to the MAPK and TOR pathways are worth doing to investigate the functions of cytoophidia. Lastly, several CTPS truncated strains are constructed, and different phenotypes are observed. The identification of critical regions for cytoophidia assembly narrows down the range of critical amino acids that regulate cytoophidia compartmentalisation and provides the basis for future structural studies of cytoophidia.

Chapter 2 Methods and Materials

To achieve our research goals and obtain experimental data, several different techniques including genetic, biochemical, microscopic and computational approaches have been applied to the investigation in this thesis. The method and materials employed in this project are as follows.

2.1 Primers design and plasmids construction using overlap PCR, In-fusion® technique and Site directed mutagenesis

In-fusion® Cloning and QuikChange® Site-Directed Mutagenesis technologies were utilised in this project to generate target point mutated plasmids. The standard commercial protocol was used for the detail manipulation of plasmid construction. The primers used in In-fusion® and QuickChange® are designed on the website. For the overlap PCR, the overlaps of the primers were designed with 25 base pairs at least. The full list of primers used in this thesis is in **Appendix 1**.

2.2 *E. coli* transformation and plasmids purification

50 μL Competent DH5α cells were used per transformation. Cells were thawed on ice for 20 minutes, then 2 μL PCR product or 0.5 μL plasmids were added. Heat shock was applied to the cells at 42°C for 30 seconds, then put back on ice for 2 minutes. 150 μL fresh LB media was added to the cells, which were then incubated at 37°C for 1 hour. Cells were plated on selective plates and incubated overnight at 37°C.

Five *E. coli* colonies were picked and transferred into 10 mL LB media with appropriate antibiotics and incubated at 37℃ in a shaker for one overnight. The following day, it was anticipated that the cells should have grown into a high concentration and the media should show a cloudy appearance. The QIAprep Spin Miniprep Kit was used and the manufactural protocol for plasmids purification was followed.

2.3 *S. pombe* transformation

The lithium acetate (LiAc) transformation procedure in Paul Nurse Lab Manual was used for *S. pombe* transformation. Cells were grown up to 10^7 cells/mL ($OD_{600} \approx 0.5$) in YES media. 10 mL of the cells was taken per transformation and washed once with an equal volume of sterile water, then re-suspended in 1 mL water and transferred to 1.7 mL Eppendorf tube. Cells were spun down cells at 4,000 rpm for 1 minute and re-suspended in 1 mL of LiAc/TE liquid, then suspended in 50 μL LiAc/TE (10mM Tris, 1mM EDTA, 0.1 M LiAc, pH7.5) liquid and mixed with 2 μL of sheared carrier DNA (10 mg/mL) and 10 μL transforming DNA. They were incubated for 10 minutes at room temperature and then 260 μL 40%PEG/LiAc/TE (400 mg/mL PEG 4000, 10 mM Tris, 1 mM EDTA, 0.1 M LiAc, pH7.5) was added, mixed gently and incubated for 30-60 minutes at 30℃. 43 μL DMSO was added and the cells were heat shocked for 5 minutes at 42℃, then washed once with 1 mL sterile water and plated on selective media plates. The plates were incubated at 32℃ for 3 days, then the clones were checked and could be observed. If G418 was used as marker, cells were firstly plated

on YES plates without selective markers and incubated at 32℃ for one overnight, then replica plated onto YES plates containing antibiotic (100 μg/mL).

2.4 Generation of CTPS-YFP expressing *S. pombe* strain

The C-terminus fragment of *cts1* gene was amplified by PCR from *S. pombe* genomic DNA with primers containing *ApaI* and Xhol restriction sites. Following digestion with *ApaI* and XholI (New England Biolab), the *cts1* fragment was ligated into plasmid pSMUY2+ (**Figure 3.2.1 A**) using T4 DNA ligase (New England Biolab). The resulting plasmid was termed pSMUY2-*cts1* (**Figure 2.1**). The pSMUY2-*cts1* plasmid was sequenced and confirmed as having no mutant within the *cts1* sequence part. The pSMUY2-*cts1* plasmid was then linearised with SpeI (New England Biolab) and transformed into the wild-type *S. pombe* strain 001 (*h+; ade6-M216, leu1-32, ura4-D18*) (**Appendix 3**) using the LiAc method (described in section 2.3). The generated *S. pombe* strain consequently expressed CTPS-YFP using the endogenous promoter at the endogenous locus.

2.5 *S. pombe* cell culture and media making

If not otherwise mentioned, all cells were cultured at 32℃ in YES media using a shaking incubator. Cell growth was monitored by OD_{600}, with a 0.1~1.0 OD_{600} value indicating exponentially growing cells and a value over 1.0 OD_{600} indicating stationary cells. Two wild type *S. pombe* strains 001 (*h+; ade6-M216, leu1-32, ura4-D18*) and 002 (*h-; ade6-M216, leu1-32, ura4-D18*) were from the Stephen Kearsey Lab. 003 (*h+; ade6-M216,*

leu1-32, ura+, cts1-yfp) was used as the control strain unless stated otherwise. A full list of the *S. pombe* strains used in this study can be found in **Appendix 3**. The media used in this project can be found in **Appendix 4**.

2.6 DNA staining and microscopy

Cells were cultured overnight before being fixed and scanned, thus guaranteed to be fresh and at an exponential stage. Cells were fixed with 4% PFA in PBS for 10 minutes and then washed with an equal volume of sterile water 3 times. Fixed cells can be kept in PBS at 4 ℃ for a month or mounted directly onto slides for later scanning. Hoescht 33342 (1 μg/ml) was used to label DNA. For mounting, 5 μL of cells was dropped on a slide, immediately mixed with 5 μL of warmed-up agarose and then covered with a coverslip and pushed with the right thumb at proper strength. The edges of the coverslip were carefully brushed with transparent nail polish and left until it dried.

All samples were imaged using a Leica TCS SP5 II confocal microscope or Nikon A1R+ confocal microscope. The 60X objective lens was used for the microscopy. All experiments were undertaken three times independently, and each time 10 pictures (each picture normally contain morethan 50 cells) were taken as samples. Cells were scanned using Z stacks.

Image processing and analysis was conducted using ImageJ (cell counting and cell length measuring) and GraphPad Prism 7 (error bar editing and p value calculating).

For the cell counting and cell length measuring using ImageJ, follow the steps shown below:

1. Open the figures you want to analyse;
2. Image –> Type –> 8-bit;
3. Image –> Adjust –> Threshold;
4. Analyse –> Set Measurements –> tick Feret's diameter;
5. Analyse –> Analyse Particles;
6. Get results.

2.7 Live cell imaging

For live imaging, *S. pombe* cells were cultured in glass-bottomed Petri dishes with YES and kept in the 32°C chamber. Imaging of time lapse for live cells was acquired under a 60 objective lens using the Leica TCS SP5 II confocal microscope or the Nikon A1R+ confocal microscope.

2.8 *S. pombe* mating and tetrad dissection

Before the mating experiment, *h*+ and *h*- cells were cultured on YES plates at 32°C overnight. *h*+ (*h*-) cells were taken from a fresh plate (the edge cells of the colony) and spread in a small circle. *h*- (*h*+) cells were taken from fresh plate (edge cells of the colony) and mixed together with the *h*+ (*h*-) cells. 5-10 μL sterile water was dropped on the mixed cells, which were mixed again to guarantee that the *h*+ cells and *h*- cells could contact each other easily. The plate was incubated at 25°C for 2-3 nights (the first

day in an upward position, and the second day in a backward position). Normally the tetrads would generate after two nights for the wild-type strains, while mutant strains would take longer.

The cells were inspected under a light microscope until the tetrad could be easily observed. Tetrads were picked up from the edge of the colony and placed into the matrix on a flat YE3S plate (**Figure 2.1**). After picking up, the plate position was recorded and cells incubated at 19°C for one night (16 hours).

To separate the four spores generated after mating, the tetrads were inspected and separated using a Singer® dissection microscope MSM 400. Each spore was put on the grid cross and left at 32°C to let the spores grow into colonies.

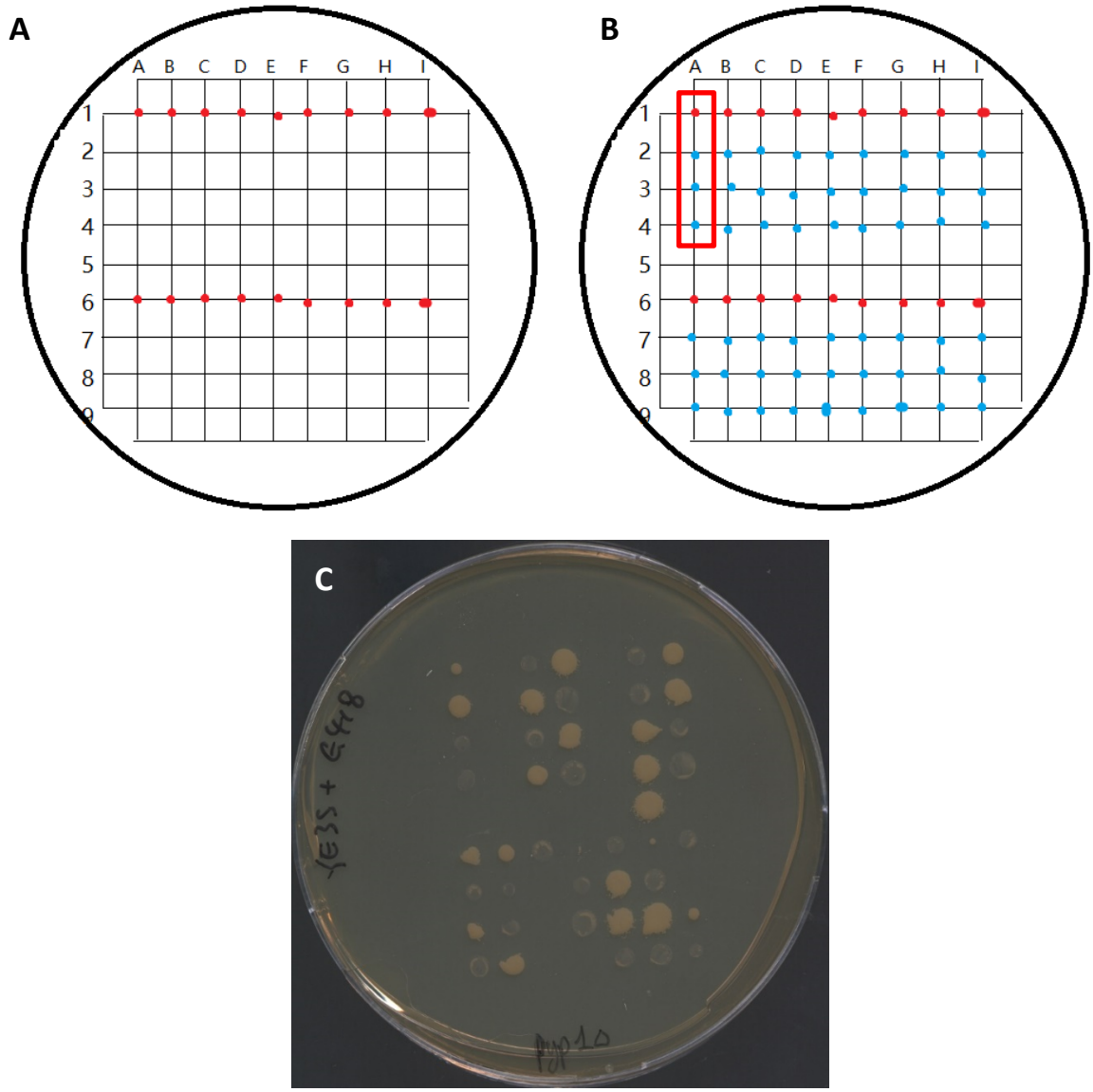


Figure 2.1 Tetrad dissection matrix. A) The tetrads were placed on the first and sixth row of the matrix (positions marked as red dots). B) When a tetrad was dissected, the four spores were separated and placed in a row. Take the tetrad at the position A1 as an example. The four spores generated from the tetrad were separated and placed on A1, A2, A3 and A4 (positions marked as blue dots). So the four spores in the red square would grow into four daughter cells generated from the same tetrad. Other tetrads have the same dissecting pattern. C) After the tetrad dissection, the plate was incubated at 32℃ for several days until visible colonies had grown. Different spores with different genotypes expressed different colony growing phenotypes. Some colonies grew healthily on the plate, while some showed a limited growth condition.

2.9 Random spore analysis

The Paul Nurse Lab Manual (Hagan, I. M., et al., 2016) was used as a standard protocol. A random spore analysis allows many more spores to be examined than in a tetrad analysis. In this way, recombination mapping and strain construction can be carried out. However, all the classes of spores should be viable to study recombination frequencies, otherwise tetrad analysis is still necessary.

A three days old mating was used to check for the presence of tetrads under the light microscope. A loopful of the mated cells was put into 1 mL sterile water, added with 2 μL Helicase (a crude snail gut enzyme that breaks down the ascus wall and kills vegetative cells) or 5 μL glusulase to the mixture and incubated at 25-29℃ for overnight. A haemocytometer was used to check the number/mL of spores. We plated 200-1000 spores per plate on YES or selective plates, then incubated them at 32℃ until colonies formed.

2.10 *S. pombe* whole genome DNA purification

S. pombe cells were grown overnight and washed once with sterile water. Cells were re-suspended in 0.2 mL Lysis buffer (2% Triton X-100 1% SDS, 100 mM NaCl, 10 mM Tris-Cl pH8, 1 mM EDTA pH8), 0.2 mL phenol:chloroform together with 0.5 mL glass beads (425-600 μm; SIGMA G8772) and vortexed for 10 minutes. 0.2 mL TE (10 mM Tris-Cl pH8, 1 mM EDTA pH8) was added and centrifuged at 13,000 rpm for 5 min. The supernatant was moved into new 1.7 mL Eppendorf tube and 1 mL 100% ethanol

was added. The cells were centrifuged at 13,000 rpm for 5 minutes and washed with 100% ethanol once. The supernatant was discarded, and the pellet was dried at room temperature. The pellet was resuspended in 50-100 μL Buffer EB.

2.11 *S. pombe* total proteins purification

The TCA protein extraction method was used. The cells were grown to OD_{600} of 0.2 and centrifuged at 4000 rpm for 5 minutes, then re-suspend in 1 mL 1.2 M Sorbitol and transferred to a 2 mL Eppendorf tube. The cells were centrifuged at full speed for 30 second and the supernatant was discarded. We added 100 μL 20% TCA (100 g TCA in 227 mL water) to re-suspend the cells, then added 0.5 mL micro beads (425-600 μm; SIGMA G8772) and put them under vortex movement using Genie2 for 10 minutes at 4℃. We let it stand for 1 minute, then put it under vortex movement for another 1 minute, then added 900 μL 5% TCA (25 g TCA in 227 mL water) and vortexed it briefly. 800 μL extract was taken and transferred to a fresh 1.7 mL Eppendorf tube and the cells were centrifuged for 10 minutes at 3000 rpm. The supernatant was discarded. The precipitate was added with 250 μL of 1X Laemmli (62.5 mM Tris, 10% glycerol, 2% SDS, 5% Beta-mercaptoethanol, 0.05% BPB) and the mixture was re-suspended (if the solution turned yellow, more 1 M Triswas added). The solution was boiled for 3 minutes and cooled on ice, then centrifuged at 3000 rpm for 10 minutes. The supernatant was transferred to a fresh 1.7 mL Eppendorf tube. The extracts could be used immediately or kept frozen at -20℃. If the frozen samples were used, the samples were thaw by boiling for 3 minutes before use.

2.12 SDS-PAGE and Western blot

The SDS-PAGE and Western blot were performed using the Invitrogen NuPAGE® MOPS System. The precast gel (NuPAGE® Novex® Bis-Tris gels) was taken out of the packet and rinsed with distilled water, then the comb and the white strip at the bottom were removed. The gel was clipped into the module (the comb facing the inside of the tank) and the inner chamber was filled with 200 mL running buffer (NuPAGE® MOPS SDS Running Buffer) added with 500 μL antioxidant. The outer chamber was filled with the running buffer to make sure the buffer level was above the bottom of the gel. Samples and ladder were carefully loaded (maximum of 25 μL in 10 well gels, 20 μL in 12 well gels, 10 μL in 15 well gels) and ran at 200 V for 48 minutes.

For transferring, we cut 4 pieces of filter paper to the same size as the sponge. The sponges and filter paper were rinsed with transfer buffer (NuPAGE® Transfer Buffer), then two sponges and two pieces of filter paper were stcked in orders. The gel was cracked open and the wells were cut off at the top and the bottom. The gel was then placed on top of the pre-rinsed sponges and filter paper. The nitrocellulose or PVDF (it should be soaked in menthol beforehand) was soaked for 30 seconds in the transfer buffer and then put on top of the gel. The pre-rinsed two pieces of filter paper and two sponges on were then placed on the top of the membrane. The stack was placed in a transfer module and the gel was closer to the negative electrode and the membrane was closer to the positive electrode. The module was sealed and topped up with transfer buffer. The transfer was done at 30 V for 1 hour.

For blocking, we rinsed and shook the membrane in TBST (0.1% Tween 20, 137 mM NaCl, 2.7 mM KCl, 19 mM Tris base) for 5 minutes. The membrane was taken out and blocked in 5% skimmed milk (5% milk in TBST), then shook for one night at 4℃.

For antibody staining, the membrane was incubated in the primary antibody solution (5% milk in TBST + 1:200 antibodies, the dilution times for antibodies can vary) for one night at 4℃. The membrane was washed with TBST 3 times, each time for 10 minutes (with shaking). Then the membrane was incubated with the secondary antibody solution (5% milk in TBST + 1:5000 antibodies) at room temperature for 1 hour. We used the Chemiluminescence Kit for the visualisation.

2.13 Prediction of CTPS structure using online databases.

Several online databases were used to predict the *S. pombe* CTPS structure. The protein database was used for the CTPS amino acid sequence (http://www.uniprot.org/) and other CTPS structural information (http://www.uniprot.org/uniprot/O42644). Pfam (http://pfam.xfam.org/) and SMART (http://smart.embl-heidelberg.de/) were also searched for domain information. The HHpred section of the Bioinformatics Toolkit (https://toolkit.tuebingen.mpg.de/hhpred) was used to generate the predicted structure of *S. pombe* CTPS.

2.14 Reconstruction of the protein 3D model using PyMOL software

We downloaded the PyMOL software from the Internet and imported the file generated from HHpred. The 3D model was produced automatically by the software. The stained colours in protein 3D structures were marked manually following the predicted protein function information.

2.15 Establishment of basic *S. pombe* libraries, protocols and equipments

To accomplish the aim of this thesis, several basic libraries were established in Liu Lab to support the cytoophidium research in *S. pombe*, including the oligos library **(Appendix 1)**, the *S. pombe* plasmids library **(Appendix 2)** and the *S. pombe* strains library **(Appendix 3).** Each library contains the full list of materials used in this thesis. Some basic protocols were also tested, corrected and reorganised. Some basic equipment was also purchased to support the research, such as the tetrad dissector and the incubator shakers. All these preparations helped establish a standard *S. pombe* lab and lay the essential foundations for future investigations of cytoophidia in Liu lab.

Chapter 3 CTPS compartmentalise into filaments (cytoophidia) in *S. pombe*

3.1 Introduction

3.1.1 *S. pombe* CTP synthase

As mentioned in Chapter 1, CTP synthase (CTPS) is a critical enzyme involved in nucleotide biosynthesis in almost all organisms and is highly conserved from bacteria to human cells. The enzyme has two major domains: the N-terminal CTP synthase domain, responsible for the synthase activity, and the C-terminal glutamine amidotransferase (GATase) domain, responsible for the amidotransferase activity **(Figure 3.1.1 B**) (Endrizzi, J. A., et al., 2004; Goto, M., et al., 2004). In human and budding yeast *S. cerevisiae*, two genes encode CTPS (*CTPS1* and *CTPS2* in humans, *URA7* and *URA8* in *S. cerevisiae*), while in fission yeast *S. pombe*, only one CTPS isoform exists, and encoded at a single locus *cts1* on chromosome I (**Figure 3.1.1 A**). This explains why *cts1*Δ show the phenotype of inviability (Kim, D. U., et al., 2010; Hayles, J., et al., 2013). The lack of CTPS redundancy in *S. pombe* gives it an advantage as a model for cytoophidia studies and makes it easier to apply to CTPS functional research. In *S. pombe*, CTPS takes the role of G0 stage maintenance and negative regulation of G0 to G1 transition (Sajiki, K., et al., 2009). CTPS can also be bound by ubiquitin and regulated through ubiquitin-mediated protein degradation (Beckley, J. R., et al., 2015).

Expression microarray evidence and the mass spectrometry evidence have demonstrated that the CTPS expression level varies with different cell conditions. The CTPS in RNA level is decreased when cells are exposed to stresses including heat, oxidative stress and osmotic stress. The treatment of cadmium sulfate or methyl methanesulfonate can also downregulate the CTPS in RNA expression (Chen, D., et al., 2003). The CTPS expression level is also regulated through the cell growing stage in *S. pombe*. The S phase cells have a decreased CTPS level by around 10% compared with the other three phases (G1, G2 and M phase) (Carpy, A., et al., 2014). Other than the microarray and the mass spectrometry results, the G1 arrest due to nitrogen deprivation can lead to a strongly decreased CTPS level by around 5 times (Marguerat, S., et al., 2012). These data demonstrates that the CTPS is regulated by stress responses and cell growth, but whether the regulation is direct or not remains unclear.

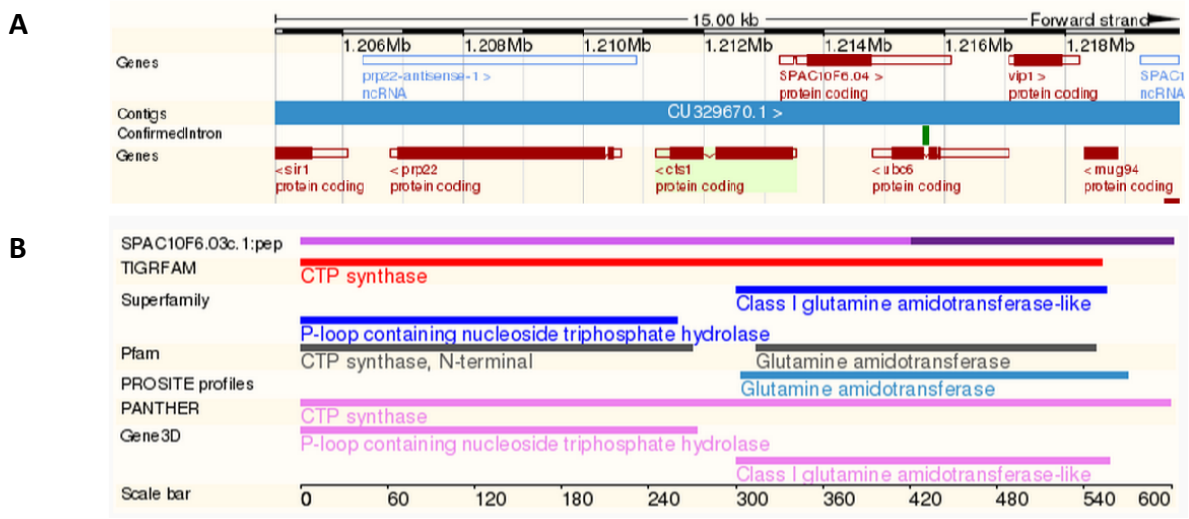


Figure 3.1.1 CTPS is encoded at single locus *cts1* and has only one isoform. (A)The CTPS protein coding gene locus *cts1* is on chromosome I. (B) CTPS has two domains: the CTP synthase domain and the glutamine amidotransferase (GATase) domain.

3.1.2 CTPS compartmentalisation is potentially regulated by different factors

Previous published papers have demonstrated that cytoophidia formation is affected by several different factors. For example, Liu lab have demonstrated that in *Drosophila*, only CTPS isoform-A is able to compartmentalise into filaments, indicating the N-terminal of CTPS is critical for cytoophidia compartmentalisation in *Drosophila* (**Figure 3.1.2**). In *C. Crescentus*, mild over-expression of CTPS gave an increased length of CTPS filaments, while the strong over-expression of CTPS resulted in the alteration of filament morphology (Ingerson-Mahar, M., et al., 2010), indicating that the abundance of CTPS protein is closely linked to cytoophidia assembly. In budding yeast *S. cerevisiae*, treatment with staurosporine, a non-specific kinase inhibitor, caused an increased number of cytoophidia (Noree, C., et al., 2010), suggesting that cytoophidia may be regulated through a phosphorylation process. The Ack (activated Cdc42 kinase) was also observed to colocalise with CTPS in *Drosophila*, supporting the speculation that cytoophidia may be regulated by phosphorylation (Strochlic, T. I., et al., 2014). Carbon source depletion showed a strong effect on cytoophidia in budding yeast, and cytoophidia can undergo rapid assembly and disassembly in response to changes in nutrient conditions, which indicates that the presence of glucose in the growth media is a central regulator of cytoophidia in budding yeast (Noree, C., et al., 2010). This phenomenon has been confirmed by another research group (Petrovska, I., et al., 2014). Besides the phosphorylation and the nutrient deprivation, cytoophidia are also regulated by several stresses in budding yeast (Noree, C., 2010).

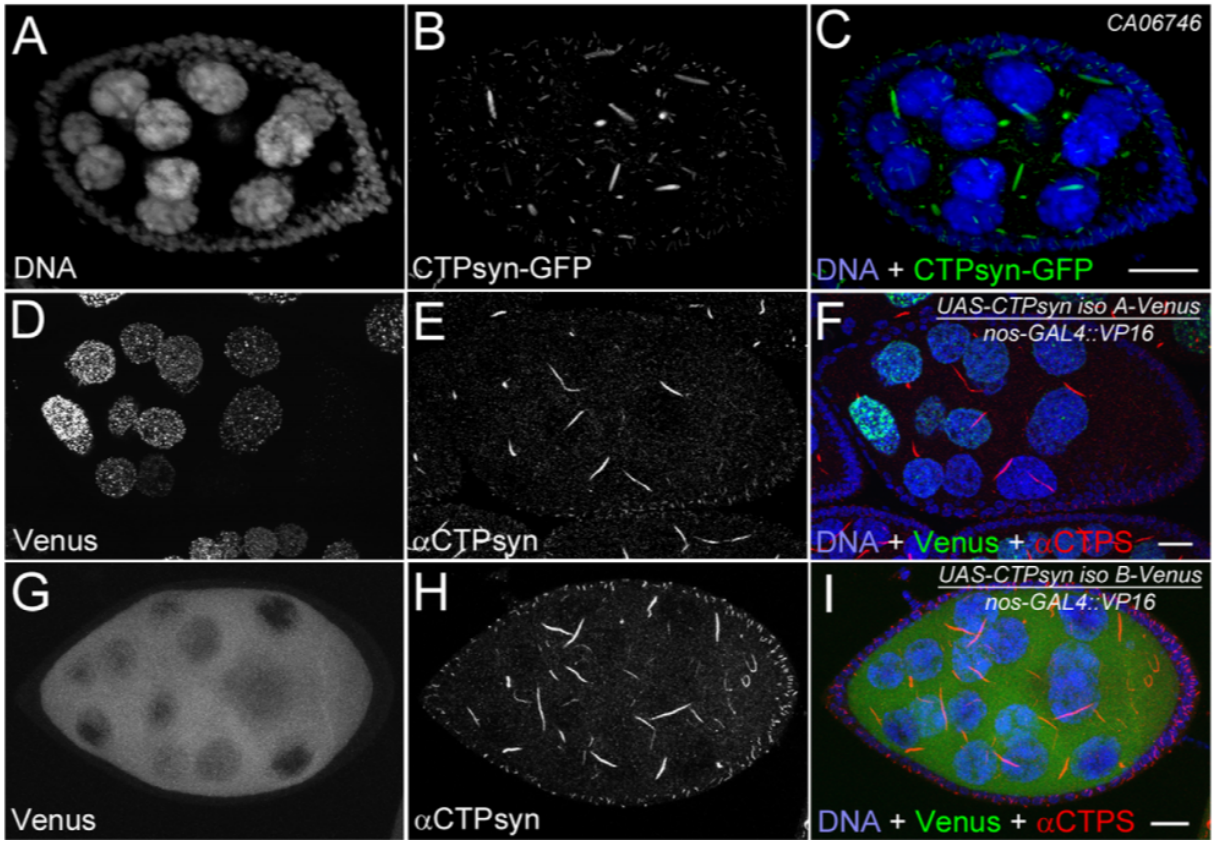


Figure 3.1.2 Only CTPS isoform-A is able to compartmentalise into filaments in *Drosophila*.

(A–C) Cytoophidia revealed by CTPS-GFP in an egg chamber from a protein trap line CA06746. (D–F) CTPS isoform A is localised in punctate structures in the nucleus and does not affect endogenous cytoophidia. (G–I) CTPS isoform B is dispersed in the cytoplasm of the egg chamber and does not affect endogenous cytoophidia. Scale bars, 20 μm. Figure adapted from (Azzam, G. & Liu, J. L., 2013).

3.1.3 The cell cycle and cell quiescence of *S. pombe*

S. pombe cells are rod-shaped and normally grow as haploid, with a rapid division cycle of 2 to 4 hours per generation. Like mammalian cells, the cell cycle of *S. pombe* is in accord with the classic model, having four phases, G1, S, G2 and M phase. In vegetative growing *S. pombe* cells, the G2 phase is particularly long, and takes up more than 60% of the division time. The G1, S and M phases each takes around 10% of the division time (**Figure 3.1.3**). Interestingly, cytokinesis and the nuclear division cycle are asynchronous: after nuclear division, the nuclei go through G1 and enter S phase before cytokinesis is completed, resulting in a short period where the cell contains 4 chromosomes. Unlike budding yeast, where the most control over the cell cycle occurs at the G1/S check point, the major control of the cell cycle in fission yeast is at the G2/M check point. The cell cycle of *S. pombe* is organised by the association of CDK(Cdc2) with various cyclins. High cdc2-cdc13 activity triggers the mitosis initiation, and the cdc2-cig2 activity drives the S phase.

Besides the standard cell cycle stages of vegetative growing condition, as in mammalian cells, fission yeast cells can also be induced into the G0 stage (also called the quiescence stage or stationary phase). To increase viability under harsh conditions, *S. pombe* cells can be induced into quiescence or sexual development by either nitrogen or carbon deprivation. Nitrogen starvation leads to G1 arrest; glucose starvation results in G2 arrest (**Figure 3.1.3**).

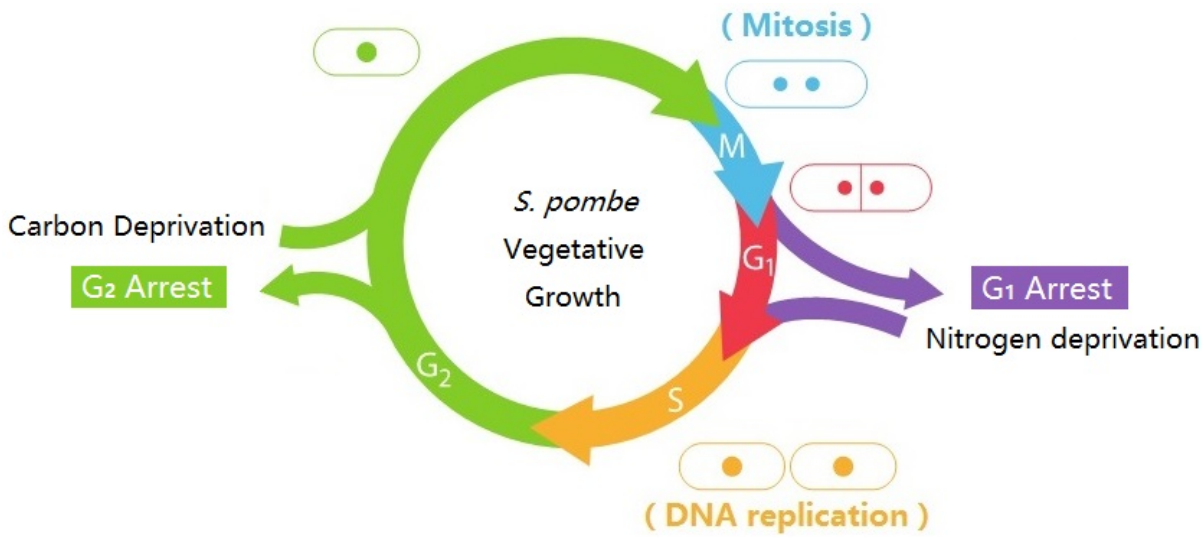


Figure 3.1.3 Abbreviated diagram of the *S. pombe* cell cycle.

3.1.4 Chapter Aims

A general description of CTPS compartmentalisation in *S. pombe* is made in this chapter. We generate several *S. pombe* strains expressing YFP tagged CTPS. With endogenous expression level of CTPS, cytoophidia can be observed in both cytoplasm and nucleus. In addition, we find that the assembly of cytoophidia is reversible and sensitive to growth conditions. We also observed that cytoophidia are not disassembled in the cytokinesis phase in *S. pombe*. The demonstration of cytoophidia morphology and the dynamic variation established the basic background of cytoophidia study in *S. pombe* and give a good platform for future analysis.

3.2 Results

3.2.1 Generation of the CTPS-YFP expressing *S. pombe* strain

To study cytoophidia systematically, we decided to investigate CTPS in a well-studied single-cell organism, *S. pombe*. For *Drosophila*, a published paper has shown that YFP fused at the C-terminus of CTPS mimics the distribution pattern of endogenous CTPS (Azzam, G., and Liu, J. L., 2013). The C-terminal tagged CTPS plasmid pSMUY2-*cts1* was therefore constructed (**Figures 3.2.1**). The plasmid was digested with *SpeI* and then integrated into the *S. pombe* chromosome. The CTPS-YFP was expressed using the endogenous promoter at the endogenous locus in *S. pombe* (**Figure 3.2.2**). The plasmid was sequenced to confirm no point mutation occur within the *cts1* fragment. The full sequences of the plasmid pSMUY2+ and pSMUY2-*cts1* are shown in the **appendix 2**.

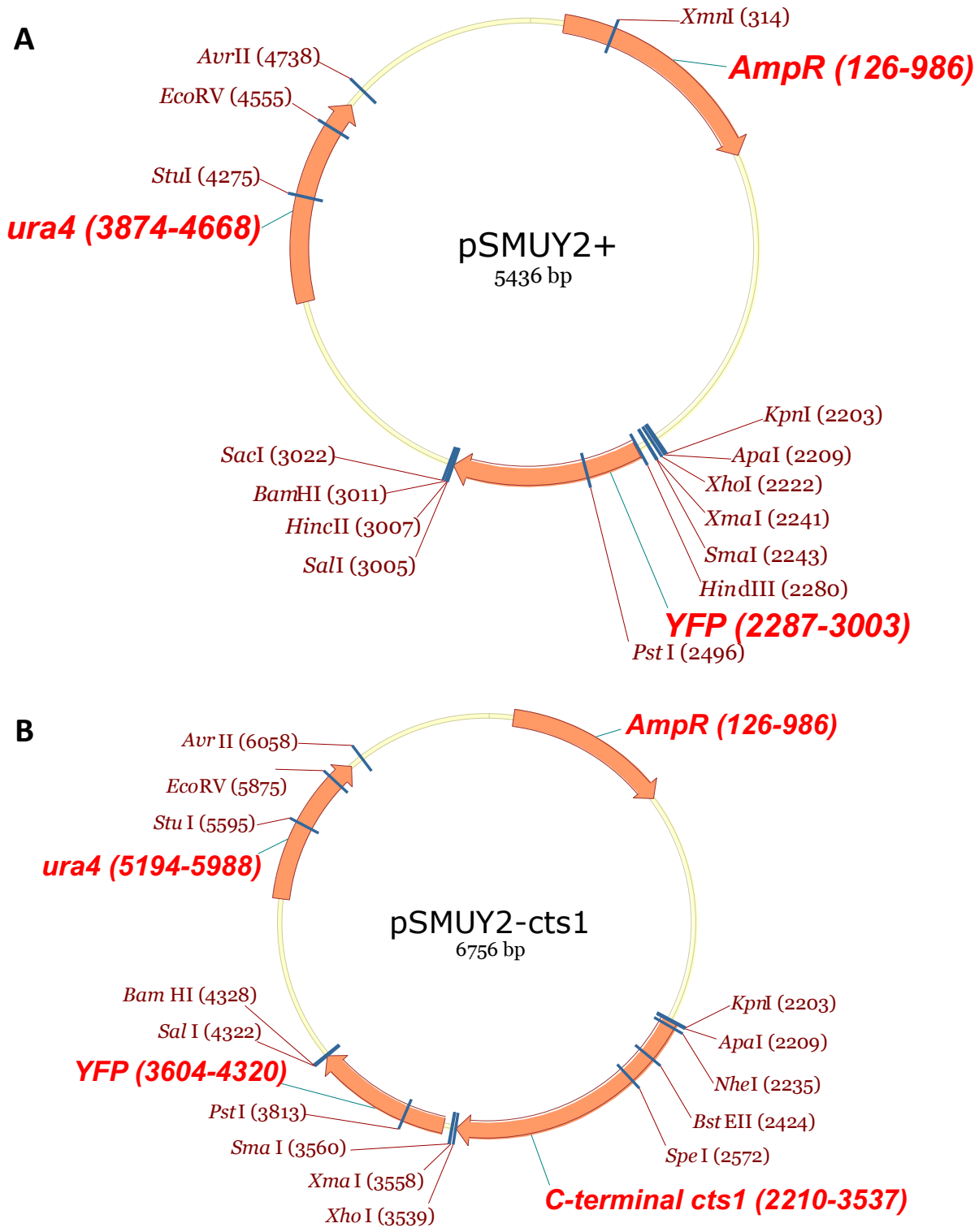


Figure 3.2.1 Construction of the pSMUY2-*cts1* plasmid. (A) Schematic of the backbone plasmid pSMUY2+. (B) Schematic of the plasmid pSMUY-*cts1* for the generation of the CTPS-YFP protein.

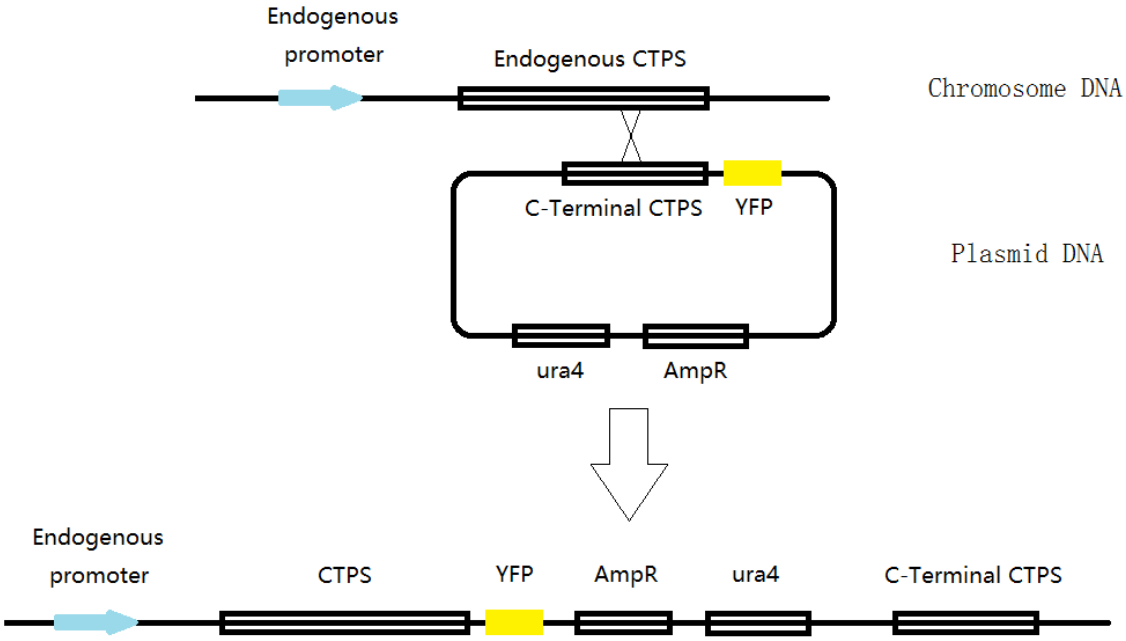
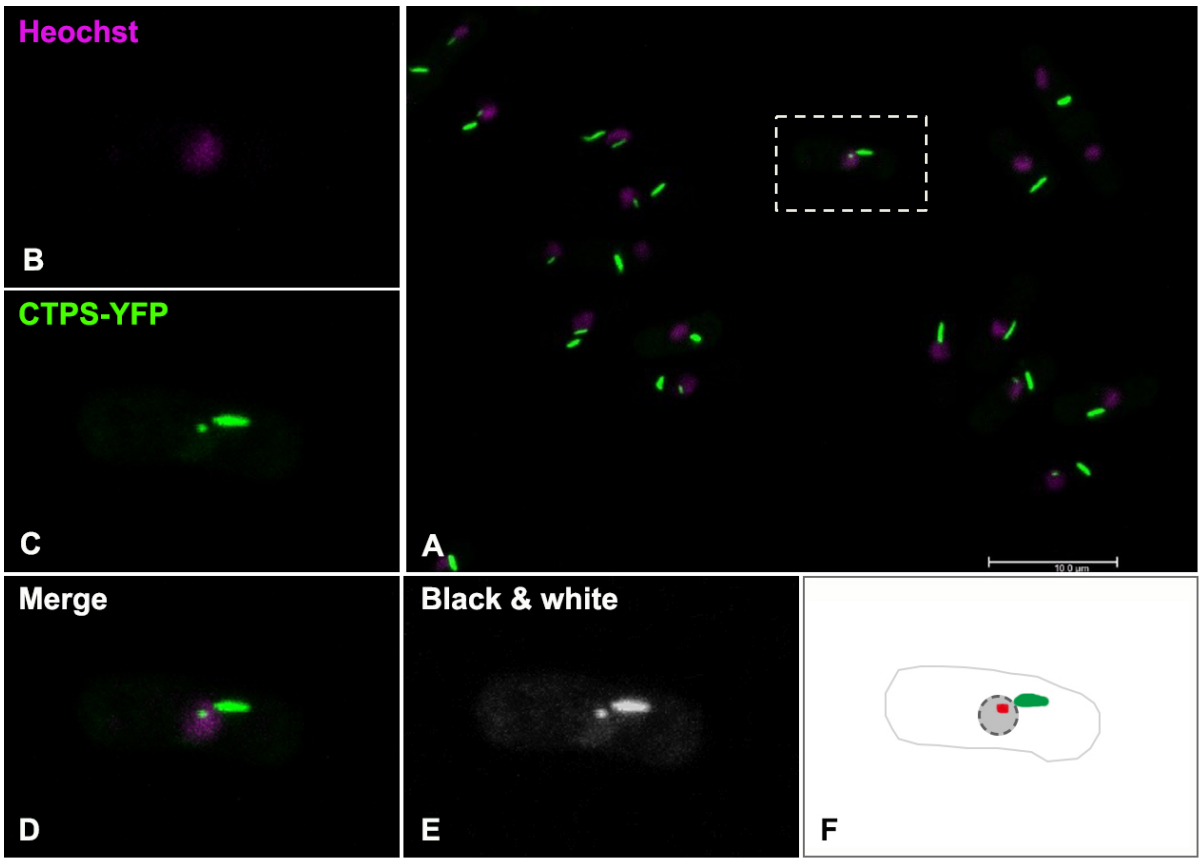


Figure 3.2.2 Generation of the CTPS-YFP expressing *S. pombe* strain. The plasmid pSMUY2-*cts1* was integrated into *S. pombe* chromosome at the endogenous locus of *cts1*. The YFP tagged CTPS was expressed using endogenous *cts1* promoter. The DNA fragment of C-terminal CTPS has no start code AUG and will not be transcribed or expressed.

3.2.2 CTPS compartmentalisation happens in both nucleus and cytoplasm in *S. pombe*

It has been described in budding yeast that the CTPS is able to assemble into cytoophidia and respond to different drug treatments and stresses (Noree, C., et al., 2010). The cytoophidium in fission yeast, however, has been little studied. To analyse the cytoophidium in *S. pombe*, the YFP-tagged CTPS strain was generated. The YFP was tagged to the C-Terminus of CTPS, and then integrated into the endogenous CTPS gene (*cts1*) locus. Therefore, CTPS-YFP was expressed using an endogenous promoter at the endogenous gene locus (**Figure 3.2.2**).

The CTPS-YFP strain was cultured in YES media at 32℃ and examined under a confocal microscope. When the cells were in the exponential stage, the CTPS filaments could be observed clearly in both nucleus and cytoplasm, normally a smaller one in the nucleus and a longer and thicker one in the cytoplasm (**Figure.3.2.3**). To have a better understanding of cytoophidia dynamic movements, live cell imaging was taken during the cells' exponential stage. In the taken video, both nuclear cytoophidia (N-cytoophidia) and cytoplasmic cytoophidia (C-cytoophidia) could be clearly observed and underwent micro-movement within cells (**Video 1**).


Heochst
B
CTPS-YFP
C
A
Merge
D
Black & white
E
F

G

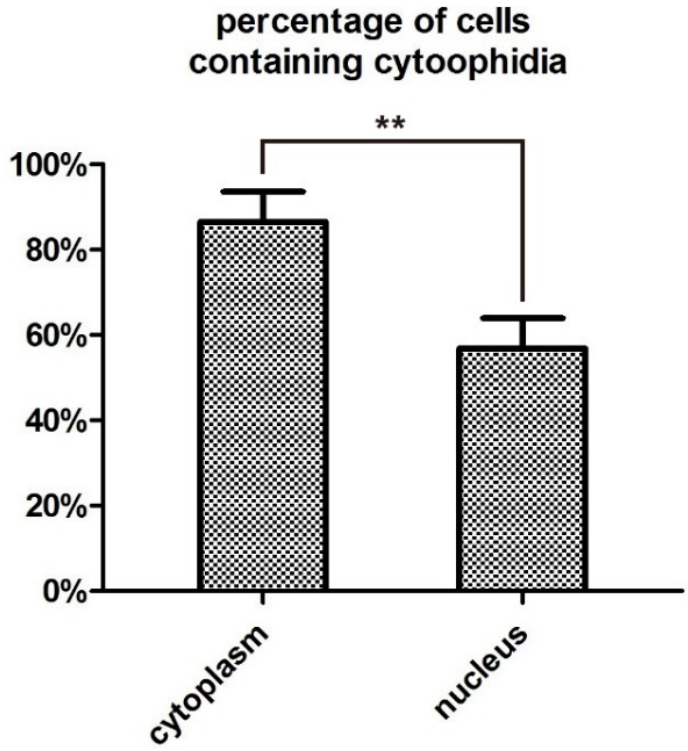

percentage of cells
containing cytoophidia
**
100%
80%
60%
40%
20%
0%
cytoplasm
nucleus

Figure 3.2.3 The compartmentalisation of CTPS can be observed in both nucleus and cytoplasm in *S. pombe.* (A) The exponential stage cells contain nucleus and cytoplasm cytoophidia. (B-F) The zoomed in cell (squared in A) with nuclear and cytoplasmic cytoophidia. Red represents nucleus cytoophidia and green represents cytoplasm cytoophidia. (G) Cell counting result shows cell percentage containing cytoplasmic cytoophidia (C-cytoophidia) and nuclear cytoophidia (N-cytoophidia). P=0.001<0.05. Scale bar, 10μm. Error bar: Mean±SEM.

Results fo the T test analysis:

Percentage of cells contain cytoophidia

Column B	nuclear
vs.	vs.
Column A	cytoplasm
Unpaired t test	
P value	0.0010
P value summary	**
Significantly different (P < 0.05)?	Yes
One- or two-tailed P value?	Two-tailed
t, df	t=5.918 df=6

3.2.3 CTPS compartmentalisation is regulated through the *S. pombe* growing stages

S. pombe cells undergo four different stages while growing: the lag phase, the exponential phase, the stationary phase and the death phase. *S. pombe* cells have different metabolic conditions during different phases. To examine whether the growth stages affect cytoophidia, the CTPS-YFP expressing cells were fixed at different growing points and observed under a confocal microscope. Cell growth was monitored by measuring the OD_{600} value using the spectrophotometer. Each counting group contains more than 500 cells. Software ImageJ was used for the cell counting and the cytoophidia length measuring (detailed in Chapter 2).

During the exponential phase, cytoophidia were highly abundant, with more than 90% of cells containing cytoophidia. As the cells grew and OD_{600} increased above 1.0, fewer cytoophidia were observed compared to the mid-exponential phase. When the cells had reached the stationary phase, most cytoophidia dispersed into the whole cell, and only rare cytoophidia remained (**Figure 3.2.3)**. This result indicates that cytoophidia regulation is closely linked to the cell growth condition in *S. pombe*.

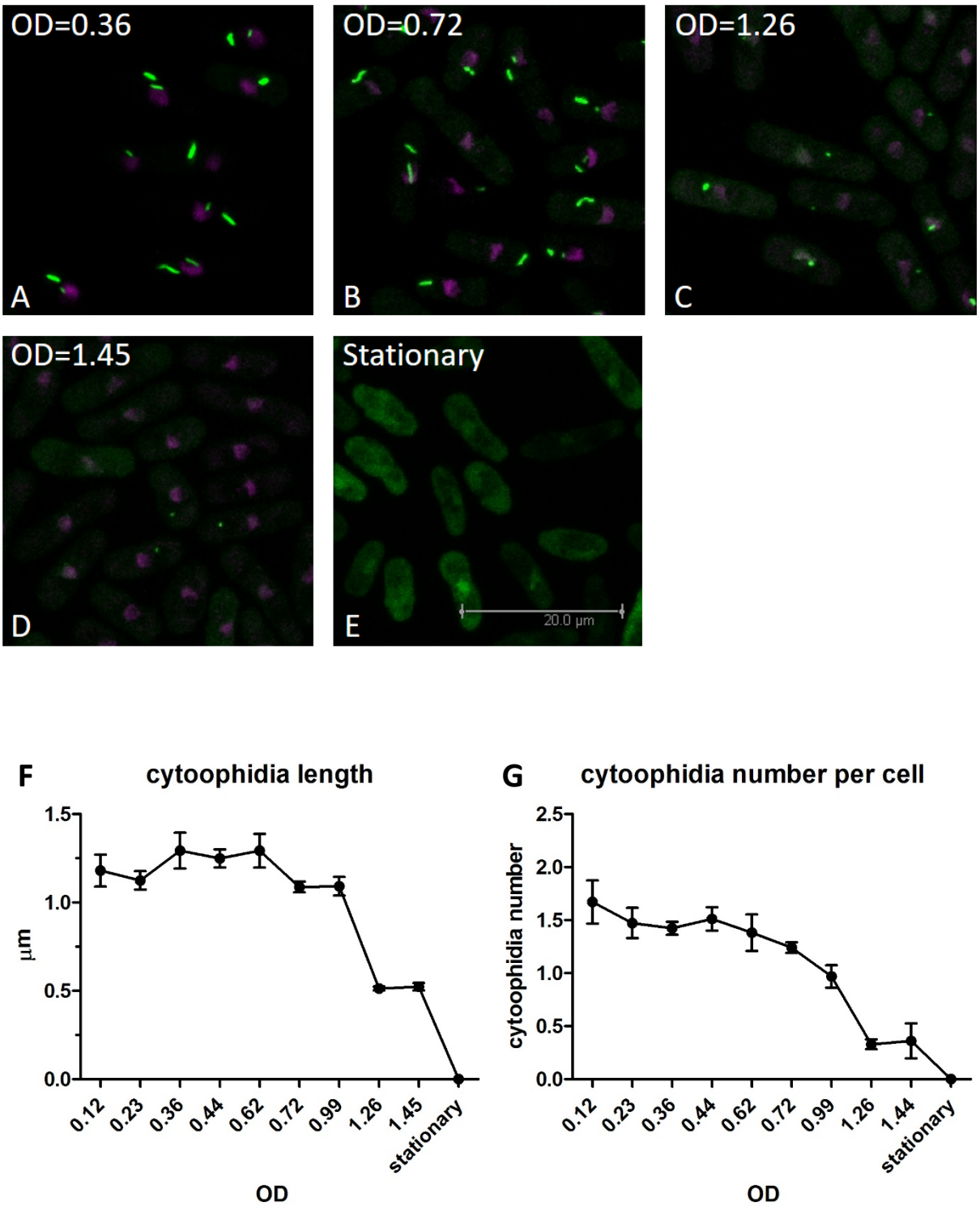


Figure 3.2.4 The cell growth condition affects cytoophidia in *S. pombe*. (A-E) The individual OD_{600} of A to E are 0.36, 0.72, 1.26, 1.45 and Stationary. Scale bar, 20µm. (F-G) cytoophidia length and number altered with the different phases of cell growth. Error bar: Mean±SD.

3.2.4 The Stationary phase induced CTPS compartmentalisation is reversible in *S. pombe*

We now know that cytoophidia are affected by the cell growth condition in *S. pombe*. The question is therefore now whether the dispersal of cytoophidia during the stationary stage is reversible. Previously published papers mention that CTPS compartmentalisation is reversible in *S. cerevisiae* (Noree, C., et al., 2010). In *S. cerevisiae*, cytoophidia were induced when cells were faced with carbon source deprivation or growing into a stationary phase. When shifted to fresh rich media, the decrease of cytoophidia caused by nutrient deprivation and a stationary phase went back to a normal level, indicating thet the CTPS assembly and disassembly are reversible (Noree, C., et al., 2010).

We performed a similar experiment on *S. pombe* and found that the cytoophidia compartmentalisation in *S. pombe* is also reversible. **Figure 3.2.3** shows, during the exponential phase (OD value from 0.1 to 1.0), that a high percentage of cells contained cytoophidia, and the average length of cytoophidia was above 1.0μm. When cells grew into the stationary phase, cytoophidia dispersed into the cytoplasm. When shifted to rich media and grown for another 2 hours, cells entered the exponential phase again, and the average length and number of cytoophidia were greatly increased (**Figure.3.2.4**), indicating that cytoophidia compartmentalisation is reversible in *S. pombe*.

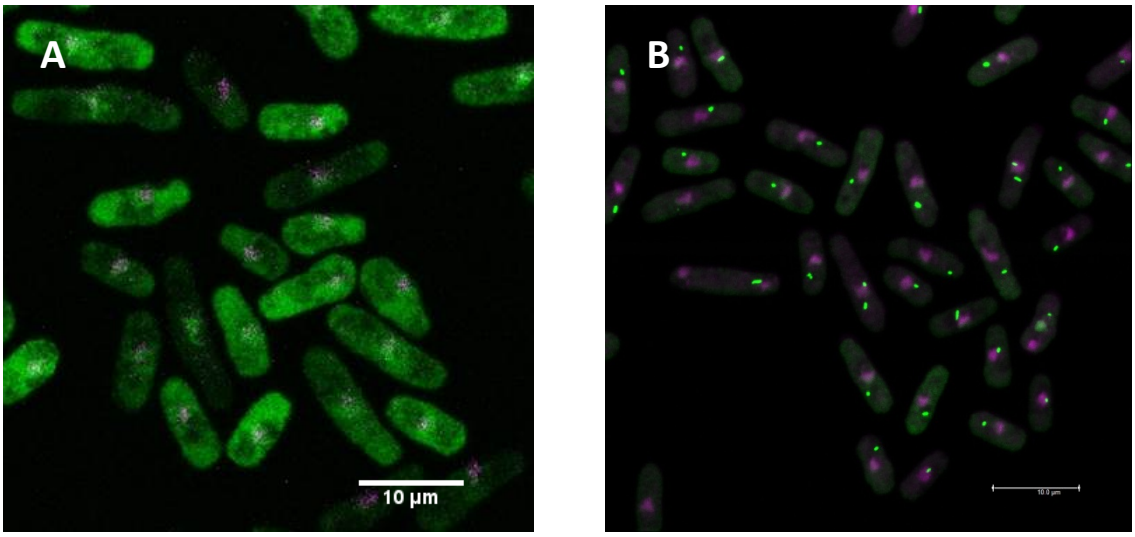


Figure 3.2.5 The stationary phase induced CTPS assembly is reversible in *S. pombe*. (A) Cytoophidia dispersed during the stationary phase. (B) CTPS assembled back into cytoophidia when cells shifted to rich media and were cultured after 2 hours. Scale bar: 10µm.

3.2.5 Cytoophidia are highly dynamic in *S. pombe*

Cytoophidia are easily observed in the exponential phase in *S. pombe* cells. To have a better understanding of cytoophidia activity, we performed the live imaging experiment under confocal microscope and observed how cytoophidia movement undergo in live cells. To make sure the cells were in the best condition for cytoophidia observation, the cells were diluted at the exponential stage and cultured overnight in YES media. The OD_{600} of cells was measured before dilution and the dilution times were calculated to guarantee that the cells would undergo a specific number of generations and grow into the exponontial stage after one night. The exponential cells were then diluted again to OD_{600} 0.1 and incubated at 32°C in the special chamber of a confocal microscope. In **Video 1**, we can see the cytoophidia did not stay in fixed positions. They moved continuously from one position to another. In **Video 2** and **Figure 3.2.6**, We can clearly see the movement of a cytoophidia from one cell tip (tip x of cell B) to another cell tip (tip y of cell B). We can also see that the cytoophidia itself trembled continually in addition to its position changes in cells (**video 1**). All these results indicate that cytoophidia are highly dynamic in *S. pombe* cells.

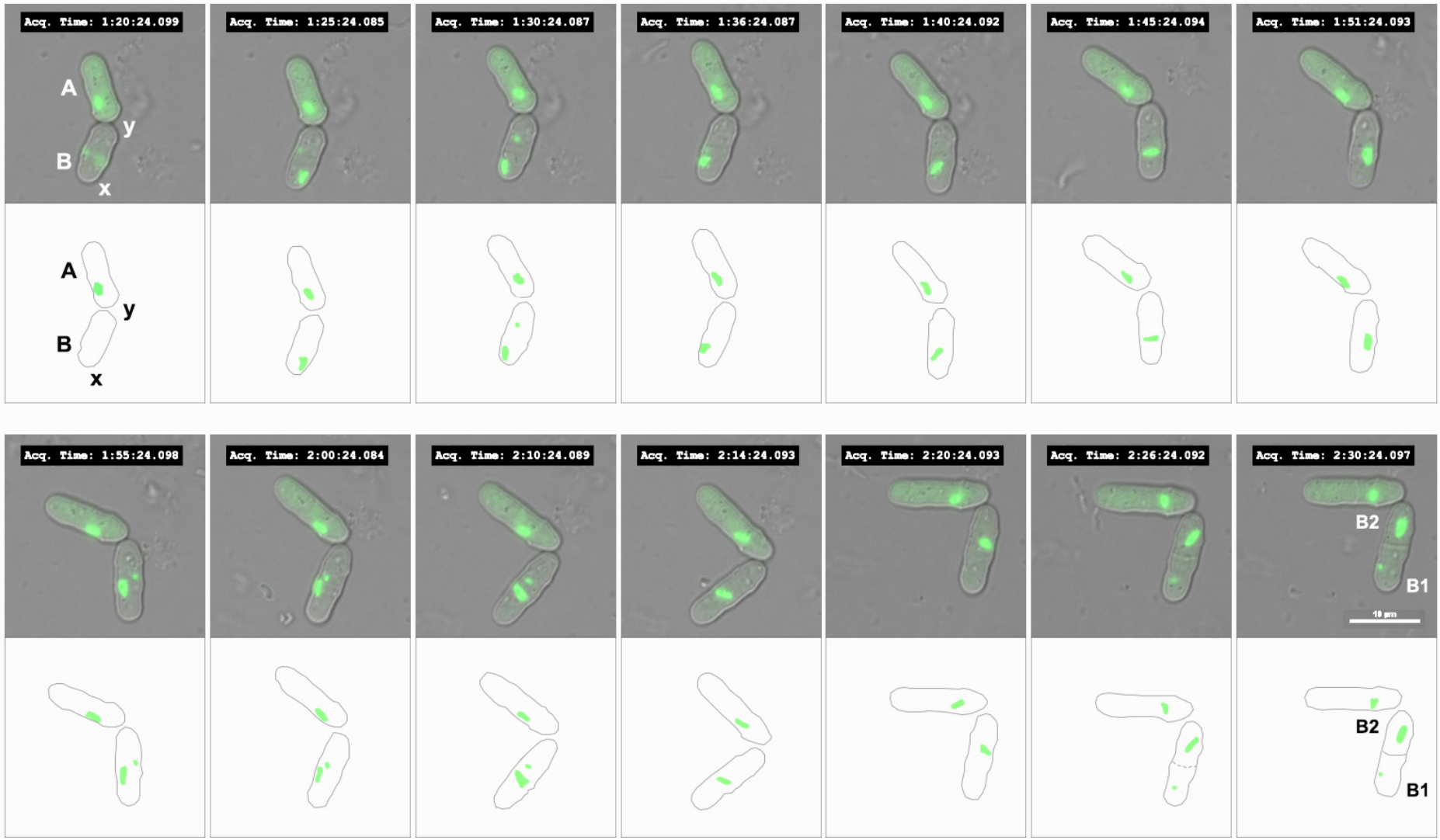

Acq. Time: 1:20:24.099
A
y
B
x
Acq. Time: 1:25:24.085
Acq. Time: 1:30:24.087
Acq. Time: 1:36:24.087
Acq. Time: 1:40:24.092
Acq. Time: 1:45:24.094
Acq. Time: 1:51:24.093
A
y
B
x
Acq. Time: 1:55:24.098
Acq. Time: 2:00:24.084
Acq. Time: 2:10:24.089
Acq. Time: 2:14:24.093
Acq. Time: 2:20:24.093
Acq. Time: 2:26:24.092
Acq. Time: 2:30:24.097
B2
B1
10 µm
B2
B1

Figure 3.2.6 Cytoophidia are highly dynamic in *S. pombe*. Time lapse video (**Video 2**) shows the easily observed cytoophidia in *S. pombe* cells. Two cells can be viewed in this video, represented as cell A and B. In cell B, the two cell tips were represented as x and y. One of the cytoophidia can be observed near the cell tip x. With time lapse the cytoophidia moved slowly from tip x to tip y. Before cytokinesis, the septum was formed and the cytoophidia was confined in the half-part of tip y (becoming daughter cell B2 in the future). From the time recorded on the top of each pictures, we can see that within as short as 1 hour, the cytoophidia moved from one cell tip to another cell tip, showing its highly dynamic movement in the *S. pombe* cell.

3.2.6 Cytoophidia remain filaments during *S. pombe* cytokinesis

Before the mitotic phase, the *S. pombe* cell grows and the cell is elongated before cytokinesis. Previously published data support the idea that the cytoplasm and most organelles will be separated evenly into two daughter cells during cytokinesis in *S. pombe.* Proteins diffuse into cytoplasm and are divided and distributed together with the cytoplasm into two daughter cells (Forsburg, S. L., 2005). This raises the question that how cytoophidia distribute during cytokinesis. To answer this, we performed a live imaging recording *S. pombe* cell activity during cytokinesis. The recorded video is presented by slices in **Figure 3.2.7**. From **Figure 3.2.7** and **Video 2**, cytoophidia can be clearly observed with bright fluorescence and long rod morphology during cell cytokinesis, indicating that cytoophidia have a unique distribution pattern in *S. pombe*. CTPS, unlike most other proteins in *S. pombe*, is distributed in filamentous form. Notably, cell A in **Figure 3.2.7** had only one cytoophidium before cytokinesis. The cytoophidium distributed into only one daughter cell, and the other daughter cell (without the inherited cytoophidium) generated a new cytoophidium soon after finishing cytokinesis. Although it is not clear yet if this difference between two daughter cells can affect the cells' fate in the future, it may be a good aspect to investigate in a future project on cytoophidia.

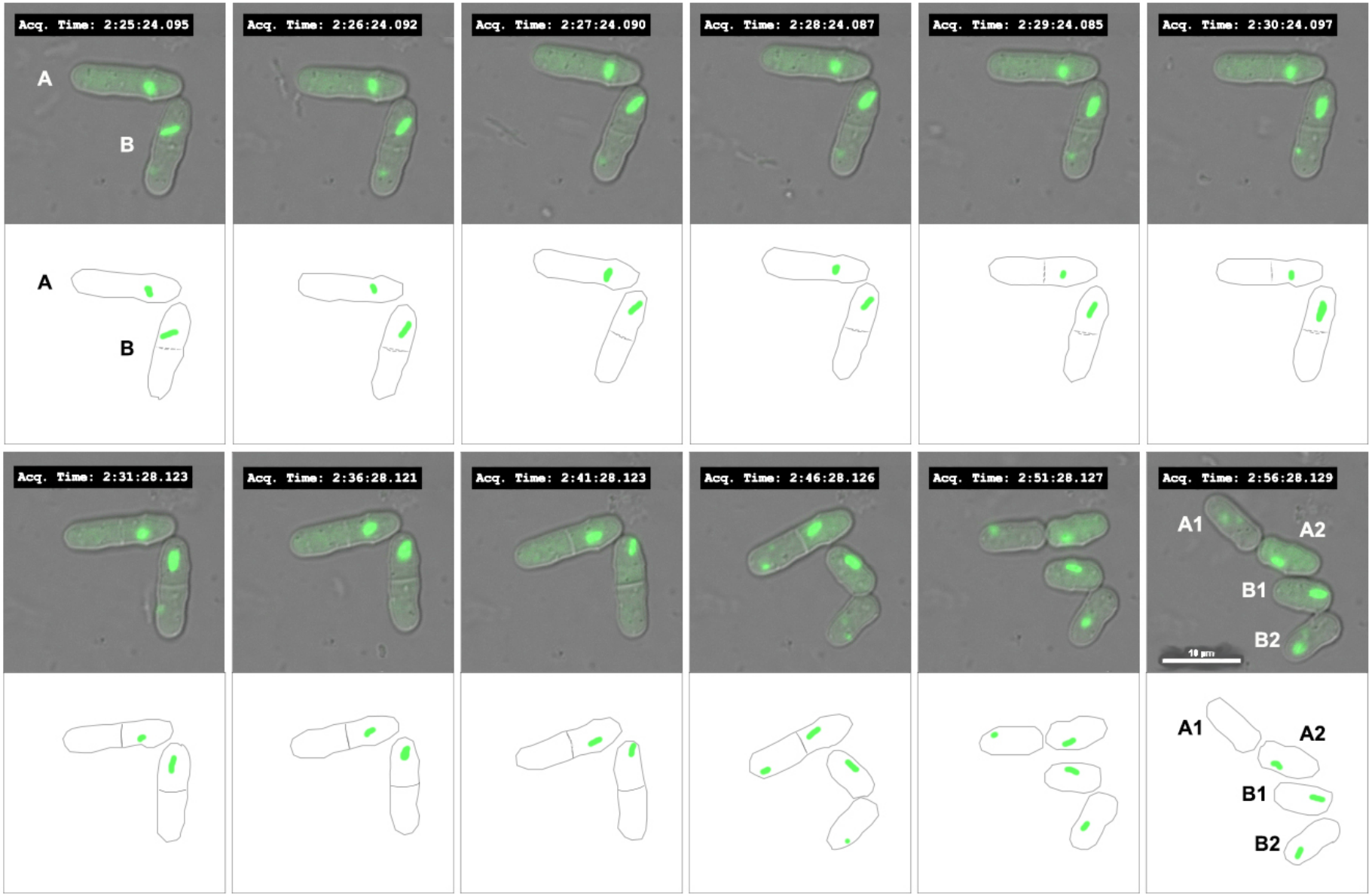

Acq. Time: 2:25:24.095
A
B
Acq. Time: 2:26:24.092
Acq. Time: 2:27:24.090
Acq. Time: 2:28:24.087
Acq. Time: 2:29:24.085
Acq. Time: 2:30:24.097
A
B
Acq. Time: 2:31:28.123
Acq. Time: 2:36:28.121
Acq. Time: 2:41:28.123
Acq. Time: 2:46:28.126
Acq. Time: 2:51:28.127
Acq. Time: 2:56:28.129
A1
A2
B1
B2
10 µm
A1
A2
B1
B2

Figure 3.2.7 Cytoophidia remain in filaments during *S. pombe* cytokinesis. The time lapse video shows the easily observed cytoophidia in *S. pombe* cells. Two cells can be viewed in this video, cell A and B. In cell A, only one cytoophidium can be observed at the beginning. Before cytokinesis, the septum was formed and separated the cell into two parts, one on the right with a cytoophidium (daughter cell A2), and another one on the left without any cytoophidium (daughter cell A1). During the process of septum generation, the left part of cell A did not inherit cytoophidium from mother cell. To make up for the lack of cytoophidium, a new cytoophidium was generated. The cytokinesis resulted in two daughter cells, A1 with a newly generated cytoophidium, and A2 with the inherited cytoophidium from mother cell. Whether this difference affects the daughter cells' future fate is not clear, but this may be a good aspect for a future project to inspect the functions of cytoophidia.

3.3 Discussion

3.3.1 Comparison of the cytoophidium between *S. cerevisiae* and *S. pombe*

Previously published papers have already shown that CTPS undergo compartmentalisation in several different organisms, including the cells of humans, rats, *Drosophila,* and bacteria (Chen, K., et al., 2011; Noree, C., 2010; Liu, J. L., 2010; Ingerson-Mahar, M., 2010). It has also been reported that cytoophidia can be observed in budding yeast (Noree, C., 2010). However, no one has examined whether CTPS assembly occurs in fission yeast. In this thesis, we identifiy that in *S. pombe*, abundant cytoophidia can be easily observed in exponential stage cells. This observation supports the hypothesis that CTPS self-assembly is a evolutionarily conserved activity in different species.

Both budding yeast and fission yeast are good model organisms to study cytoophidia. It has already been reported that the expression products of both CTPS genes (Ura7 and Ura8) can compartmentalise into filaments (Noree, C., 2010). In budding yeast, cytoophidia respond to several drugs and environmental stresses (Noree, C., 2010). This is supported by our observation that, in fission yeast, cytoophidia are also sensitive to drugs and stresses. However, some results still differ from the known data collected from budding yeast.

In budding yeast, cytoophidia can mostly be detected mostly in the stationary

phase (Noree, C., 2010). However, in fission yeast, only the exponential cells contain large numbers of cytoophidia; in contrast, no cytoophidium can be observed during the stationary stage. In budding yeast, also, cytoophidia are highly induced by carbon deprivation (Noree, C., 2010), while in contrast, in fission yeast, the lack of a carbon source causes the dispersal of cytoophidia. These completely opposing results indicate that the mechanism of cytoophidia regulation differs between *S. pombe* and *S. cerevisiae*.

Another difference of the cytoophidium between budding yeast and fission yeast is that cytoophidia can be observed in both the nucleus and cytoplasm in *S. pombe,* while only cytoplasmic cytoophidia have been reported detected in *S. cerevisiae*. Despite the difference between the two yeast species, fission yeast shares this feature with human cells: nuclear cytoophidia have also been reported in human HeLa cells, supporting the hypothesis that cytoophidia are evolutionary conserved structures and indicating that nuclear cytoophidia may have potential functions separate from its cytoplasmic functions.

3.3.2 Cytoophidia regulation is related to the cell growing phase, and the stationary phase caused cytoophidia dispersal is reversible

In exponential stage *S. pombe* cells, more than 90% cells contain cytoophidia (**Figure 3.2.3**). As cells grow and the cell concentration becomes higher, the OD_{600} is increased. When the OD_{600} became higher than 1.0, the percentage of

cells containing cytoophidia started decreasing (**Figure 3.2.3**). When *S. pombe* cells were cultured for more than two nights and all cells were in the stationary stage, cytoophidia can be merely detected. This suggestes that cytoophidia were regulated through the cell growing phase in *S. pombe*.

Previously published data have demonstrated that both the CTPS protein level and the mRNA level were decreased to around 20% during the cell quiescence induced by nitrogen deprivation (Marguerat, S., et al., 2012), which is very similar to the stationary phase. The dispersal of cytoophidia during the stationary phase may be due to the decrease of CTPS protein concentration in cells. When the cells were shifted back to fresh YES media, cytoophidia emerged again, indicating that the stationary phase induced dispersal of cytoophidia is reversible (**Figure 3.2.4)**.

In this case, we can reasonably speculate that cytoophidia take the role of storage for CTPS. In accordance with this hypothesis, during the *S. pombe* exponential growing stage, CTPS had a high expression level and was promoted and stored as cytoophidia. When cells approached stationary phase, the CTPS was downregulated, leading to the disassembly of cytoophidia and the release of CTPS into cytoplasm. When the cells shifted back into rich media and the cell reinitiated cell division and cell growth, the CTPS expression level increased and was stored as cytoophidia again.

3.3.3 Cytoophidia remain filaments during cytokinesis

During cytokinesis, unlike most proteins that diffuse into cytoplasm and separate evenly into two daughter cells, CTPS remained in a filamentous form and distributed asymmetrically (detailed data and discussion will be given in Chapter 5). CTPS is the critical enzymes responsible for *de novo* CTP biosynthesis. CTP is not only the building block of nucleic acid but also the essential precursor of all membrane phospholipids in *S. pombe*, which means the CTPS is critical for DNA replication and transcription as well as for vegetative cell growth. In the *S. pombe* cell cycle, the S phase is the phase most involved in DNA duplication; a 10% decrease of CTPS protein level has been observed during the S phase in fission yeast (Carpy, A., et al., 2014). The G2 and G1 phases are the phases when cell growth happens and high protein levels of CTPS are detected (Carpy, A., et al., 2014). The M phase needs high CTPS activity for septum generation. All four different cell cycle phases require CTPS activity for different reasons. In *S. pombe*, after nuclear division occurs, the nuclei go through G1 and enter S phase before the cells complete cytokinesis, which means CTPS activity is highly in demand for the ribonucleic acid, even during cytokinesis (Knutsen, J. H., et al., 2011).

The existence of cytoophidia during fission yeast cytokinesis has led to the theory that cytoophidia may serve as metabolic stabiliser and maintain the cellular homeostasis during cytokinesis, preventing the over-activation of

CTPS. Cytoophidia can respond quickly to environmental changes and reversibly assemble and disassemble, making them a good metabolic buffer.

3.3.4 The nuclear cytoophidium

As one of the central metabolic enzymes, CTPS appears in both the cytoplasm and the nucleus in *S. pombe.* It is not surprised that the CTPS compartmentalisation happens in both the cytoplasm and the nucleus. However, the compartmentalisation occurs not only under normal growth conditions but also during cytokinesis, which is not a commonly observed phenomenon. There is no clear evidence for why CTPS forms into filaments in the nucleus, or of what roles they are playing. Several theories can be proposed.

C-cytoophidia can be considered as a pool of metabolic enzymes regulating enzymatic activities and affected by enzyme concentration, and therefore can help regulate cellular metabolism processes (Ingerson-Mahar, M., et al., 2010; Noree, C., et al., 2010; Barry, R. M., et al., 2014; Noree, C., et al., 2014; Aughey, G. N., et al., 2010). N-cytoophidia may perform a similar function in the nucleus. N-cytoophidia may act as the metabolic buffer to help regulate enzyme activity and concentration in the nucleus. Although no investigation has been made testing the relationship between N-cytoophidia and C-cytoophidia, communication between them is highly expected. The existence of cytoophidia in both nucleus and cytoplasm suggests that they may undertake the function

of balancing the regulation of metabolic enzymes between nucleus and cytoplasm. All these hypotheses need to be confirmed in the future research, and it is worth continuing to dig into the functions of N-cytoophidia.

Chapter 4 Cytoophidia respond to different environmental stresses in *S. pombe*

4.1 Introduction

4.1.1 DON is an unspecific CTPS inhibitor and induces cytoophidia in *Drosophila* and HeLa cells

6-Diazo-5-oxo-L-norleucine (DON) is a non-standard amino acid and a glutamine antagonist, making it a good inhibitor for many glutamine-utilising enzymes. It can easily get access to the glutamine binding site of enzyme catalytic centre due to its similarity to a glutamine, and inhibits the function of an enzyme by covalent binding (Pinkus, L. M., 1977; Lebedeva, Z. I., et al., 1986). **Table 4.1.1** gives a list of DON inhibit targets. DON is used in some clinical trials for cancer therapy. Even though it is not approved as a pharmaceutical treatment reagent, DON still has its great value in cancer studies. It is tested in combination with a recombinant glutaminase in treatments with several different solid tumours (Catane, R., et al., 1979).

Enzyme	Involved Metabolic Pathway
CTP synthase (CTPS)	Pyrimidine-De-Novo-Synthesis
Carbamoyl phosphate synthase (CAD)	Pyrimidine-De-Novo-Synthesis
FGAR amidotransferase	Purine-De-Novo-Synthesis
Guanosine monophosphate synthetase (GMPS)	Purine-De-Novo-Synthesis
PRPP amidotransferase	Purine-De-Novo-Synthesis
Mitochondrial glutaminase	First step of glutaminolysis
NAD synthase	Coenzyme of the electron transport chain
Asparagine synthetase	Amino acid synthesis

Table 4.1.1 List of enzymes inhibited by DON (Pinkus, L. M., 1977)

DON is a good inducer of cytoophidia in many different organisms. In *Drosophila*, cytoophidia can be observed in several different cell types, although not all tissues. *Drosophila* ovaries, optic proliferation centres of larval CNS, larval lymph glands and testes have abundant amount of cytoophidia that can be easily detected (Liu, J. L., 2010). However, cytoophidia can be induced by DON in all cell types studied so far in *Drosophila*, including multiple tissues and *Drosophila* S2 cells (Aughey, G. N., et al., 2014). It has also been reported that, in human HeLa cells, adding DON can induce cytoophidia compartmentalisation in both cytoplasm and nucleus (**Figure 4.1.1**) (Gou, K. M., et al., 2014).

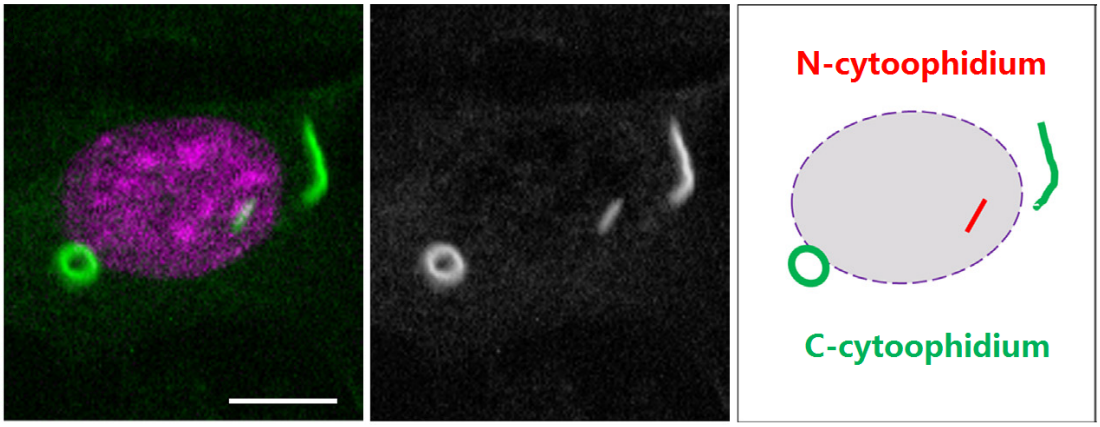


Figure 4.1.1 The CTPS can form filaments in both cytoplasm and nucleus in HeLa cells (Guo, K. M., et al., 2014). Nuclear cytoophidia are presented as N-cytoophidia in red; Cytoplasmic cytoophidia are presented in green as C-cytoophidia. Scale bar: 10μm.

4.1.2 Cytoophidia respond to environmental stresses in *S. cerevicae* and *Drospophila*

According to previous studies, cytoophidia respond to several different stresses in budding yeast and fruit flies (Noree, C., et al., 2010). In budding yeast, the CTPS filaments are strongly induced by glucose depletion and the kinase inhibitor staurosporine while over-expression of Hsp104 or 4°C temperature has no effect on cytoophidia (Noree, C., et al., 2010). The CTPS filaments are also strongly influenced by the presenting of azide (Noree, C., et al., 2010). The formation of cytoophidia induced by glucose depletion is reversible in as little as 15 minutes or less, giving rise to the conclusion that cytoophidia undergo an efficient response to nutrient condition changes (Noree, C., et al., Biol 2010). In *Drosophila* S2R+ cells, nutrient depletion led to an increase in the number of cytoophidia (Aughey, G. N., et al., 2014), which is parallel to the results for budding yeast. Similarly, cytoophidia assembly can also be induced in *Drosophila* larvae by nutrient restriction (Aughey, G. N., et al., 2014). In *Drosophila* larval post embryonic neuroblasts (pNbs), cytophidia can be induced by nutritional restriction during the late L2 stage (Aughey, G. N., et al., 2014). Cytoophidia can also be observed in imaginal discs (eye, wing, leg and haltere) of third instar larvae when fed with nutrient-deprivated food (Aughey, G. N., et al., 2014).

4.1.3 Heat shock proteins and temperature stress responses

The heat shock proteins (HSPs) are a family of proteins that are expressed and regulated in response to different stressful conditions. They were first described involved in heat shock response, and are now being widely-known to have critical functions during other stress responses, including cold shock response, UV light induced DNA damage response, and wound healing processes or tissue remodelling (Li, Z. & Srivastava, P., 2004). The increase of HSPs expression under stress is transcriptionally regulated. The dramatic upregulation of the heat shock proteins is a key part of the heat shock response and is induced primarily by the heat shock factor (HSF) (Wu, C., 1995). Many HSP family members perform a chaperone function to ensure the proteins correct folding by binding and stabilising newly synthesised proteins, or binding and guiding the degradation of aberrant proteins generated under stress conditions. They also play critical roles in several other metabolic activities, including multi-protein complexes assembly, protein transportation and proper sequestering into correct subcellular compartments, cell-cycle and cell signalling control, and protection of cells against stresses and apoptosis (Li, Z. & Srivastava, P., 2004).

HSPs are found in virtually all living organisms, from bacteria to humans (Li, Z. & Srivastava, P., 2004). They are named according to their molecular weight.

In human cells, Hsp60, Hsp70 and Hsp90 are the most widely studied HSPs (Li, Z. & Srivastava, P., 2004). In *S. pombe*, several heat shock proteins are conserved with human cells and budding yeast. For instance, in *S. pombe*, Sks2 and Ssz1 (two heat shock proteins belonging to the Hsp70 family) are highly conserved with human Hsp70 family proteins and budding yeast HSP70 family members (Ensembl, UniProt & SGD). Proteins hsp16 and hsp20 are highly conserved with human small heat shock protein (sHSP) family and budding yeast sHSP family members (Ensembl, UniProt & SGD).

Gene	Product	Functions in *S. pombe*	References
hsp9	Hsp9	Negative regulation of G2/M transition; cellular response to heat	Ahn, J., et al., 2012
hsp16	Hsp16	Cellular response to midfolded and unfolded proteins; chaperone-mediated protein folding; mRNA export from nucleus in response to heat stress	Hirose, M., et al., 2005 Coelho, M., et al., 2014 Yoshida, J. & Tani, T., 2005
SPCC338.06c	Hsp20(predicted)	Chaperon-mediated protein folding (Predicted)	Unpublished data
hsp104	Hsp104	Chaperon-mediated protein folding cofactor; cellular heat acclimation and heat shock protein binding; response to misfolded proteins (*gln1* & *cts1*) and undergo protein unfolding and refolding	Senechal, P., et al., 2009 Coelho, M., et al., 2013 Coelho, M., et al., 2014 Oberti, D., et al., 2015
pdr13 *(ssz1)*	Ssz1	Unfolded protein binding during cytoplasmic translation; ER-associated ubiquitin-dependent protein catabolic process	Predicted functions
sks2	Sks2	*De novo* co-translational protein folding; chaperone-mediated protein folding; involved in ribosome biogenesis of rRNA processing;	Predicted functions & unpublished data

Table 4.1.2 Heat shock proteins and their functions in *S. pombe*

Gene	Product	Orthologues	References
hsp9	Hsp9	*Saccharomyces cerevisiae* HSP12 (plasma membrane protein involved in maintaining membrane organisation);	Ensembl, UniProt, SGD.
hsp16	Hsp16	*Homo sapiens* heat shock protein family B (small); *Saccharomyces cerevisiae* Small heat shock protein (sHSP) with chaperone activity	
SPCC338.06c	Hsp20 (predicted)	*Homo sapiens* heat shock protein family B (small); *Saccharomyces cerevisiae* Small heat shock protein (sHSP) with chaperone activity;	
hsp104	Hsp104	*Homo sapiens* CLPB (ClpB homolog, mitochondrial AAA ATPase chaperonin) *Saccharomyces cerevisiae* HSP104 (Disaggregase)	
pdr13 (ssz1)	Ssz1	Homo sapiens heat shock protein family A (Hsp70); *Saccharomyces cerevisiae* SSZ1 (Hsp70 protein that interacts with Zuo1p)	
sks2	Sks2	*Homo sapiens* heat shock protein family A (Hsp70); *Saccharomyces cerevisiae* SSB1 and SSB2 (Cytoplasmic ATPase that is a ribosome-associated molecular chaperone)	

Table 4.1.3 Heat shock proteins and their orthologues

4.1.4 The MAPK pathway and stress responses

Mitogen-activated protein kinases (MAPKs, originally called ERKs, extracellular signal-regulated kinases) are a group of serine/threonine protein kinases belonged to the same protein family that are evolutionarily conserved and ubiquitously expressed in eukaryotes from yeast to human cells. The typical MAPK pathway consists of a set of three sequentially acting kinases: a MAP kinase (MAPK), a MAPK kinase (MAPKK) and a MAPKK kinase (MAPKKK). These kinase-signalling cascades can be triggered by a vast range of stimuli, and are involved in a variety of fundamental cellular processes including proliferation, differentiation, motility, stress response, apoptosis, and survival (Pearson, G., et al., 2001).

Three conserved MAPK pathways have been identified in *S. pombe*: the stress activated protein kinase Sty1 pathway, which is homologous to the mammalian JNK pathway; the cell integrity Pmk1 pathway, which is homologous to the mammalian ERK1/ERK2 pathway; and the pheromone signalling Spk1 pathway (**Figure 4.1.2**). The Sty1 pathway and the Pmk1 pathway can be induced by specific stresses separately as well as by shared environmental stresses, and the cross-talks between two pathways have been demonstrated (Perez, P. & Cansado, J., 2010; Sanso, M., et al., 2011). The former, Sty1 pathway, is the major metabolic mechanisms responsible for the stress response in *S. pombe*, which is also called the Stress-activated protein kinase pathway (SAPK pathway). When

cells are under stress (osmotic stress, oxidative stress, heat shock, cadmium stress and stationary phase), the stress sensor histidine kinases (Mak 1, 2, 3) are activated, triggering the cascade process in MAPK pathway (**Figure 4.1.3**). This chain reaction process involved in a succession of genes like *spy1, mca4, wak1/win1* (MAPKKK), *wis1* (MAPKK), *sty1* (MAPK), *atf1* and *pcr1*. The Pyp1 and Pyp2 are also involved in this series reaction (**Figures 4.1.2 and 4.1.3**) (Alao, J. P. & Sunnerhagen, P., 2008; Perez, P. & Cansado, J., 2010; Sanso, M., et al., 2011).

The MAPK pathway is also involved in cold shock response. The cold stress transiently triggeres the MAPK pathway and then rapidly abolishes it, shifting to the Wak1p/Win1p-Wis1p-Sty1p stress-activated protein kinase (SAPK) pathway, partially depending on Wak1p (MAPKKK) and fully depending on Wis1p (MAPKK). Cold-induced SAPK pathway is dispensable for yeast growth in cold conditions, and Atf1/Pcr1 are critical for cell survival under cold conditions (Soto, T., et al., 2002).

A published paper demonstrates that the MAPK (Sty1-Wis1) pathway also plays a major role in the entry into quiescence. The Sty1 and Wis1 are involved in the response of nitrogen starvation and the downstream target of sty1 for arresting cell growth in the nitrogen deprivation progress is neither Rum1 (CDK inhibitor) or Atf1 (Sajiki, K., et al., 2009). In support of the work of Sajiki,

K., et al., the induction of Sty1 transient activation has been observed (Shiozaki, K. & Russell, P., 1996), which is carried through the TOR signalling pathway (Petersen, J. & Nurse, P., 2007). These data suggest the link between the MAPK pathway and the TOR pathway. Although there is no direct evidence showing the relationship between Sty1 and Cdc2-Cdc13, the requirement of Sty1-Wis1 for Rum1 upregulation (Sajiki, K., et al., 2009) suggests that MAPK is potentially involved in nitrogen deprivation triggered cell quiescence.

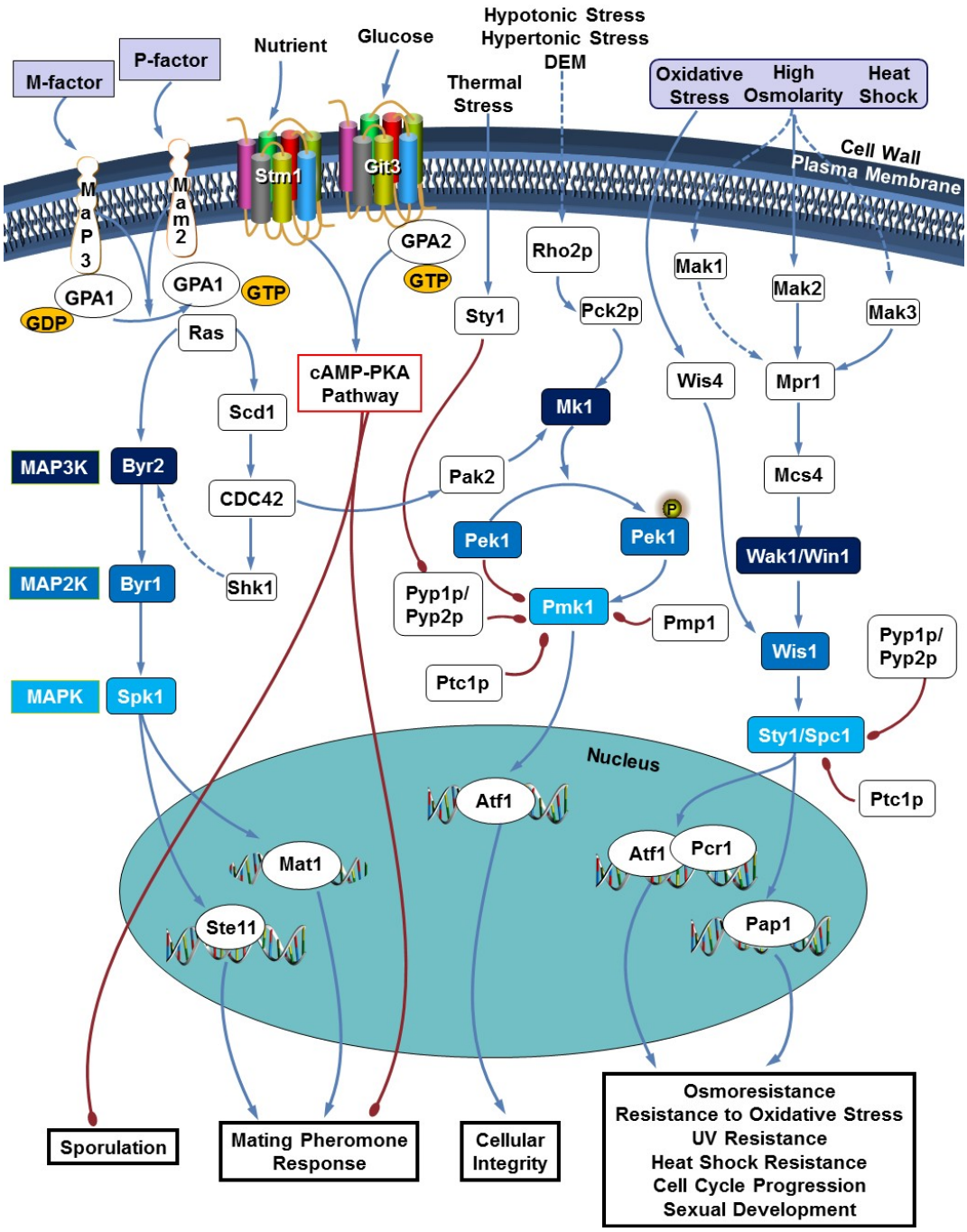


Figure 4.1.2 Components and signalling model of the MAP kinase pathway in *S. pombe*

There are three MAPK pathways: the pheromone signalling pathway, the stress-activated pathway (SAPK), and the cell integrity pathway (CIP). The Sty1 pathway is the major stress response pathway in *S. pombe*. Figure adapted from QIAGEN's original image (https://www.qiagen.com/gb/shop/genes-and-pathways/pathway-details/?pwid=280).

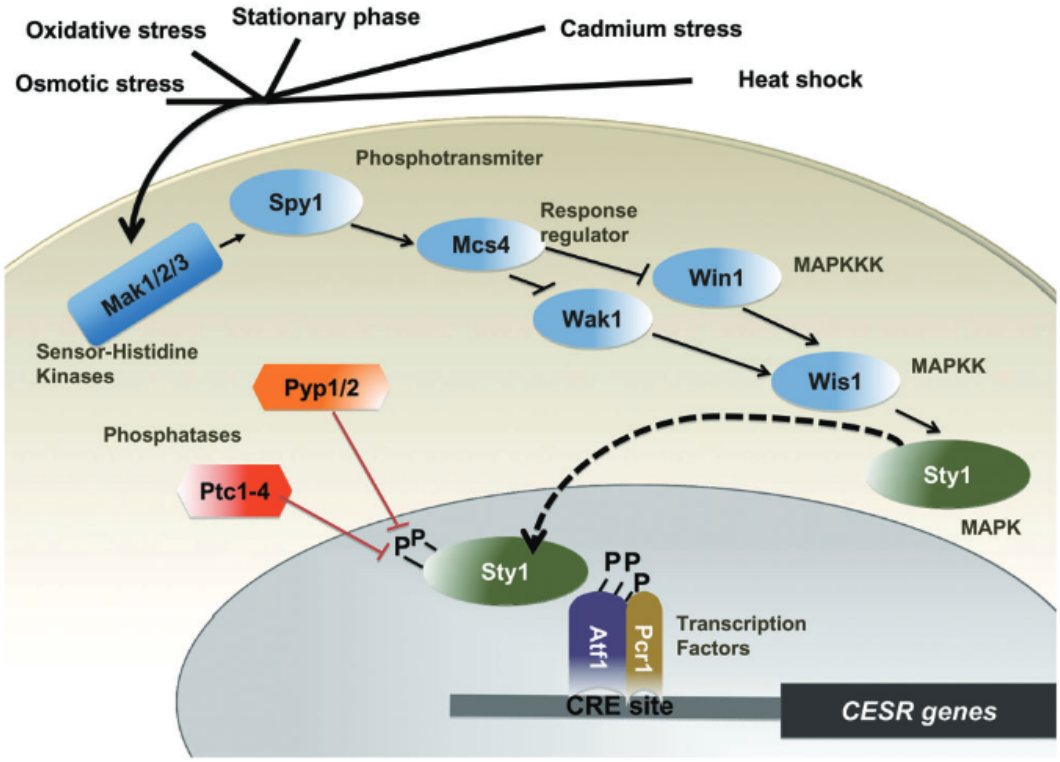


Figure 4.1.3 Stress activation of the Sty1 MAP kinase pathway. Figure adapted from Sanso, M., Vargas-Perez, I., Garcia, P., Ayte, J. and Hidalgo, E. (2011).

4.1.5 The TOR pathway and nitrogen starvation

The highly conserved TOR (target of rapamycin) pathway is essential for the regulation of cell growth and responds to nutrient deprivation. Deregulation of TOR has been reported to lead to several human diseases, including cancer, immune dysfunction and autism. In *S. pombe*, there are two TOR homologs, Tor1 and Tor2. Tor2 plays a major role in cellular growth control and compose the TOR complex 1 (TORC1), together with Mip1 and other conserved proteins. Tor1 is mainly associated with Ste20 and Sin1, forming the TOR complex 2 (TORC2). TORC1 takes the major role of regulating cellular growth under natural growth condition; TORC1 disruption mimics the wild-type cells response to nitrogen starvation. TORC2 is not required under normal growth conditions but is essential in response to stresses and nutrient starvation. TORC2 is also important for gene silencing, telomere length maintenance and DNA damage response (**Figure 4.1.4**). (Hall, Michael N., 2010)

TOR1 and TOR2 are have the opposite regulation in response to nitrogen starvation. The TORC1 defect mutant shows a mimic phenotype of the wild type cells' response to nitrogen starvation, while the TORC2 depletion shows a phenotype of 'never starved for nitrogen'. The Tor2Δ mutant has shortened cell morphology and can be induced into sexual development on rich media. The Tor1Δ results in an elongated cell morphology, has no cell-size response to nitrogen starvation and is highly sterile. The Tor2Δ mutant also has an

increased expression of nitrogen-starvation specific genes, while Tor1Δ lead to reduced expression of these genes. TORC1 negatively regulates the entrance of cytokinesis, while TORC2 undergoes positive regulation of mitotic entry. Both TORC1 and TORC2 deficiency cause the defect of amino acid uptake. (Hall, Michael N., 2010)

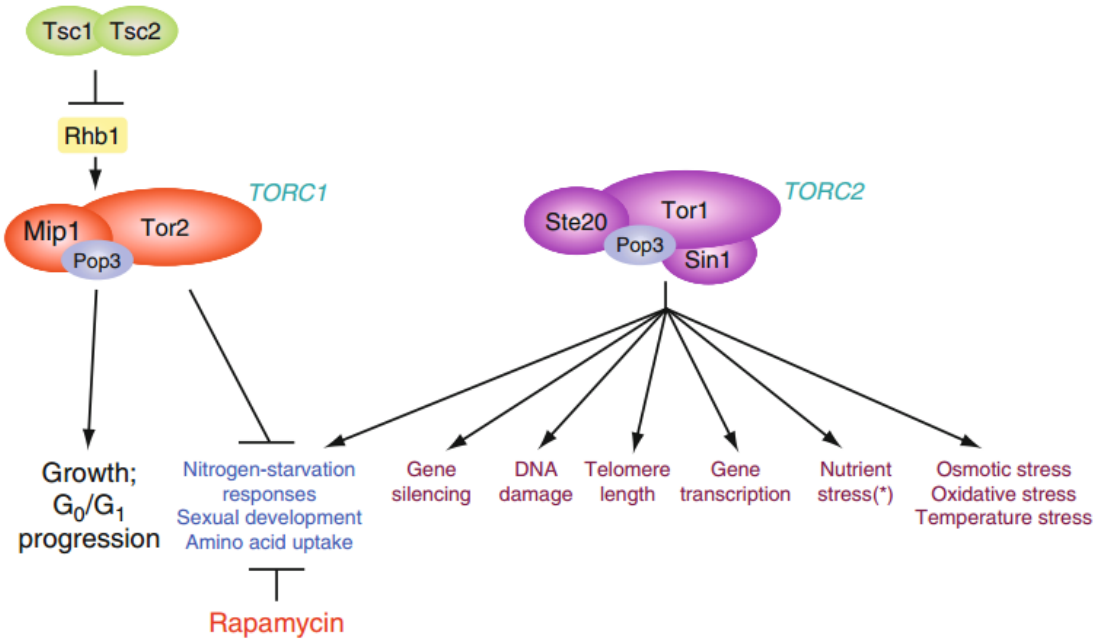


Figure 4.1.4 Schematic model of TORC1 and TORC2 in *S. pombe*. TORC1 is mainly responsible for the cell growth. TORC2 takes the roles of stress response, gene silencing and DNA damage response. TORC1 and TORC2 regulate nitrogen starvation response in opposite ways. (*): Properly enter and maintain for the stationary phase following nutrient depletion. Figure adapted from Hall, Michael N. (2010)

When cells OD_{600} grow higher than 1.0, which indicates that the cells are approaching the stationary phase and the nutrients in the media are largely consumed, the nutrient limitation will arrest the cells at the G1 or G2 stage, depending on whether the limiting factor is nitrogen or carbon. The nitrogen limitation arrests cells at the G1 stage and consequently triggers the G1 to G0 transition. whereas carbon deprivation results in G2 arrest and finally undergoes the G2 to G0 transition (Su, S. S., et al., 1996).

Previously published papers have demonstrated that CTPS is required for G0 phase entry and maintenance, and negatively regulates the G0 to G1 transition (Sajiki, K., et al., 2009). It has also been reported that the downregulation of both the CTPS protein and the mRNA level during cell quiescence is induced by nitrogen deprivation in *S. pombe* (Marguerat, S., et al., 2012). An undefined mutation in the *S. pombe cts1* gene locus resulted in the loss of viability in nitrogen deprivation media (Sajiki, K., et al., 2009), indicating that CTPS is critical for the cell survival during nitrogen starvation. Nitrogen starvation also downregulates the purine biosynthesis intermediates and decreases the cellular level of several amino acid including glutamine and glutamate (**Figure 4.1.5**) (Sajiki, K., et al., 2013). One of the downregulated purine biosynthesis intermediates is AICAR, which is the activator for the cellular energy homeostasis regulator AMPK (Sajiki, K., et al., 2013).

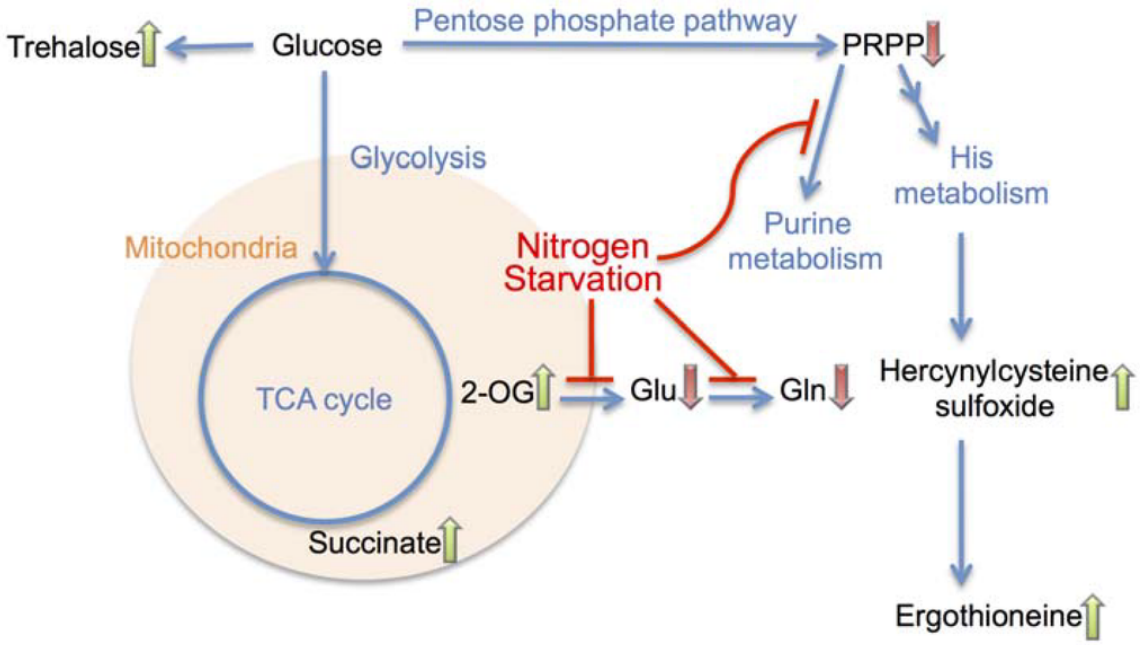


Figure 4.1.5 A summary diagram of metabolic changes in *S. pombe* cells at the onset of nitrogen starvation. Adapted from Sajiki, K., Pluskal, T., Shimanuki, M. and Yanagida, M. (2013)

4.1.6 Chapter aims

Several drug treatments have been undertaken on *S. pombe* in this chapter, including DON (CTPS inhibitor), MPA (IMPDH inhibitor), Hydroxyurea (shortened as HU, an RNR inhibitor and the DNA damage inducer) and sodium azide (inhibitor of cytochrome oxidase and therefore decrease cellular ATP level). I have also demonstrated the responses of cytoophidia to different environment stresses. These experimental results suggest that cytoophidia formation is regulated by environmental conditions. Several theories have also been raised regarding the potential links between cytoophidia and several different metabolic processes. All these primary data establish a brief background to the cytoophidium research in *S. pombe* and can benefit the future cytoophidium study.

4.2 Results

4.2.1 Cytoophidia respond to several drugs in *S. pombe*, including DON

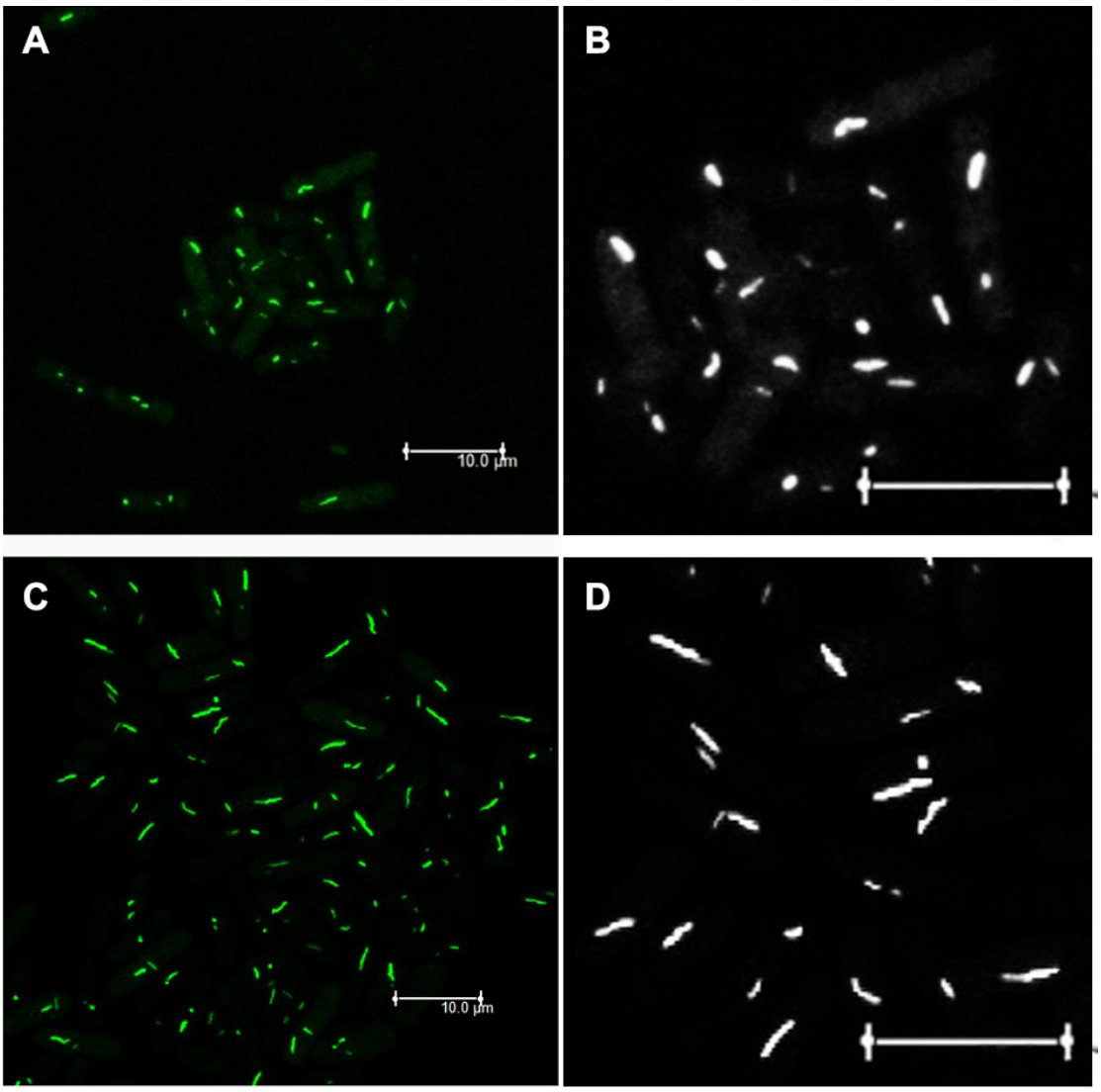
Different drugs have been tested on *S. pombe* cells, including DON, MPA, Azide and Hydroxyurea (**Table 4.2.1**). *S. pombe* cells were awakened and cultured in YE3S media at 32°C. The drug concentration and treatment time used in the experiments are shown in **Table 4.2.2**. Experiments were taken at 32°C. Notably, after treatment with the CTPS inhibitor DON, the average length of cytoophidia was increased (**Figure 4.2.1**), which is similar to the situation observed in *Drosophila* and human HeLa cells (Chen, K., et al., 2011; Aughey, G. N., et al., 2014). However, unlike the published data in the *Drosophila* and human cell line, the cytoophidia number per cell in *S. pombe* decreased after DON treatment. Interestingly, the CTPS protein level was unchanged after DON treatment (**Figure 4.2.2)**. Treatment with IMPDH inhibitors MPA increased the average numbers of cytoophidia from 1.8 to 3.17 (**Figure 4.2.3 A**), while the RNR inhibitor and the DNA damage inducer Hydroxyurea (HU) downregulated the assembly of cytoophidia, decreasing the cytoophidia containing cell percentage from 98.10% to 60.77% (**Figure 4.2.3 B**). Notably, azide treatment resulted in an increased, then a decreased cytoophidia number per cell, and consequently lead to a total disassembly of cytoophidia after treatment for 4 hours (**Figure 4.2.3 C and D**).

Name	Functions	Mechanism	References
DON	Unspecific inhibiting of CTPS	Acts as the glutamine analogue and covalently binds with CTPS at the glutamine binding site	Pinkus, L. M., 1977; Ortlund, E., et al., 2000
Mycophenolic Acid (MPA)	Reversibly inhibits IMPDH; immunosuppressant that prevents rejections in organ transplantation	Covalently binds with IMPDH, acting as a replacement of nicotinamide portion of the nicotinamide adenine dinucleotide cofactor and as a catalytic water molecular	Fulton, B. & Markham, A., 1996; Kitchin, J. E., et al., 1997; Ransom, J. T., 1995
Sodium azide	Depletes intracellular ATP level by inhibiting cytochrome oxidase	Inhibits cytochrome oxidase by binding irreversibly to the heme cofactor	Harvey, J., et al., 1999; Tsubaki, M., 1993
Hydroxyurea (HU)	Inhibits RNR and cause DNA damage	Arrests DNA replication and stalls the Replication Forks	Feng, W., et al., 2011

Table 4.2.1 Effects and mechanisms of drugs tested in experiments.

Drug	Concentration used	Duration
DON	10 μM	4 hours
MPA	10 μM	4 hours
Sodium azide*	10 mM	4 hours
Hydroxyurea (HU)	18 mM	4 hours

Table 4.2.2 List of drugs and conditions that tested in experiments. Cells were taken as samples and fixed every hour. *Samples of the sodium azide treatment experiment were taken at 15, 30, 60 minutes and 2 and 4 hours for different time points.


A
10.0 µm
B
C
10.0 µm
D

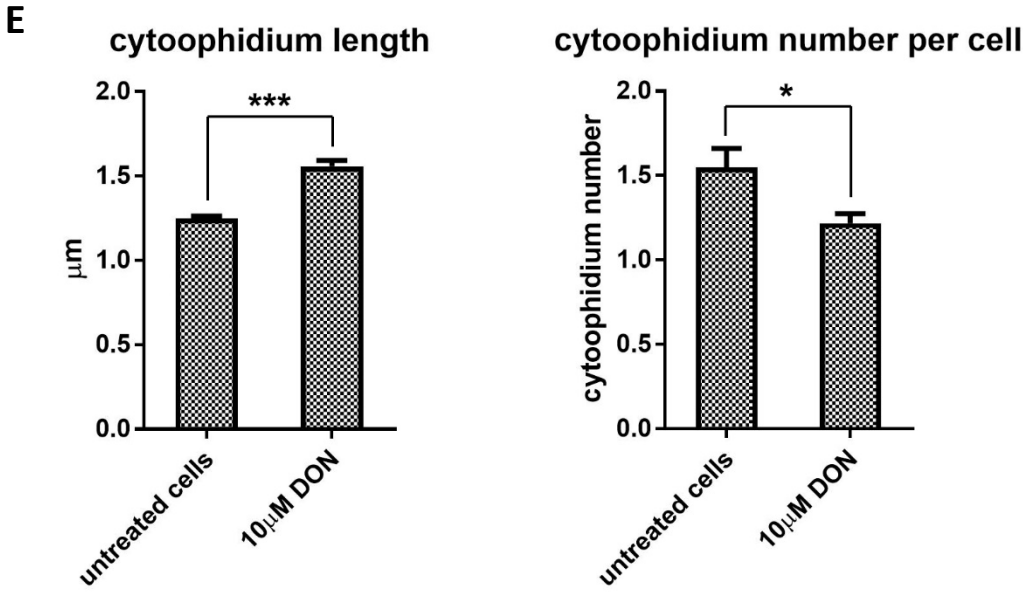

E
cytoophidium length
***
µm
2.0
1.5
1.0
0.5
0.0
untreated cells
10µM DON
cytoophidium number per cell
*
cytoophidium number
2.0
1.5
1.0
0.5
0.0
untreated cells
10µM DON

Figure 4.2.1 DON treatment caused an increase of cytoophidia length in *S. pombe*. (A) Cells without DON treatment; (B) Zoomed-in cells for detail from A; (C) DON treatment for 4 hours; (D) Zoomed-in cells for detail from C; (E) DON increased the average length of cytoophidia, and interestingly decreased the number of cytoophidia. Scale bar: 10μm. Error bar: Mean±SEM.

Results fo the T test analysis:

Average length of cytoophidia

Column A	untreated cells
vs	vs
Column B	10μM DON
Paired t test	
P value	0.0002
P value summary	***
Are means signif. different? (P < 0.05)	Yes
One- or two-tailed P value?	Two-tailed
t, df	t=8.335 df=6

Average number of cytoophidia per cell

Column B	10μM DON
vs.	vs.
Column A	untreated cells
Unpaired t test	
P value	0.0351
P value summary	*
Significantly different (P < 0.05)?	Yes
One- or two-tailed P value?	Two-tailed
t, df	t=2.374 df=12

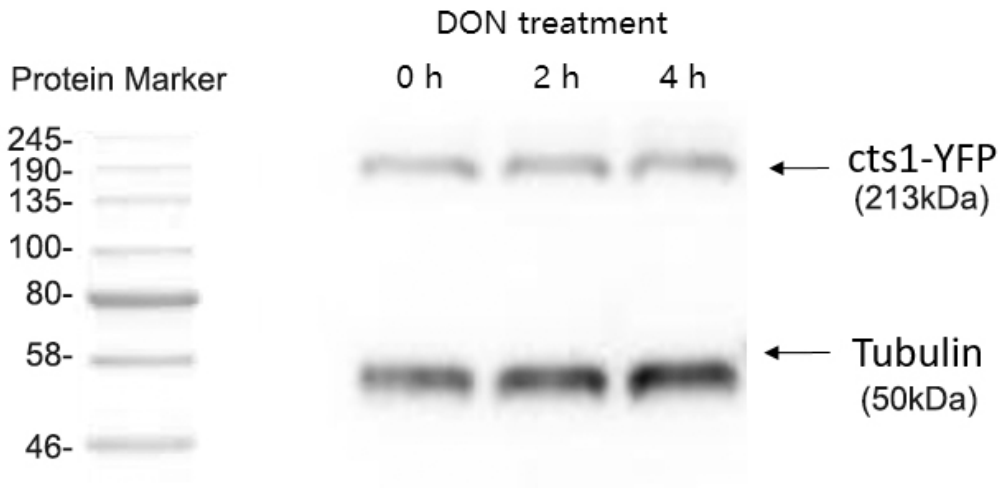


Figure 4.2.2 DON treatment did not affect the CTPS protein level. Cells were treated with DON for 2 hours and 4 hours. The results showed that DON treatment did not increase the CTPS protein expression level. Scale bar: 10μm.

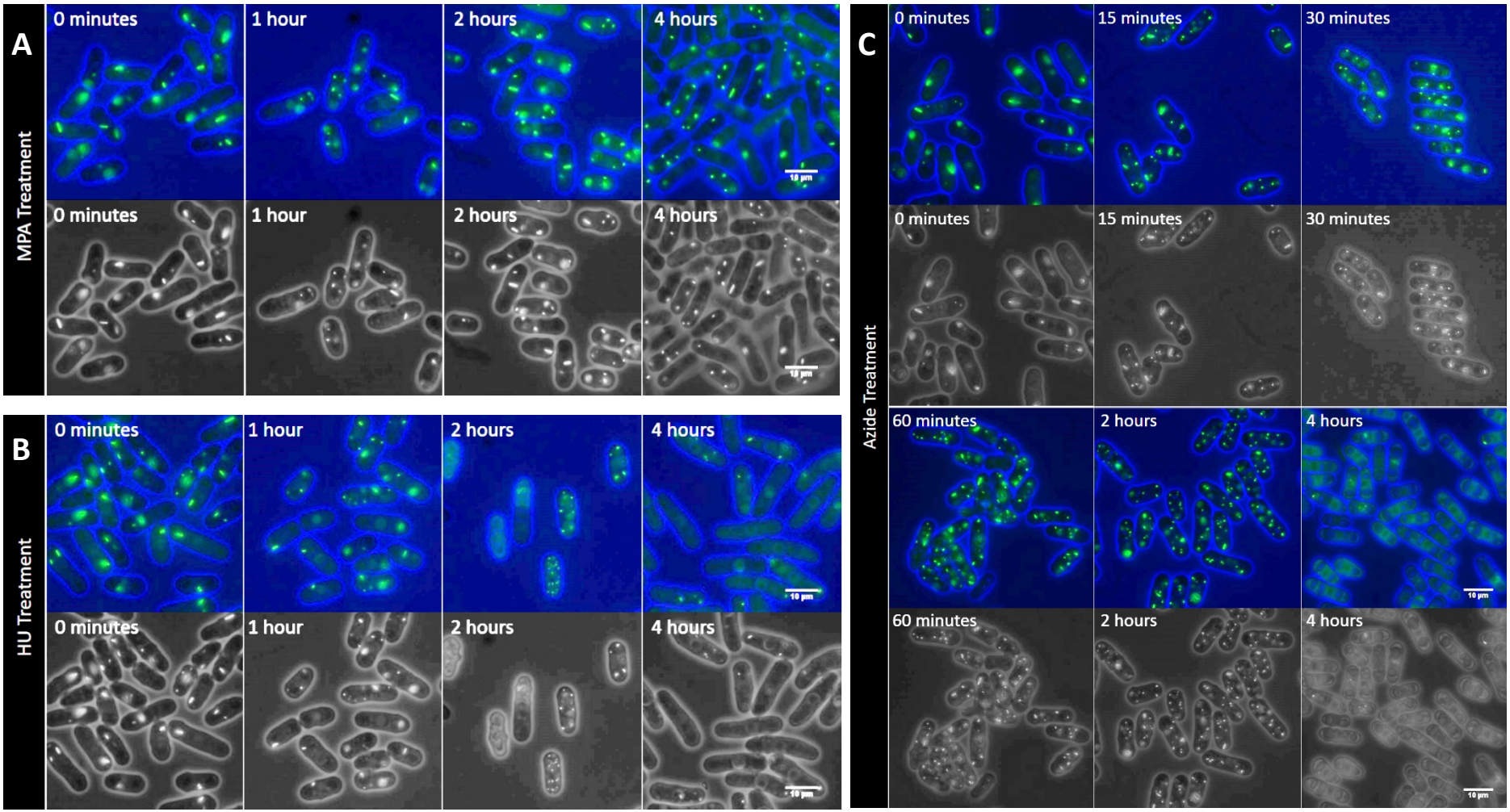


Figure 4.2.3 (part1) Cytoophidia respond differently with different drugs

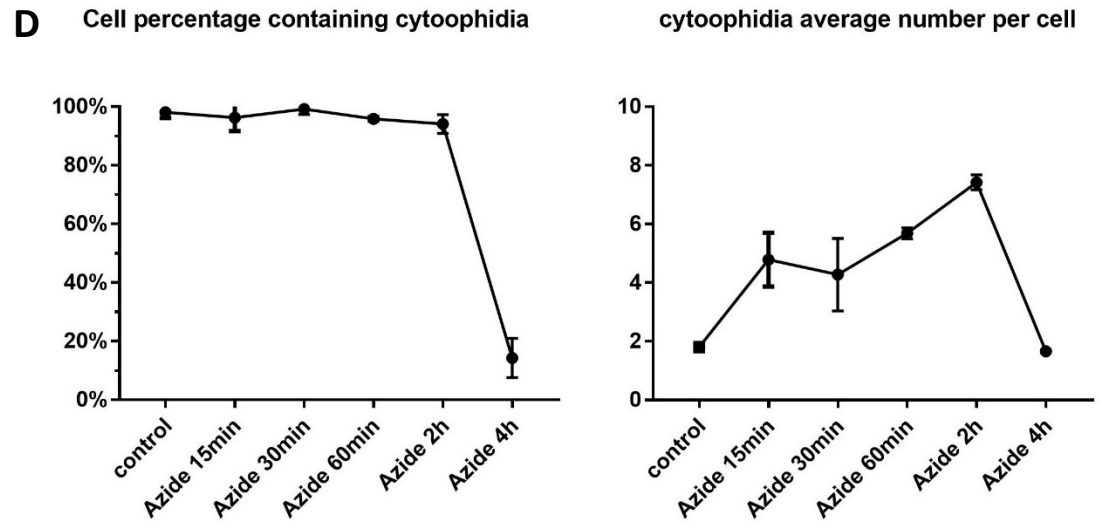


Figure 4.2.3 (part 2) Cytoophidia respond differently with different drugs. (A) MPA treatment upregulated cytoophidia average numbers per cell from 1.8 (n=392) to 3.17 (n=202); (B) HU treatment downregulated cytoophidia containing cell percentage from 98.10% (n=392) to 60.77% (n=315); (C-D) Azide treatment led to an increased, then a decreased, cytoophidia number per cell, and consequently led to a total disassembly of cytoophidia after treatment for 4 hours. Scale bar: 10μm. Error bar: Mean±SEM.

4.2.2 Cytoophidia respond to different environmental stresses

Aside from drugs, cytoophidia respond to several different environmental stresses including temperature stress, osmotic stress, oxidative stress, and nutrient deprivation. Experiments were taken under certain conditions (**Table 4.2.3**). Different experiments led to different effects on either cytoophidia numbers or cytoophidia average length. According to the confocal read-outs, nutrient deprivation (glucose and nitrogen starvation) caused a decrease in the average length of cytoophidia, while osmotic stress and oxidative stress had no significant effect on the average length of cytoophidia. However, both osmotic stress and oxidative stress caused a decrease in the number of cytoophidia, while glucose starvation caused no significant change. Unlike glucose starvation, nitrogen deprivation resulted in a decrease in the number of cytoophidia, resembling the oxidative stress and osmotic stress. The stationary stage growing condition cause a complete dispersal of cytoophidia, with either cytoophidia number or cytoophidia average length becoming zero under this condition **(Figure 4.2.4**).

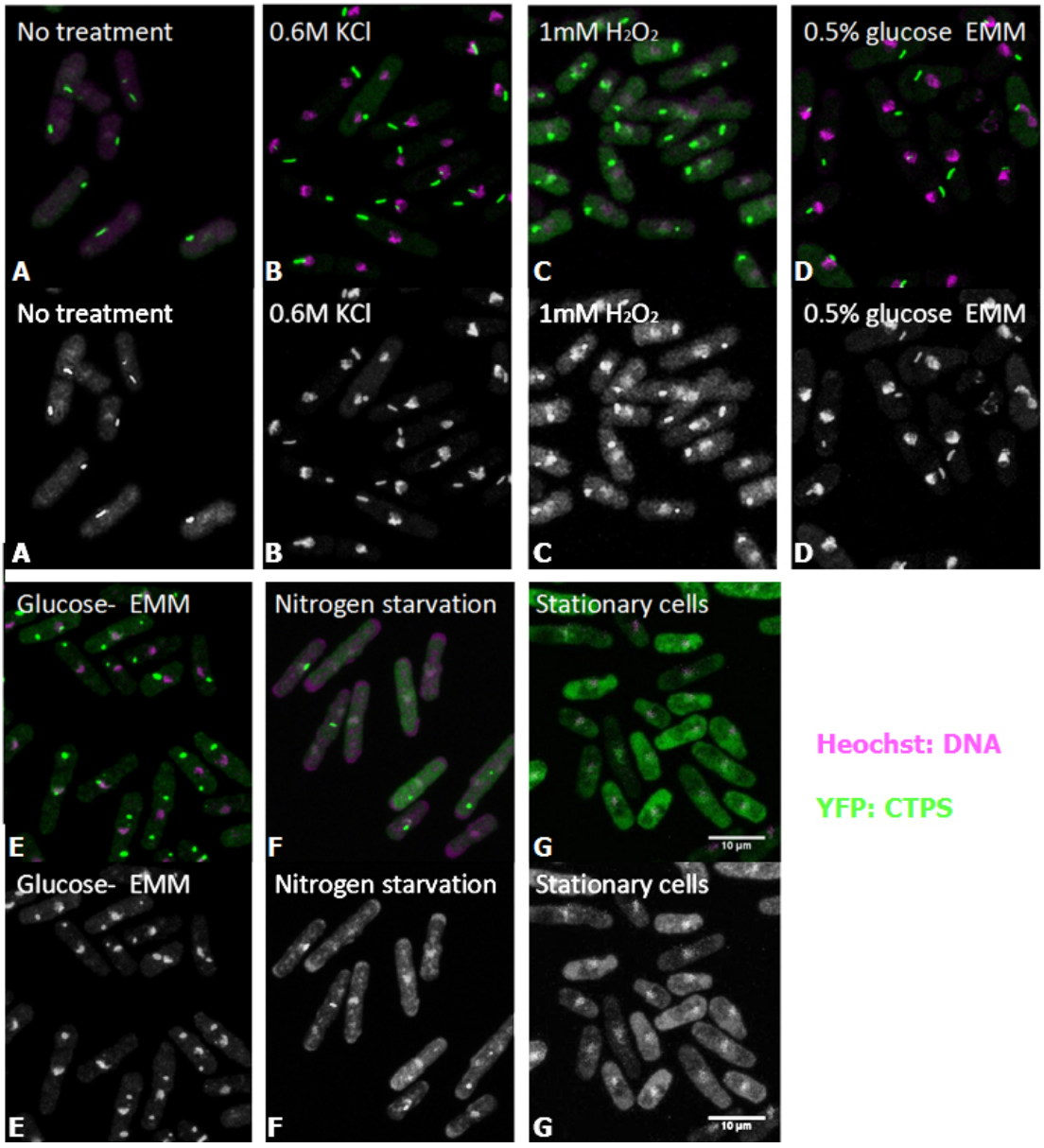
When grown in pure water, *S. pombe* cells also showed a response. Cells were washed with sterile water three times and then cultured in water at 32°C. After as little as 5 minutes, the cytoophidia appeared to have a decrease in both number and average length (not shown in figures). After 2 hours, most of the cytoophidia disappeared (**Figure 4.2.5**), indicating that washing with water can

distinctly affect cytoophidia in live cells.

Most stresses, including osmotic stress, oxidative stress and nutrient deprivation, can affect cytoophidia in live cells. The cell fixation process should thus be taken very carefully to guarantee that cytoophidia remain in the same pattern after a fixation. The best solution we worked out was to add 4% PFA directly into the cell culture for 10 minutes and wash three times with PBS.

	Concentration	Sample collection
KCl (Osmotic stress)	0.6M	Samples were taken after 2 hours' treatment under different conditions; cells were fixed with 4% PFA for 10 minutes and then washed with PBS twice and kept in PBST at 4°C. *PBST: PBS+0.25%NHS+0.3% Triton-X
H_2O_2 (Oxdative stress)	1mM	
Glucose starvation	0.5% Glucose EMM medium	
Nitrogen Starvation	Nitrogen-EMM medium	
H_2O		
Stationary stage		

Table 4.2.3 List of stresses tested


No treatment
0.6M KCl
1mM H2O2
0.5% glucose EMM
A
B
C
D
No treatment
0.6M KCl
1mM H2O2
0.5% glucose EMM
A
B
C
D
Glucose- EMM
Nitrogen starvation
Stationary cells
E
F
G
10 μm
Glucose- EMM
Nitrogen starvation
Stationary cells
E
F
G
10 μm
Heochst: DNA
YFP: CTPS

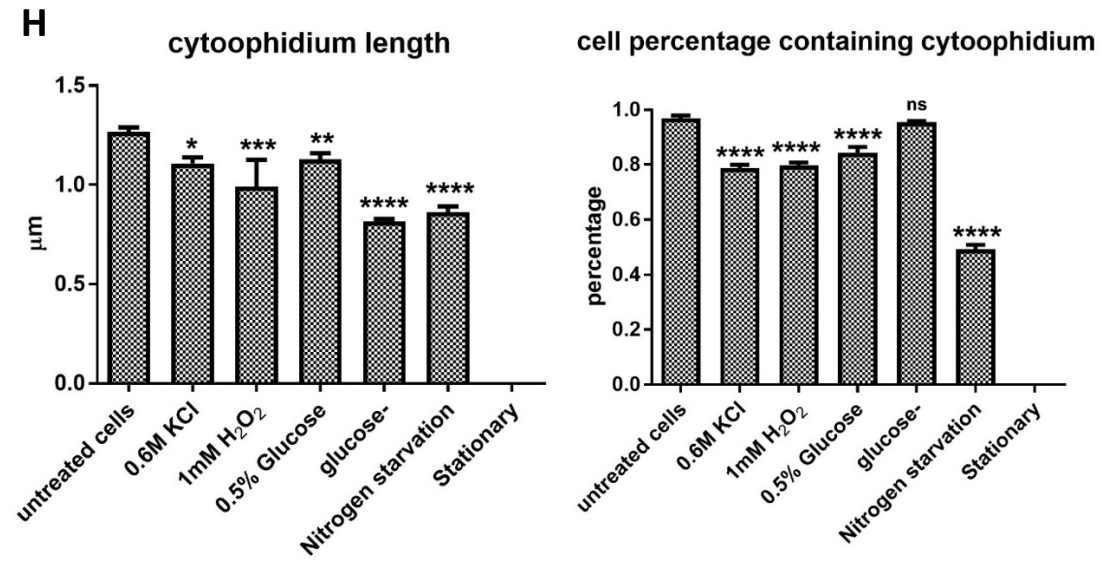

H
cytoophidium length
μm
1.5
1.0
0.5
0.0
*
***
**
****
****
untreated cells
0.6M KCl
1mM H2O2
0.5% Glucose
glucose-
Nitrogen starvation
Stationary
cell percentage containing cytoophidium
percentage
1.0
0.8
0.6
0.4
0.2
0.0
****
****
****
ns
****
untreated cells
0.6M KCl
1mM H2O2
0.5% Glucose
glucose-
Nitrogen starvation
Stationary

Figure 4.2.4 *S. pombe* cytoophidia respond to different environmental stresses. (B-G) show how cytoophidia respond to the osmotic stress (0.6M KCl), oxidative stress (1mM H_2O_2), low carbon source (0.5% glucose), carbon deprivation, nitrogen deprivation and stationary stage. Scale bar: 10 μm. Error bar: Mean±SEM.

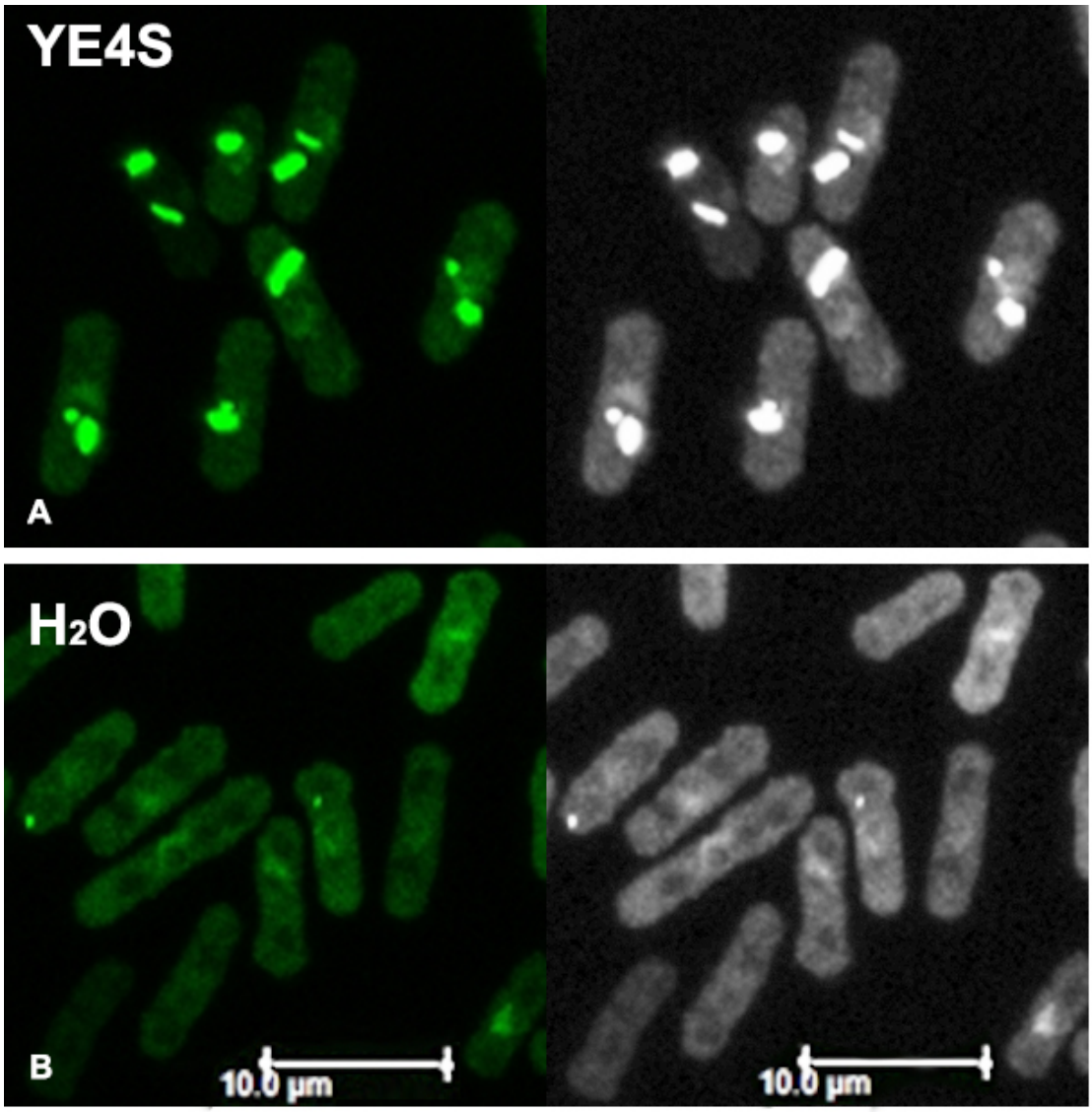
Results fo the T test analysis:

Average length of cytoophidia

	Mean Diff.	95.00% CI of diff.	Significant?	Summary	P Value
untreated cells vs. 0.6M KCl	0.1581	0.01768 to 0.2986	Yes	*	0.0219
untreated cells vs. 1mM H_2O_2	0.2746	0.1194 to 0.4299	Yes	***	0.0002
untreated cells vs. 0.5% Glucose	0.137	0.02824 to 0.2458	Yes	**	0.0086
untreated cells vs. glucose-	0.4498	0.3351 to 0.5644	Yes	****	<0.0001
untreated cells vs. Nitrogen starvation	0.4041	0.2895 to 0.5188	Yes	****	<0.0001
untreated cells vs. Stationary	1.267	1.136 to 1.397	Yes	****	<0.0001

Average number of cytoophidia per cell

	Mean Diff.	95.00% CI of diff.	Significant?	Summary	P Value
untreated cells vs. 0.6M KCl	0.1806	0.1102 to 0.2511	Yes	****	<0.0001
untreated cells vs. 1mM H_2O_2	0.1694	0.09152 to 0.2473	Yes	****	<0.0001
untreated cells vs. 0.5% Glucose	0.1247	0.07015 to 0.1793	Yes	****	<0.0001
untreated cells vs. glucose-	0.01296	-0.04456 to 0.07047	No	ns	0.9774
untreated cells vs. Nitrogen starvation	0.476	0.4185 to 0.5335	Yes	****	<0.0001
untreated cells vs. Stationary	0.9675	0.902 to 1.033	Yes	****	<0.0001


YE4S
A
H2O
B
10.0 µm
10.0 µm

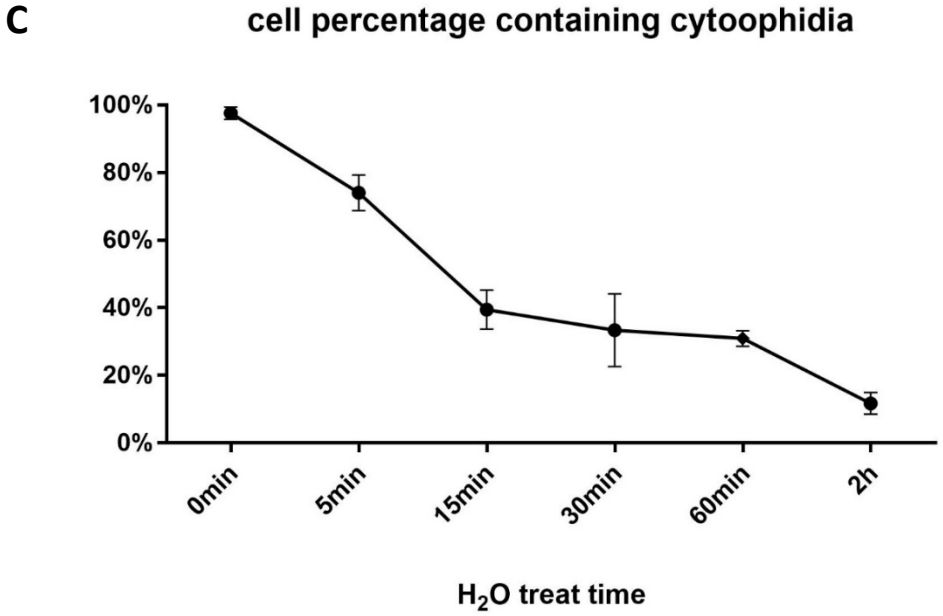
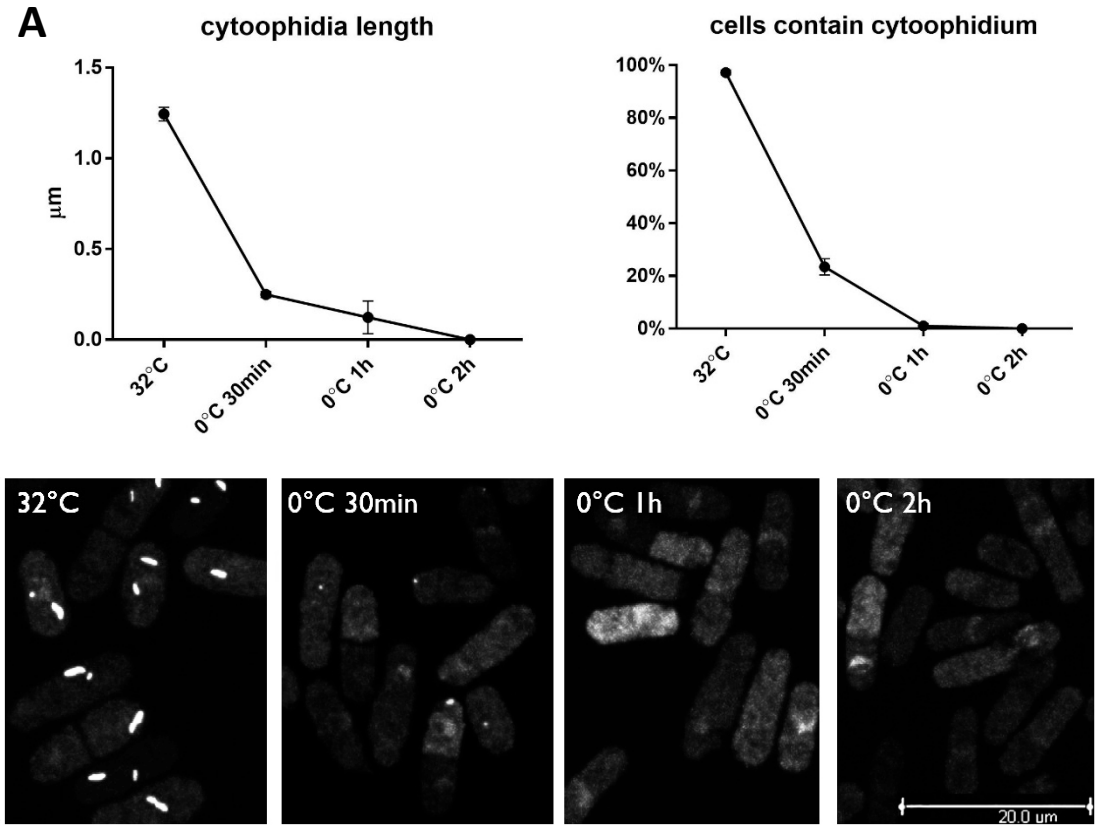

C
cell percentage containing cytoophidia
100%
80%
60%
40%
20%
0%
0min
5min
15min
30min
60min
2h
H2O treat time

Figure 4.2.5 H_2O decrease the number of cytoophidia in *S. pombe*. (A) Cells are cultured in normal condition (YE4S media). (B) After being cultured in sterile water for 2 hours, most cytoophidia disappear and CTPS-YFP filaments disperse into cytoplasm. Scale bar: 10 μm. (C) Cell counting result showed a dramatically decrease of cell percentage of containing cytoophidia after *S. pombe* cells were cultured in water for 2 hours. Error bar: Mean±SEM.

4.2.3 Both cold stress and heat stress disperse cytoophidia

To test whether cytoophidia respond to temperature stress in *S. pombe*, both heat shock and cold shock experiments were undertaken. At the starting time point, cells were cultured in YE3S media at 32°C at the exponential stage. When put on ice, the number and average length of cytoophidia significantly decreased (**Figure 4.2.6**). After ice-cold treatment for 2 hours, all cytoophidia disappeared. When exposed to high temperature (37°C), the number and average length of cytoophidia also decreased (**Figure 4.2.6**). These results indicate that cytoophidia are sensitive to temperature changes and respond to both high and low temperatures.


A
cytoophidia length
μm
1.5
1.0
0.5
0.0
32°C
0°C 30min
0°C 1h
0°C 2h
cells contain cytoophidium
100%
80%
60%
40%
20%
0%
32°C
0°C 30min
0°C 1h
0°C 2h
32°C
0°C 30min
0°C 1h
0°C 2h
20.0 um

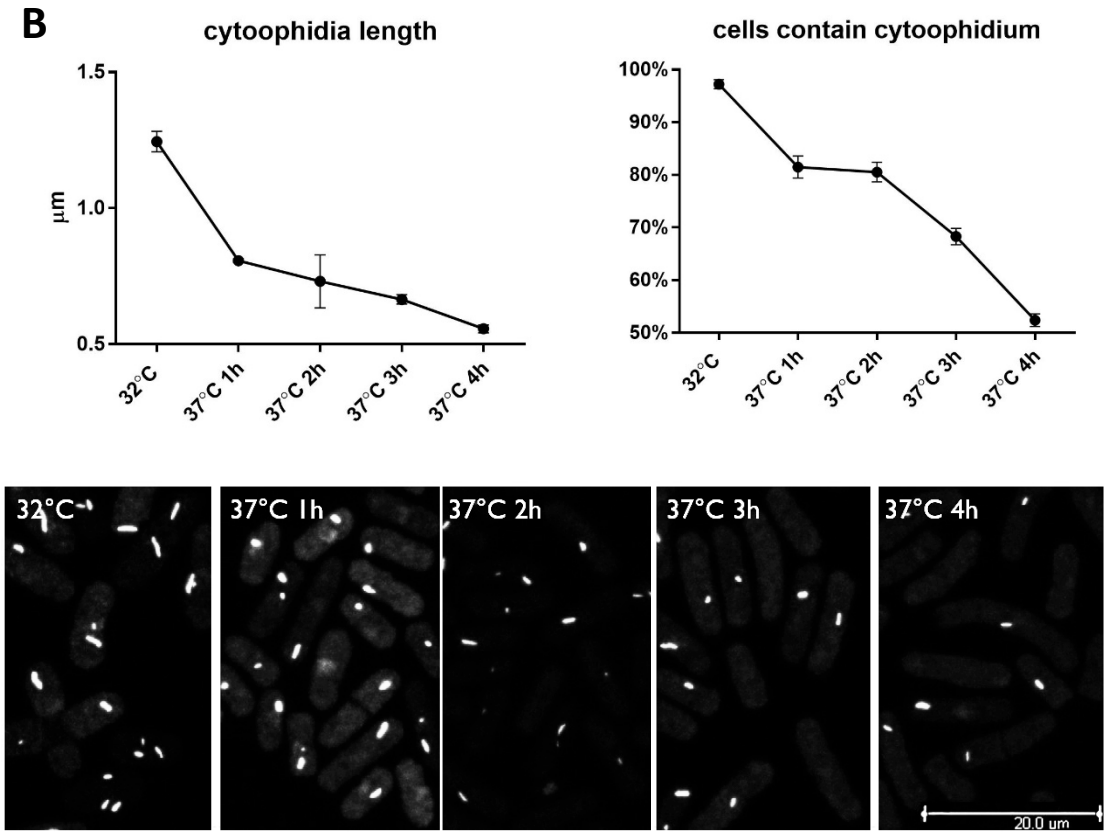

B
cytoophidia length
μm
1.5
1.0
0.5
32°C
37°C 1h
37°C 2h
37°C 3h
37°C 4h
cells contain cytoophidium
100%
90%
80%
70%
60%
50%
32°C
37°C 1h
37°C 2h
37°C 3h
37°C 4h
32°C
37°C 1h
37°C 2h
37°C 3h
37°C 4h
20.0 um

Figure 4.2.6 Both high and low temperatures cause the decrease of cytoophidia in *S. pombe*. Cytoophidia are sensitive to both 0℃ and 37℃. (A) When exposed to an ice-cold environment, cytoophidia dramatically decreased in number and average length. Most cytoophidia disassembled after 30 minutes and totally disappeared after 2 hours. (B) When exposed to 37℃, both the average length of cytoophidia and the cell percentage containing cytoophidia decreased. Scale bar: 20 μm. Error bar, Mean±SEM

4.2.4 Cytoophidia respond very quickly to both heat and cold stresses

Cytoophidia in *S. pombe* cells respond to both heat and cold stresses. Since the decrease in the number and length of cytoophidia after cold and heat stresses were so dramatic, a shorter time treatment (5 minutes) was taken. As shown in **Figure 4.2.7**, after as little as 5 minutes, both the number and average length of cytoophidia decreased in response to cold (0°C) and heat (42°C) shocks, indicating that cytoophidia are extremely sensitive and respond very fast to temperature changes.

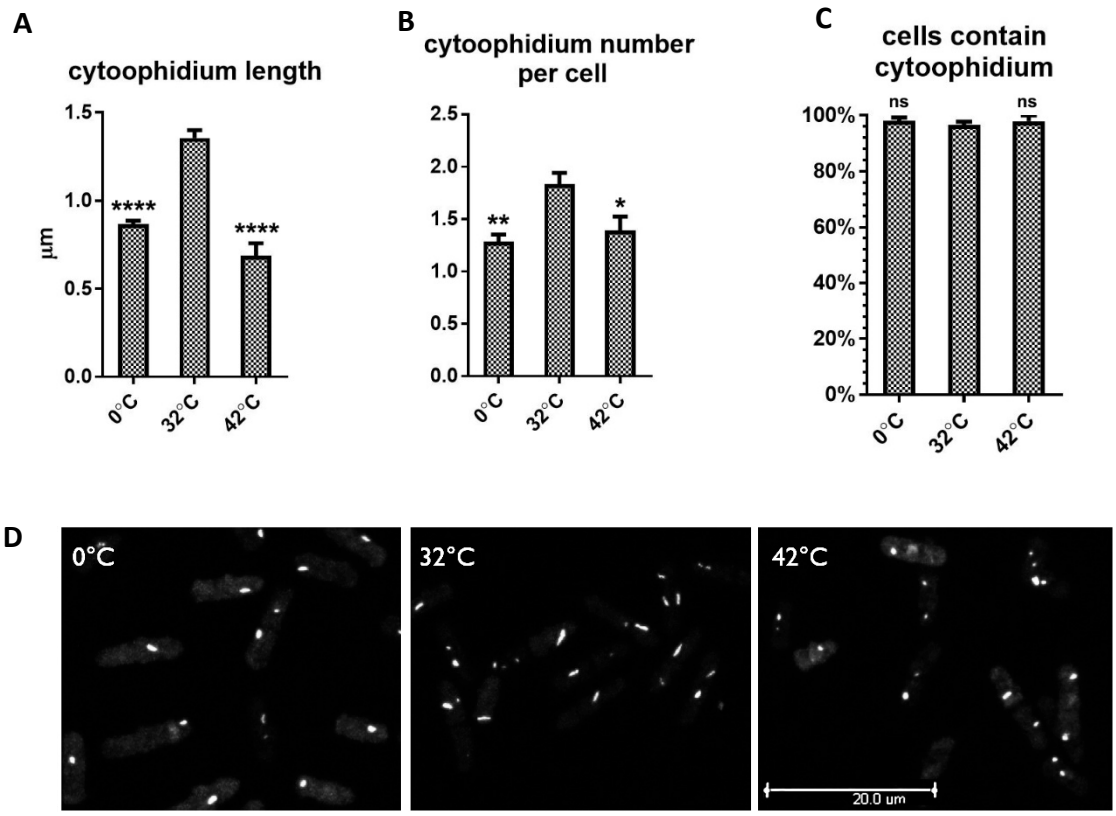


Figure 4.2.7 Cytoophidia respond quickly to both heat and cold stresses. After as little as 5 minutes, the number and average length of cytoophidia decreased in response to cold and heat shocks, indicating that cytoophidia are extremely sensitive to temperature changes. (A) The average length of cytoophidia are decreased significantly when *S. pombe* cells were exposed to 0°C or 42°C. (B) The number of cytoophidia decreased when *S. pombe* cells were exposed to 0°C or 42°C. (C) The cell percentage containing cytoophidia stayed unchanged after 5 minutes of heat or cold shock. Scale bar: 20µm. Error bar: Mean±SEM.

Results fo the T test analysis:

Average length of cytoophidia

Dunnett's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
32°C vs. 0°C	0.4887	0.3308 to 0.6465	Yes	****	<0.0001
32°C vs. 42°C	0.6667	0.4971 to 0.8362	Yes	****	<0.0001

Average number of cytoophidia per cell

Dunnett's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
32°C vs. 0°C	0.5488	0.1858 to 0.9118	Yes	**	0.0044
32°C vs. 42°C	0.442	0.05205 to 0.8319	Yes	*	0.0268

Percentage of cells contain cytoophidia

Dunnett's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
32°C vs. 0°C	-1.566	-6.356 to 3.224	No	ns	0.6449
32°C vs. 42°C	-1.352	-6.497 to 3.793	No	ns	0.7480

4.2.5 Cold-induced cytoophidia disassembly is reversible

We already know that stationary phase induced cytoophidia dispersal is reversible in *S. pombe* (Chapter 3). To examine whether temperature changes induced cytoophidia disassembly is also reversible, we performed a recovery experiment in live imaging. *S. pombe* cells were put on ice for more than 2 hours to guarantee that all cytoophidia disassembled. The cells were then transferred to a glass-bottomed dish containing 32℃ fresh YES media in a 32℃ chamber, and then inspected under confocal microscopy. It took 3 minutes and 45 seconds to prepare the sample and set up the microscopy before starting the recording. We already know cytoophidia respond to cold treatment very fast (within 5 minutes) in *S. pombe*. In the recovery experiment, we observed that cytoophidia reassembly was also very fast (**Figure 4.2.8**) (**Video 3**).

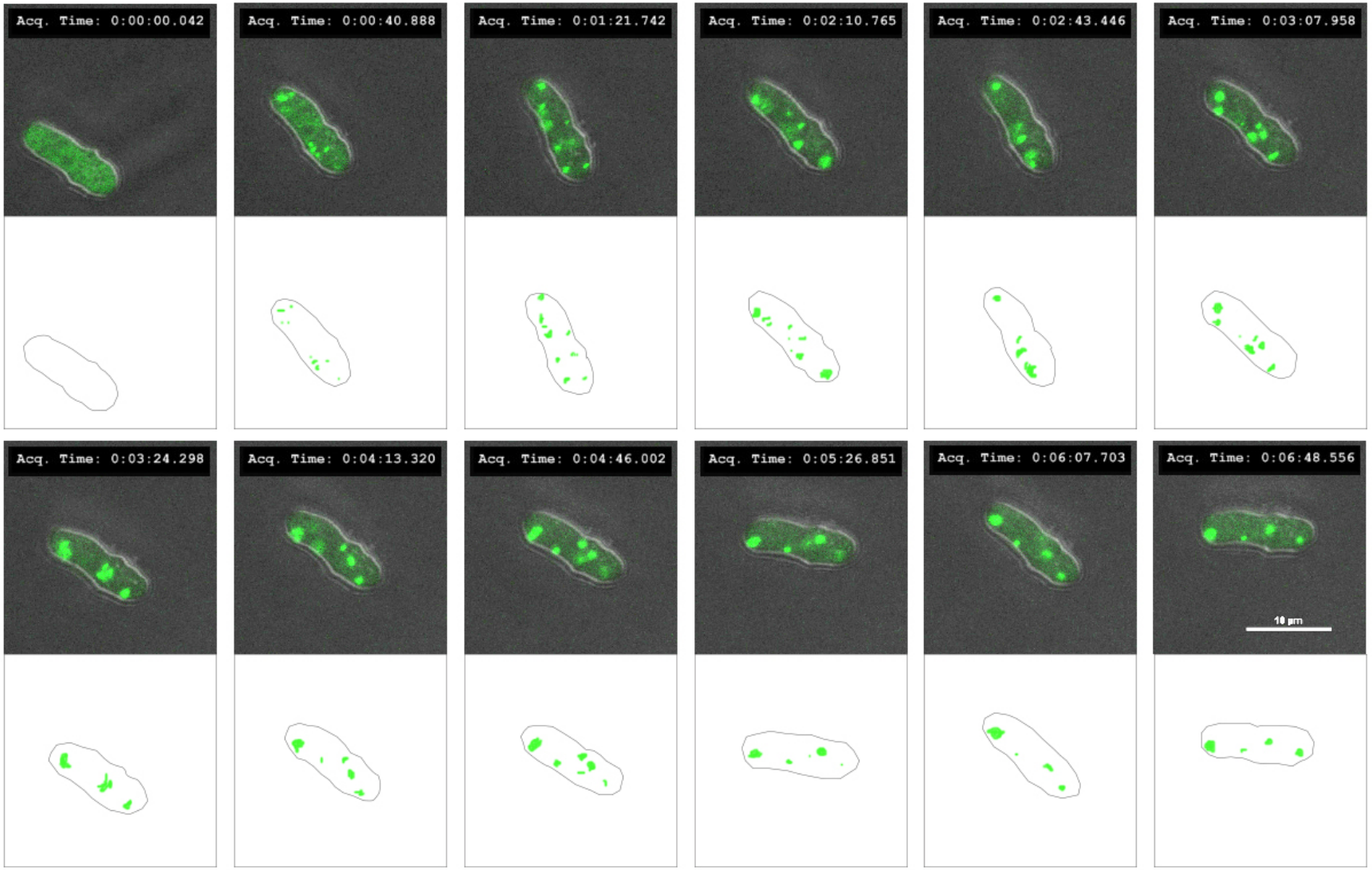

Acq. Time: 0:00:00.042
Acq. Time: 0:00:40.888
Acq. Time: 0:01:21.742
Acq. Time: 0:02:10.765
Acq. Time: 0:02:43.446
Acq. Time: 0:03:07.958
Acq. Time: 0:03:24.298
Acq. Time: 0:04:13.320
Acq. Time: 0:04:46.002
Acq. Time: 0:05:26.851
Acq. Time: 0:06:07.703
Acq. Time: 0:06:48.556
10 µm

Figure 4.2.8 Cold-induced cytoophidia dispersal is reversible and fast. It took 3 minutes and 45 seconds to prepare the sample before the confocal captureing of this video. The starting time point should be added to this 3 minutes and 45 seconds. The cells were transferred from 0℃ to 32℃ to perform the recovery experiment. We can see that within 5 minutes cytoophidia had started to emerge, indicating that cytoophidia reponse to temperature changes quickly in *S. pombe*. Scale bar: 10μm.

4.2.6 Protein level of CTPS is not changed by cold stress

To test whether CTPS protein level is changed by cold stress, a western blot was performed. Anti-CTPS antibodies were used for the western blot and Histone H3 was used as an endogenous control. As shown in **Figure 4.2.9**, samples were taken at 5, 15, 30, 60 minutes and 2 hours after the cells were put on ice, and the CTPS protein level remained unchanged.

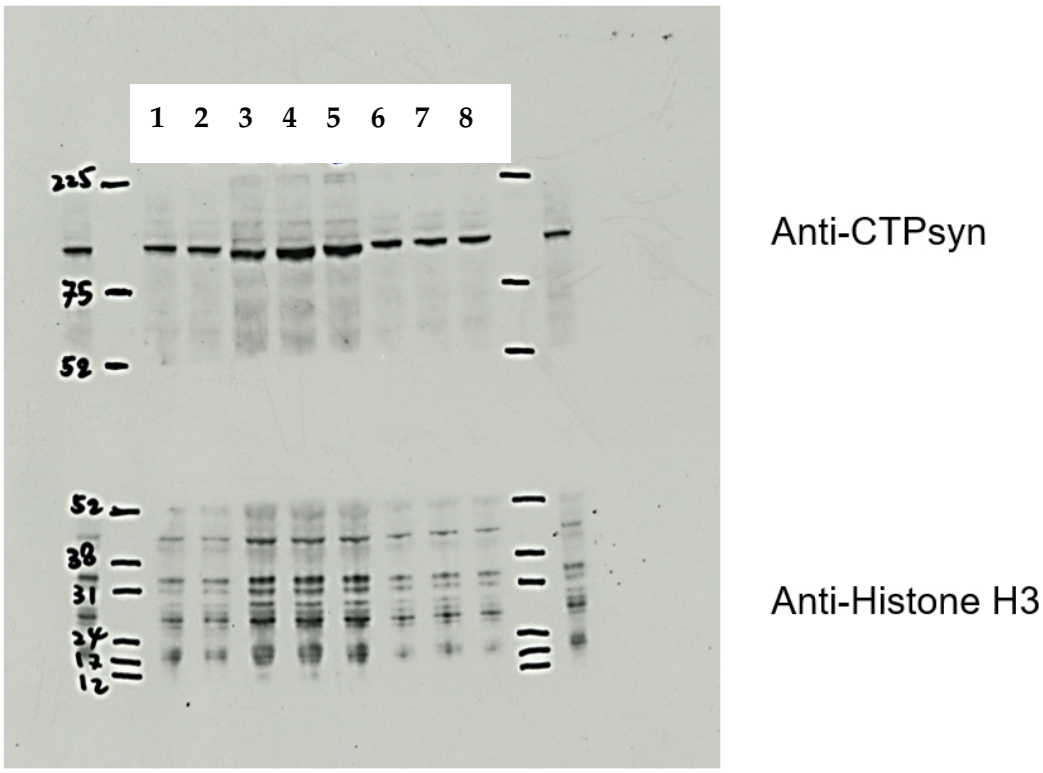


Figure 4.2.9 CTPS protein level remains unchanged under cold stress. (1-5) Samples taken after cells put on ice for 0, 15, 30, 60 minutes. (6-8) Samples taken after cells put on ice for 2 hours.

4.2.7 DON cannot prevent cold shock induced cytoophidia disassembly

As shown in a previous experiment result mentioned in this chapter, DON can induce cytoophidia and cause an irreversibly increased average length of cytoophidia. To test whether DON induced cytoophidia stay undispersed under low temperature stress, cells were grown in YE3S at 32℃ and treated with DON for 2 hours, and then put on ice. As shown in **Figure 4.2.10**, DON induced cytoophidia disappeared after 2 hours of ice-cold treatment, showing that DON cannot prevent cold-induced cytoophidia disassembly.

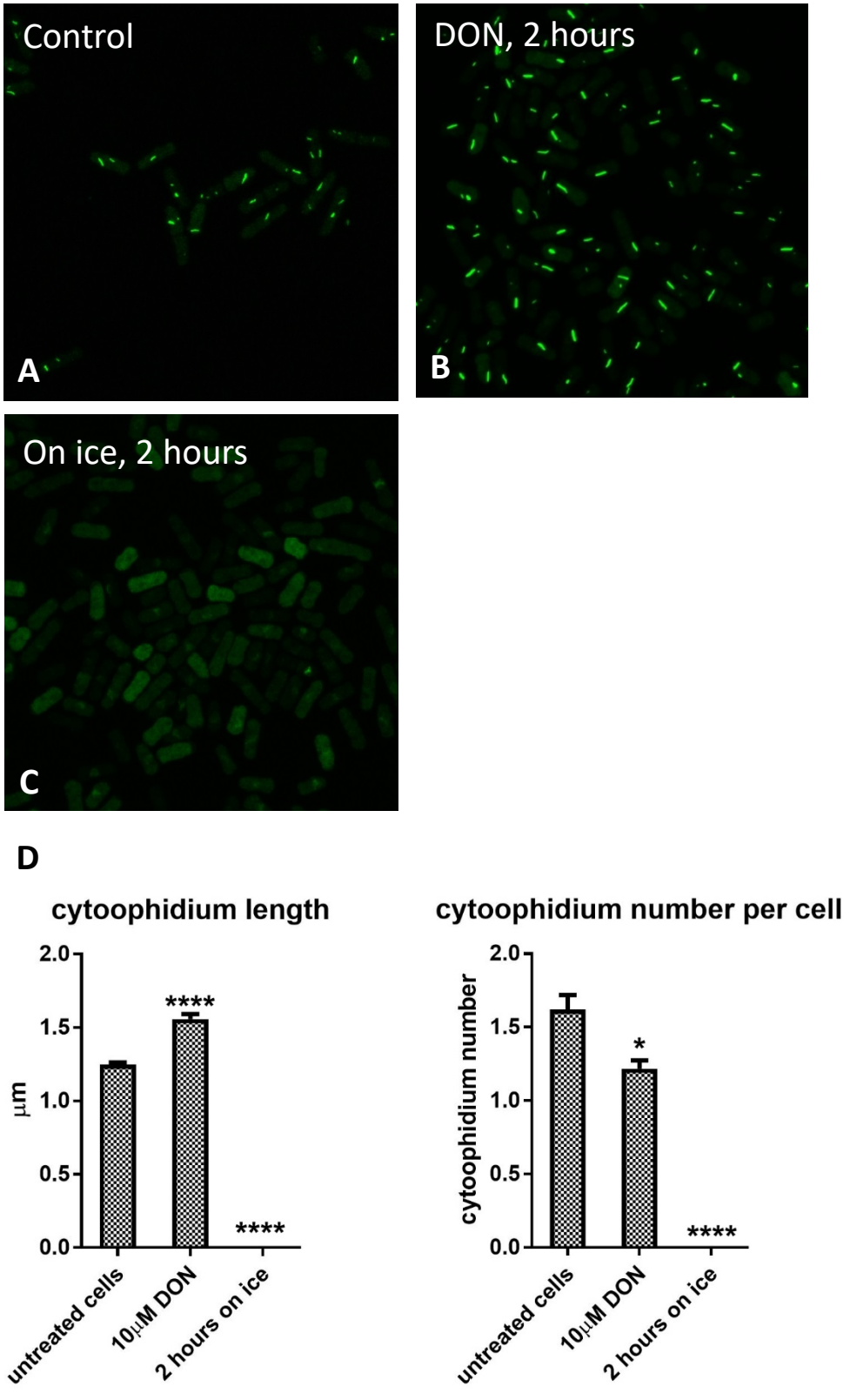

Control
A
DON, 2 hours
B
On ice, 2 hours
C
D
cytoophidium length
2.0
1.5
1.0
0.5
0.0
µm
****
****
untreated cells
10µM DON
2 hours on ice
cytoophidium number per cell
2.0
1.5
1.0
0.5
0.0
cytoophidium number
*
****
untreated cells
10µM DON
2 hours on ice

Figure 4.2.10 DON induced cytoophidia can be disassembled by cold stress. (A) Control group, with cells grown in 32℃ in YES media. (B) Cytoophidia had increased length after cells were treated with DON for 2 hours. (C) Cytoophidia were disassembled after being put on ice for 2 hours. Error bar: Mean±SEM.

Dunnett's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
untreated cells vs. 10μM DON	-0.3075	-0.3769 to -0.238	Yes	****	<0.0001
untreated cells vs. 2 hours on ice	1.249	1.09 to 1.408	Yes	****	<0.0001

Dunnett's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
untreated cells vs. 10μM DON	0.4038	0.1017 to 0.7059	Yes	*	0.0113
untreated cells vs. 2 hours on ice	1.621	1.04 to 2.201	Yes	****	<0.0001

4.2.8 Heat shock proteins are related to cytoophidia assembly

Heat shock proteins are a family of proteins formed in response to environmental stresses, especially heat shock stress. We already know that cytoophidia respond to temperature changes. To inspect whether small heat shock proteins are related to cytoophidia, we selected several small heat shock proteins in *S. pombe* cells and constructed a series of deletion strains expressing CTPS-YFP, including *hsp9Δ*, *hsp16Δ*, *hsp20Δ*, *hsp104Δ*, *pdr13Δ* and *sks2Δ*. All these strains showed decreased average length of cytoophidia and cell percentage containing cytoophidia (**Figure 4.2.11**). *sks2Δ* cells showed a very strange distribution pattern of CTPS-YFP (**Figure 4.2.11 G**). *ppk15* is a serine/threonine protein kinase and not related to hsp proteins or heat shock response. The *ppk15Δ* strain is as an extra control aside from the wild type cells control group.

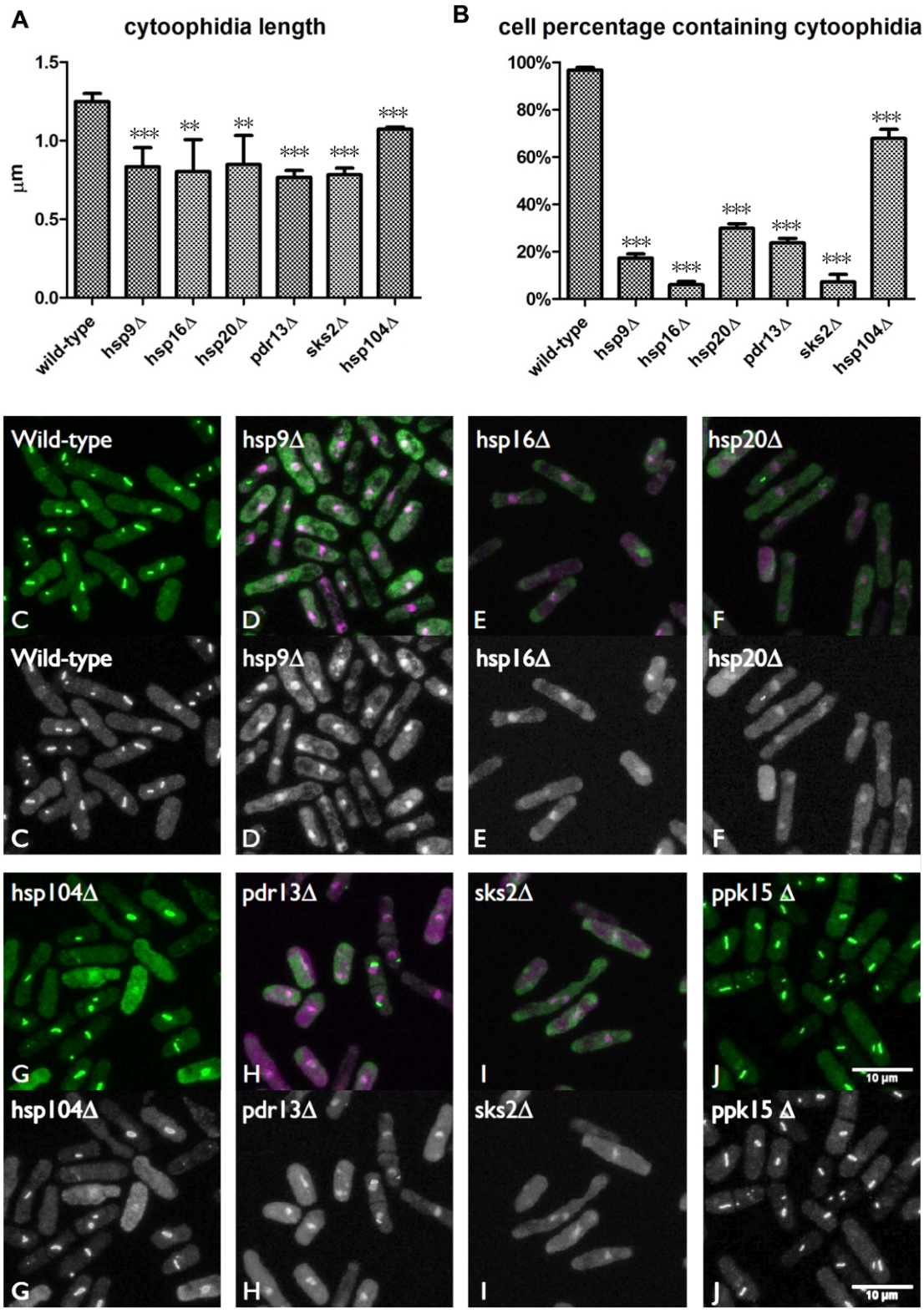

A
cytoophidia length
µm
1.5
1.0
0.5
0.0
***
**
**
***
***
***
wild-type
hsp9Δ
hsp16Δ
hsp20Δ
pdr13Δ
sks2Δ
hsp104Δ
B
cell percentage containing cytoophidia
100%
80%
60%
40%
20%
0%
***
***
***
***
***
wild-type
hsp9Δ
hsp16Δ
hsp20Δ
pdr13Δ
sks2Δ
hsp104Δ
Wild-type
hsp9Δ
hsp16Δ
hsp20Δ
C
D
E
F
Wild-type
hsp9Δ
hsp16Δ
hsp20Δ
C
D
E
F
hsp104Δ
pdr13Δ
sks2Δ
ppk15 Δ
G
H
I
J
10 µm
hsp104Δ
pdr13Δ
sks2Δ
ppk15 Δ
G
H
I
J
10 µm

Figure 4.2.11 Cytoophidia morphology in heat shock protein deletion strains. (A-B) Decreased average length of cytoophidia and cell percentage containing cytoophidia in all tested heat shock protein deletion strains. *ppk15Δ* cells were used as second control group. (C-J) Different deletion strains showed different CTPS-YFP distribution patterns. Scale bar: 10μm**.** Error bar: Mean±SEM.

Results fo the T test analysis:

Average length of cytoophidia

Dunnett's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
w.t. vs. ppk15Δ	0.05086	-0.06968 to 0.1714	No	ns	0.7729
w.t. vs. hsp9Δ	0.4146	0.2987 to 0.5305	Yes	****	<0.0001
w.t. vs. hsp16Δ	0.4455	0.2892 to 0.6017	Yes	****	<0.0001
w.t. vs. hsp20Δ	0.4009	0.2921 to 0.5097	Yes	****	<0.0001
w.t. vs. hsp104Δ	0.1744	0.04811 to 0.3006	Yes	**	0.0028
w.t. vs. pdr13Δ	0.4826	0.3706 to 0.5947	Yes	****	<0.0001
w.t. vs. sks2Δ	0.4656	0.2895 to 0.6416	Yes	****	<0.0001

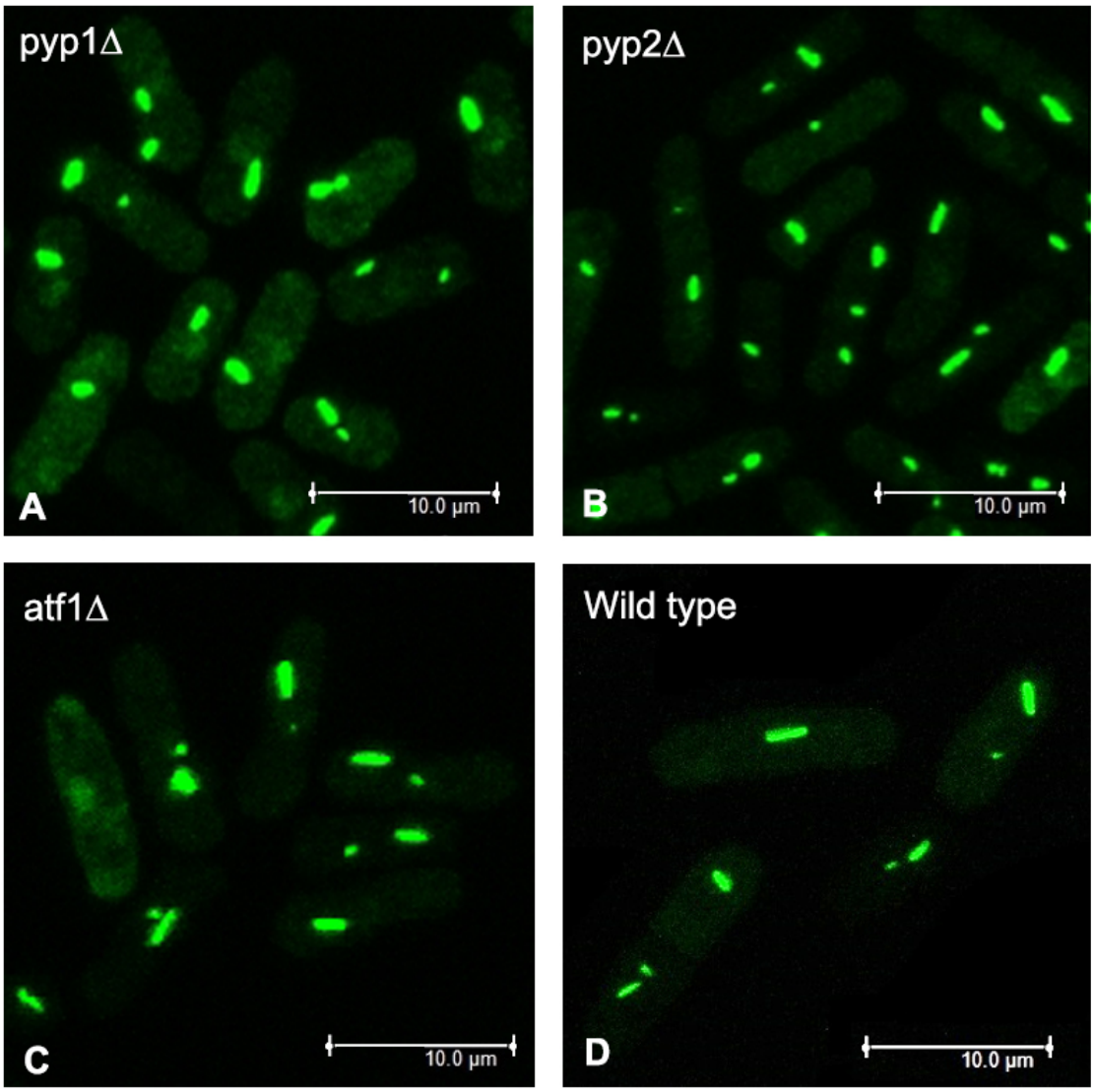
Average number of cytoophidia per cell

Dunnett's multiple comparisons test	Mean Diff. (%)	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
w.t. vs. ppk15Δ	3.979	-1.909 to 9.866	No	ns	0.3157
w.t. vs. hsp9Δ	79.46	72.38 to 86.53	Yes	****	0.0001
w.t. vs. hsp16Δ	90.73	82.43 to 99.04	Yes	****	0.0001
w.t. vs. hsp20Δ	66.92	60.15 to 73.69	Yes	****	0.0001
w.t. vs. hsp104Δ	28.84	21.3 to 36.38	Yes	****	0.0001
w.t. vs. pdr13Δ	73.04	66.13 to 79.95	Yes	****	0.0001
w.t. vs. sks2Δ	89.53	79.67 to 99.39	Yes	****	0.0001

4.2.9 Do MAPK pathway regulate cytoophidia?

In *S. pombe*, the MAP Kinase (MAPK) pathway is responsible for stress response. We know cytoophidia respond to several different stresses, so the MAPK pathway should be a good candidate to test. Three deletion strains were constructed, *pyp1*Δ CTPS-YFP, *pyp2*Δ CTPS-YFP, and *atf1*Δ CTPS-YFP. Cytoophidia could be observed in all three strains. Cell counting result showed both *pyp2*Δ and *atf1*Δ stains had a decreased cell percentage of cytoophidia, indicating that *pyp2* and *stf1* are possibly related to cytoophidia regulation (**Figure 4.2.12**).

However, whether the whole MAPK pathway is involved in cytoophidia regulation still remains unknown. More deletion strains (*spy1Δ, wis1Δ, sty1Δ*) should be constructed to confirm if the MAPK pathway regulates cytoophidia.


pyp1Δ
A
10.0 μm
pyp2Δ
B
10.0 μm
atf1Δ
C
10.0 μm
Wild type
D
10.0 μm

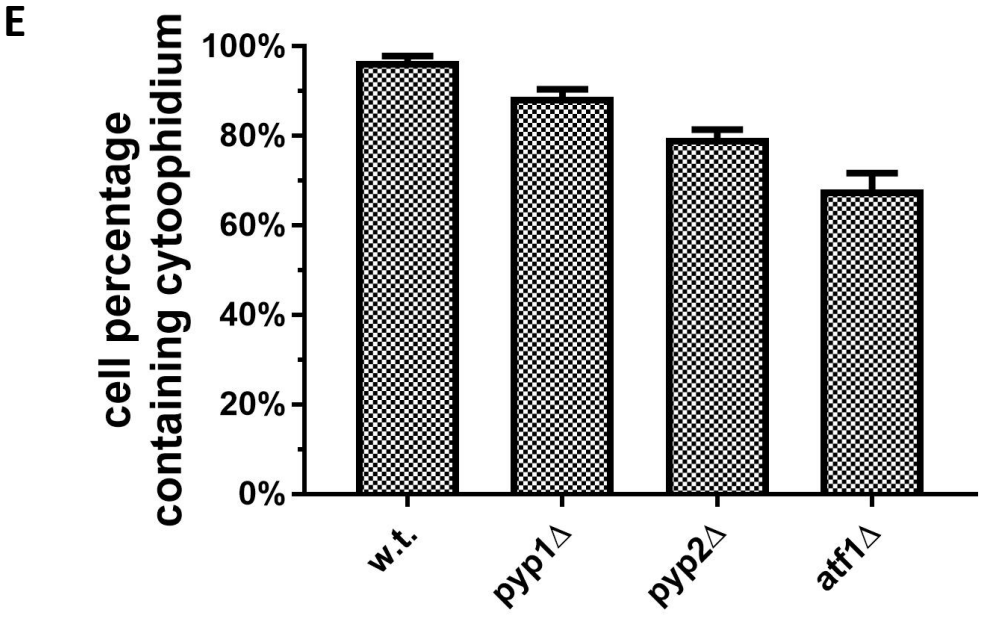

E
cell percentage containing cytoophidium
100%
80%
60%
40%
20%
0%
w.t.
pyp1Δ
pyp2Δ
atf1Δ

Figure 4.2.12 *atf1* and *pyp2* are potential regulators of cytoophidia. (A) *pyp1* gene deleted *S. pombe* cells. (B) *pyp2* gene deleted *S. pombe* cells. (C) *atf1* gene deleted *S. pombe* cells. (D) Control group with wild type cells. (E) Both *pyp2*Δ and *atf1*Δ cells showed a decreased percentage of cells containing cytoophidia. Scale bar: 10μm. Error bar: Mean±SEM.

Results fo the T test analysis:

Percentage of cells contain cytoophidia

Dunnett's multiple comparisons test	Mean Diff. (%)	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
w.t. vs. *pyp1*Δ	8.014	-0.3942 to 16.42	No	ns	0.0645
w.t. vs. *pyp2*Δ	17.22	9.012 to 25.43	Yes	****	<0.0001
w.t. vs. *atf1*Δ	28.71	20.31 to 37.12	Yes	****	<0.0001

4.3 Discussion

4.3.1 Cytoophidia are promoted by DON and respond to several different drugs

DON is an inhibitor for many glutamine utilising enzymes and can non-specifically inhibit CTPS. It has been reported that the majority of the content of cytoophidia in human cells and *Drosophila* is made up of an inactive form of CTPS (Barry, R. M., et al., 2014; Aughey, G. N., et al., 2014; Noree, C., et al., 2014), leading to a hypothesis that cytoophidia are a CTPS storage depot as well as a regulator of CTPS metabolic activities. This hypothesis accords with our results for *S. pombe*. When treated with DON, *S. pombe* showed an increase in the average length of cytoophidia (**Figure 4.2.1**). Similar results can be detected in human HeLa cells, *Drosophila* S2 cells and *Drosophila* tissues (Chen, K., et al., 2011; Aughey, G. N., et al., 2014), indicating that the promotion of cytoophidia by DON is globally conserved. We can speculate that after *S. pombe* is treated with DON, the CTPS in cells became inactive and therefore caused cytoophidia filaments to be enhanced, though it is still not clear whether the CTPS assembly enhancement is caused directly by CTPS inhibition, or by other enzymes inhibition that then affecta CTPS assembly, or both.

Interestingly, although the average length of cytoophidia increased after DON treatment, the average cytoophidia number per cell decreased (**Figure 4.2.1 C)**. The speculation is made that small cytoophidia may fuse into longer and

thicker filaments and therefore the numbers decreased, the filament length increased. Notably, the azide treatment also gave rise to an interesting result: the cytoophidia number per cell increased first, then decreased (**Figure 4.2.3**). From the confocal microscope observation, instead of filaments, plenty of punctate structures were observed after azide treatment **(Figure 4.2.3**). This can be explained by the fact that long cytoophidia disassembled into smaller and shorter filaments, thus increasing the number of cytoophidia. Small cytoophidia then dispersed, resulting in the total disappearance of cytoophidia and the decrease in the number of cytoophidia. In this theory, cytoophidia undergo fusion or disruption when treated with DON or azide respectively. This can be supported by the live imaging results: small cytoophidia fused into large cytoophidia when cells recovered from cold stress (**Video 3**). There is a published paper demonstrating the fusion and disruption actions of cytoophidia in human cells (Gou, K. M., et al., 2014), and this supports our hypothesis in *S. pombe*.

In *Drosophila*, the kinase Ack (DAck) colocalised with CTPS in female germline cells and the Ack mutant only partially disrupted cytoophidia assembly, leading to fragmented short cytoophidia but increased numbers of cytoophidia (Strochlic, T. I., et al., 2014). This indicates that Ack acts as the 'glue' of cytoophidia integration. All these data promote the hypothesis that cytoophidia undergo assembly by forming small filaments first, then fusing

into large filaments. During cytoophidia disassembly, arge cytoophidia are fragmented first into small filaments and then dispersed into smaller units.

A previous study demonstrated that the CTP inhibiter DON can induce IMPDH assembly, and IMPDH inhibitor can also induce CTPS assembly (Chang, C. C., et al. 2015; Carcamo, W. C., et al., 2011). Here we show a similar phenomenon in *S. pombe*: IMPDH inhibitor MPA can induce CTPS filaments, suggesting the links between CTPS and IMPDH, although it is still not clear if the two enzymes are functionally or structurally, or directly or indirectly, related. Given that CTPS and IMPDH are responsible for the critical step of pyrimidine and purine *de novo* synthesis respectively, and CTPS filaments and IMDPH filaments are partially colocalised in mammalian cells (Keppeke, G. D., et al., 2015), it can be speculated that the two enzymes are sequestered in the common structure, the cytoophidium, and this may facilitate their functions and increase the efficiency of nucleotide metabolism. It can also be proposed that the sequestration of two enzymes in the same structure is simply due to the storage of inactive enzymes. DON and MPA have all been proposed as drugs of cancer treatment therapy. Cytoophidia's capability of respondance to both of them indicates that cytoophidia may be a potential future target for treatment of tumours.

Azide can irreversibly bind to the heme cofactor and inhibits cytochrome

oxidase, which means the cell's energy will be depleted in the presence of azide. Adding azide resulted in a significant downregulation of cytoophidia (**Figure 4.2.3**), suggesting that the maintenance of cytoophidia filaments requires the presence of cell energy. Interestingly, after treatment with azide, cytoophidia numbers increased and then dispersed totally. This can be explained by the hypothesis that the breakage of long cytoophidia into smaller and shorter cytoophidia happened first, and that small cytoophidia then dispersed into CTPS monomers, dimers, or tetramers. However, it is not clear if the CTPS filaments need ATP directly or not. Further experiments need to be done to confirm this speculation.

Cytoophidia also responded to hydroxyurea (HU), the ribonucleotide reductase (RNR) inhibitor, leading to the DNA replication stress and initiating the DNA damage response signalling. It has also been reported in mouse embryos that the HU caused DNA damage is by inducing oxidative stress, and the DNA damage response depends on the p38 mitogen-activated protein kinase (MAPK) signalling (Banh, S. & Hales, B. F., 2013). This supports the results what we observed in *S. pombe*, that both oxidative stress and HU treatment downregulated cytoophidia.

4.3.2 Cytoophidia and stress response

The CTPS mRNA expression level decreased when faced with heat stress, osmotic stress and oxidative stress (Chen, D., et al., 2003), which explained the downregulation of cytoophidia when cells were placed under these stresses. It was also observed that cytoophidia disassembled during the stationary phase, which can be explained by the decreased CTPS protein level (4 folds) and the CTPS mRNA level (5.5 folds) (Marguerat, S., et al., 2012). These data suggest that cytoophidia are regulated by CTPS cellular concentration in *S. pombe*, which accords with the previous study in *Caulobacter crescentus* showing that knocking down CTPS led to cytoophidia downregulation and that the mild-overexpression resulted in an increase in the length of cytoophidia (Ingerson-Mahar, M., et al., 2010). The *in vitro* study of *E. coli* CTPS also showed that CTPS filament formation has a linear relationship with CTPS concentration (Barry, R. M., et al., 2014).

Cytoophidia also responded to both high and low temperature stresses. Both temperature stresses caused significant downregulation of cytoophidia, which occurred rapidly in as little as 5 minutes. The role of temperature stress response is majorly taken by the MAPK pathway, also involving the TOR pathway. Both pathways are activated by stresses and go through the chain reactions by kinase phosphorylation. The cascade of phosphorylation results in the promotion or suppression of different downstream genes and triggers the different responses. The phosphorylation actions normally take 1 to 2 hours

and rarely take effect in minutes, suggesting that there is another mechanism underlying the role of temperature stress response in cytoophidia.

4.3.3 Heat shock proteins and temperature stress response

Heat shock proteins are a family of proteins produced by cells in response to exposure to stressful conditions. They were first described in relation to heat shock, but are now known to be expressed during other stresses, including exposure to cold, UV light, and during wound healing or tissue remodelling. Many members of this protein family perform chaperone function by stabilising new proteins to ensure correct folding or by helping to refold proteins that were damaged by the cell stress. This increase of expression is transcriptionally regulated. The dramatic upregulation of the heat shock proteins is a key part of the heat shock response and is induced primarily by the heat shock factor (HSF). HSPs are found in virtually all living organisms, from bacteria to humans.

Our study has revealed that cytoophidia maintenance is sensitive to heat shock. The response is quick and profound. We also confirmed the involvement of heat shock proteins, including hsp9, hsp16, hsp20, hsp104, pdr13(ssz1) and sks2 in cytoophidia assembly by yeast genetics. Among these 6 proteins, only hsp9 has been reported as being involved in vegetative growth metabolism under normal growing temperature conditions, while the other 5 proteins are only

described undertaking different roles during stress responses (**Table 4.1.2**). It is still unlear whether the downregulation of cytoophidia due to the lack of heat shock proteins is direct or indirect. Further studies are needed to figure out how various heat shock proteins cooperate in the processes of assembly, maintenance and disassembly of cytoophidia.

4.3.4 Improvement of the cytoophidium analysis procedure.

Yeast cells have evolved to acclimatise to diverse environments and are able to cope with different osmotic conditions. In *S. pombe*, cells respond to osmotic stress through the MAPK pathway and stimulate the High Osmolarity Glycerol (HOG) pathway (Hohmann, S., 2002). We found that when *S. pombe* cells were exposed to a high osmotic environment, although the average length of cytoophidia did not change, the percentage of cells with visible cytoophidia dropped significantly, showing cytoophidia to be related to the *S. pombe* osmotic stress response.

Although the response of yeast cells to high osmolarity is relatively well known, the signalling involved in their response to low osmolarity has been less studied (Hohmann, S., 2002). In this chapter we found that cytoophidia have a rapid response to low osmolarity, that even 5 minutes of water treatment led to cytoophidia dispersal.

In *S. pombe*, using PBS or sterile water to wash cells before cell fixation is a routine process. In this thesis, howeverm, we found even a quick exposure to water induced a stress response in the cells and led to cytoophidia dispersal. We therefore suggest that care must be taken when processing cells for the cytoophidium analysis.

4.3.5 Cytoophidia assembly may be an energy consuming process

The stationary phase induced cytoophidia dispersal suggests that cytoophidia assembly may be regulated by CTPS cellular concentration, which is supported by the study in *Caulobacter crescentus* showing that the overexpression of CTPS enlongated cytoophidia length and CTPS knock-down led to cytoophidia becoming shorter (Ingerson-Mahar, M., et al., 2010). This introduced the question: is CTPS the only factor affecting cytoophidia?

Cytoophidia dispersal caused by azide suggested that cytoophidia assembly is not solely regulated by the cellular concentration of CTPS protein; instead of a passive aggregating action, it might be the energy consuming process. In support of this hypothesis, the CTPS inhibitor DON significantly enhanced cytoophidia assembly while leaving the CTPS protein level unchanged, and cold-induced cytoophidia disassembly did not change the CTPS expression level either. In *Caulobacter crescentus*, the mild-overexpression also promotes

cytoophidia forming, and severe-overexpression also interfered with cytoophidia forming (Ingerson-Mahar, M., et al., 2010). This is also observed in *S. pombe*: cytoophidia dispersed when CTPS is overexpressed.

All these data suggest that the assembly and maintenance of cytoophidia is not solely dependent on the CTPS protein concentration and might be an active and energy consuming process. The confirmation of this hypothesis needs further experimental data in its support.

4.3.6 Potential functions of cytoophidia in *S. pombe*

An increasing number of metabolic enzymes have recently been identified with the ability to self-assemble into filamentous structures, including CTPS. This suggests that intracellular compartmentalisation of metabolic enzymes is more general than previously thought. A large-scale screen of the yeast GFP library identified that over 20% examined proteins are able to form distinct intracellular structures (Narayanaswamy, R., et al., 2009). Another screen of 40% of the GFP collection of budding yeast showed that 9 proteins form 4 types of filamentous structures (Noree, C., et al., 2010). More recently, several studies have demonstrated that polymerisation of CTPS into cytoophidia downregulates enzymatic activity in bacteria (Barry et al., 2014), budding yeast (Noree, C., et al., 2014), fruit flies and human cell lines (Aughey, G. N., et al.,

2014). Although not yet confirmed, it can be speculated that the fission yeast CTPS is inactive when forming into filaments, since the CTPS inhibitor, DON, can promote cytoophidia in *S. pombe.* Thus cytoophidia formation seems to be a general strategy to regulate enzymatic activity. The assembly and disassembly of cytoophidia and its kind provides a swift and robust buffer mechanism to maintain cellular homeostasis during development and in mediating adaptive metabolic responses (Aughey, G. N., et al., 2014; Barry, R. M., et al., 2014; Noree, C., et al., 2014; Petrovska, I., et al., 2014).

Cytoophidia can also be proposed as a potential depot for CTPS. Barry and his colleagues promoted a hypothesis that *E. coli* CTPS is stored as 'ready-to-use' tetramer form in filaments and can therefore be rapidly reactivated when needed by enzyme depolymerisation (Barry, R. M., et al., 2014). This strategy can store enzymes in a conformationally restricted form and have a quicker response when CTPS is demanded, giving cells the advantage of maintaining metabolic homeostasis more easily and more quickly. In *S. pombe*, temperature stress induced cytoophidia disassembly is fast and reversible, supporting the idea that cytoophidia can act as a storage depot for CTPS.

Chapter 5 Asymmetric distribution of CTPS in *S. pombe* during cytokinesis

5.1 Introduction

5.1.1 *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*

The budding yeast *Saccharomyces cerevisiae* and the fission yeast *Schizosaccharomyces pombe* are the two most studied yeast species (Forsburg, S. L., 2005). Both two yeast species provide excellent models in much fundamental biological research, including on cell growth, cell cycle, intracellular trafficking, cell damage response, metabolism, organelles and cell division (Mitchison, J. M., 1957; Mitchison, J. M., 1958). *S. cerevisiae* was the first eukaryotic genome to be fully sequenced and published in 1996. The rod-shaped fission yeast *S. pombe* was first isolated in 1893 by Paul Lindner and for at least six decades it has been a powerful model system for studying fundamental biological processes including growth control and cell division (Rose, Anthony H. & Harrison, J. S., 1987). The complete sequence of the *S. pombe* genome was published on 2002, becoming the sixth fully genome sequenced model eukaryotic organism.

S. pombe has been a popular system for cell growth and cell division studies due to its regular shape and size. Like most other systems, the central events of *S. pombe* cell reproduction are chromosome replication in S phase, followed by

chromosome segregation and nuclear division (cytokinesis) and cell division (cytokinesis) in M phase. While *S. cerevisiae* has an extended period at the gap between M and S phases (G1), *S. pombe* remains in the G2 phase of the cell cycle for an extended period (Hartwell, L. H., et al., 1974; Mitchison, J. M., 1972; Nurse, P., et al., 1976). In *S. pombe*, two daughter cells remain associated with each other physically after cytokinesis and cytokinesis at the G1/S phases. The abscission of the paired cells occurs afterwards.

5.1.2 Symmetric and asymmetric inheritance during cytokinesis

Although fission yeast and budding yeast share many features and diverged from the same ancestor, they have evolved separately over 1,000 million years and become very different nowadays (Heckman, D. S., et al., 2001; Hedges, S. B., 2002). The most notable feature shared by these two organisms is that *S. cerevisiae* divides by budding (thus called budding yeast), while *S. pombe* does fission (thus called fission yeast). Budding yeast undergoes asymmetric division morphologically and the mother cell keeps most of the cytoplasmic content, while the bud (the daughter cell) contains very little cytoplasmic material. Fission yeast is believed to undergo symmetric division. The cylindrical *S. pombe* cells grow by elongation at the tip extension and divide by binary fission at the medial septum generation, resulting in two same-sized daughter cells (Mitchison, J. M., 1957; Mitchison, J. M., 1958; Hayles, J. & Nurse, P., 2001; Forsburg, S. L., 2005). Although it is believed that the two daughter

cells inherit their components symmetrically from the mother cell, there are a few exceptions. For example, the spindle pole bodies appear to be non-equivalent after division and the asymmetry of the spindle pole bodies may be established early during cytokinesis (Cerutti, L. and Simanis, V., 1999). Another asymmetric example in *S. pombe* is the switch between two cell mating types, the plus (P) and the minus (M). The asymmetric inheritance of mating type switching is due to a strand specific and site-specific imprint, which is a programmed single stranded nick that initiates the recombination event following replication at the mat1 locus. The break can be repaired using either the transcriptionally silent mat2P or mat3M as a template for recombination (Klar, A. J., 1987).

5.1.3 Chapter aims

It is widely believed that most proteins in *Schizosaccharomyces pombe* undergo symmetric cytokinesis, and that two daughter cells share equal content from the mother cell after division. In this chapter, we demonstrate that CTPS and cytoophidia display asymmetric inheritance pattern during cytokinesis in *S. pombe*. Both cytoplasmic and N-cytoophidia were observed to be asymmetrically inherited during cell division. In addition, the cytoplasmic and N-cytoophidia were inherited independently into only one of the two daughter cells respectively during cell division. The time-lapse studies also showed the dynamic activities of cytoophidia. Our quantitative analysis indicated that the

partition of cytoophidia is a stochastic process. Our discovery on cytoophidia asymmetric inheritance in *S. pombe* establishes a good platform for the research of metabolic enzyme inheritance in *S. pombe*.

5.2 Results

5.2.1 Both cytoplasmic and cytoplasmic cytoophidia are inherited asymmetrically during cytokinesis

In Chapter 3, we already demonstrated the presence of both cytoplasmic and nuclear cytoophidia (C- and N- cytoophidia) in *S. pombe* cells. Here we presented further studies on the asymmetric partition of C- and N-cytoophidia. The frequency and numbers of C- and N-cytoophidia in *S. pombe* cells was examined, giving a result that the majority of *S. pombe* cells have one C-cytoophidium and one N-cytoophidium. This raised a fundamental question: how are C- and N-cytoophidia inherited by the next generation after cell division? We analysed cells at the G1/S phase, when two daughter cells were still clearly attached to each other (after cytokinesis but before scission) and observed that both C- and N-cytoophidium are asymmetrically inherited during *S. pombe* cytokinesis. Among all the C-cytoophidia detected cells, 99.5% (n = 358) showed the C-cytoophidium was inherited by only one of the two daughter cells (**Figure 5.2.1**). Similarly, N-cytoophidium was also detectable in only one daughter cell. Interestingly, the N-cytoophidum was not always inherited together with the C-cytoophidium by the same cell (**Figures 5.2.1 and 5.2.2**). Instead, the distributions of C- and N-cytoophidia varied from cell to cell (**Figure 5.2.2**), indicating that C- and N-cytoophidia are inherited independently during cell division.

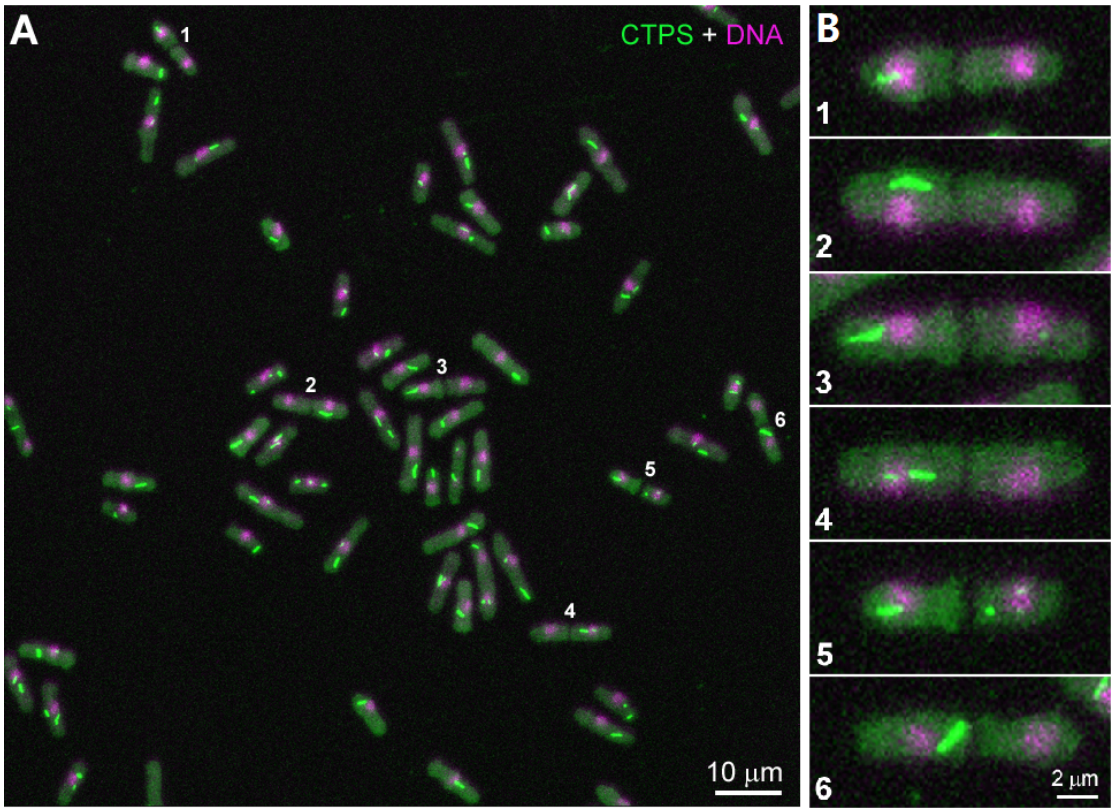


Figure 5.2.1 The C-cytoophidium is inherited by only one daughter cell during *S. pombe* cytokinesis. (A) CTPS is tagged with YFP to C-terminus (green) and DNA is stained with Hoechst (magenta). Most cells in this field contain both C- and N-cytoophidia. Six pairs of cells are labeled by numbers. (B) Zoomed-in images of the six paired cells in A. Note the different distribution pattern of cytoophidia between the two cells in each pair. The zoomed-in images have been rotated so the cells on the left hand contain large C-cytoophidia in the cytoplasm, while the cells on the right hand either lack of C-cytoophidium or contain small-sized C-cytoophidia.

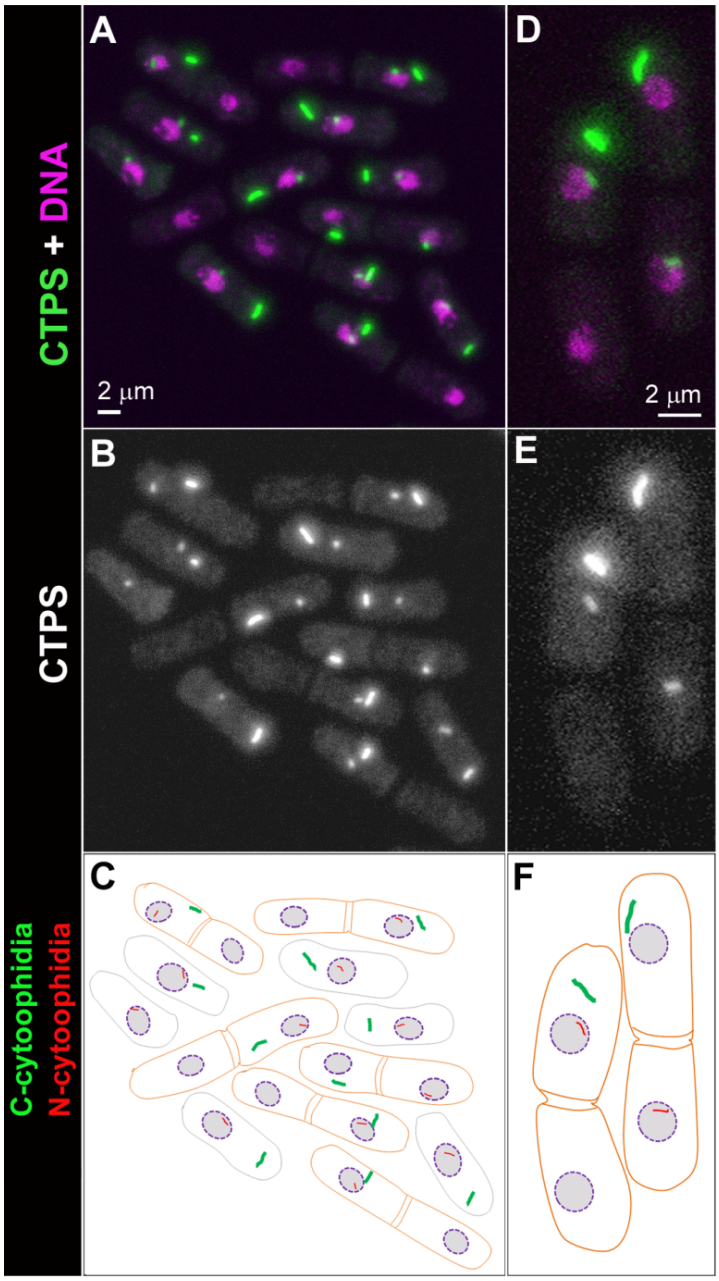


Figure 5.2.2 Differential inheritance of cytoophidia in *S. pombe*. YFP fused CTPS was expressed using the endogenous *cts1* promoter. (A-C) Among the six pairs of cells (outlined in orange in C), both C-cytoophidium and N-cytoophidium are inherited respectively by only one of the two daughter cells. (D-F) Zoomed in image of two pairs of *S. pombe* cells showing the different inheritance pattern of the cytoophidia. The C-cytoophidium and N-cytoophidium can be distributed into the common cells or the different cells during cytokinesis.

5.2.2 Cytoophidia inheritance is a stochastic process. (this part was performed together with Professor Ji-long Liu)

To have a better understanding of cytoophidia inheritance in *S. pombe*, we focused on cells in pairs for further analysis. After mitosis and cytokinesis, the paired cells are still physically associated for a certain period before the abscission occurs. Each of the two daughter cells inherits one end of the mother cells. To observe and distinguish the two daughter cells more easily, calcofluor white staining was used, which is widely employed in *S. pombe* experiments for cell wall labelling. A 405 nm laser was used as the excitation beam and the emission signal was collected at 420-470 nm for the cell wall (**Figures 5.2.3 A and B**) and at 620-670 nm for cytoplasm (**Figure 5.2.3 C**).

Under microscopy, the birth scars can be detected as dark rings crossing the *S. pombe* cell wall (**Figure 5.2.3 B**). Since the old end has a different growth rate from the new end, the distances between the birth scar and the two ends are different, and the two daughter cells can be easily distinguished (**Figure 5.2.3 G and H**). The two daughter cells were referred to as the 'Smooth cell', for the cell inheriting the old end of the mother, and the 'Rough cell', for the one inheriting the new end of the mother cell (**Figure 5.2.3 G-L**). Since the old end of the mother cell grows extensively before the cell division, the smooth cell would be mostly free from birth scars (**Figure 5.2.3 H**). In contrast, the rough cell contains one or more birth scars from previous generations.

Given the morphological differences between the two daughter cells (**Figure 5.2.3**), we next ask whether the cytoophidium is differentially inherited by the two daughter cells. We focused on C-cytoophidia because of their large sizes. Our quantificational analysis showed that the chances of inheriting the C-cytoophidia are similar from the smooth cell and the rough cell (49.4% vs. 50.6%, n = 178). These data suggest that the inheritance of C-cytoophidia is stochastically passed into one of the two daughter cells.

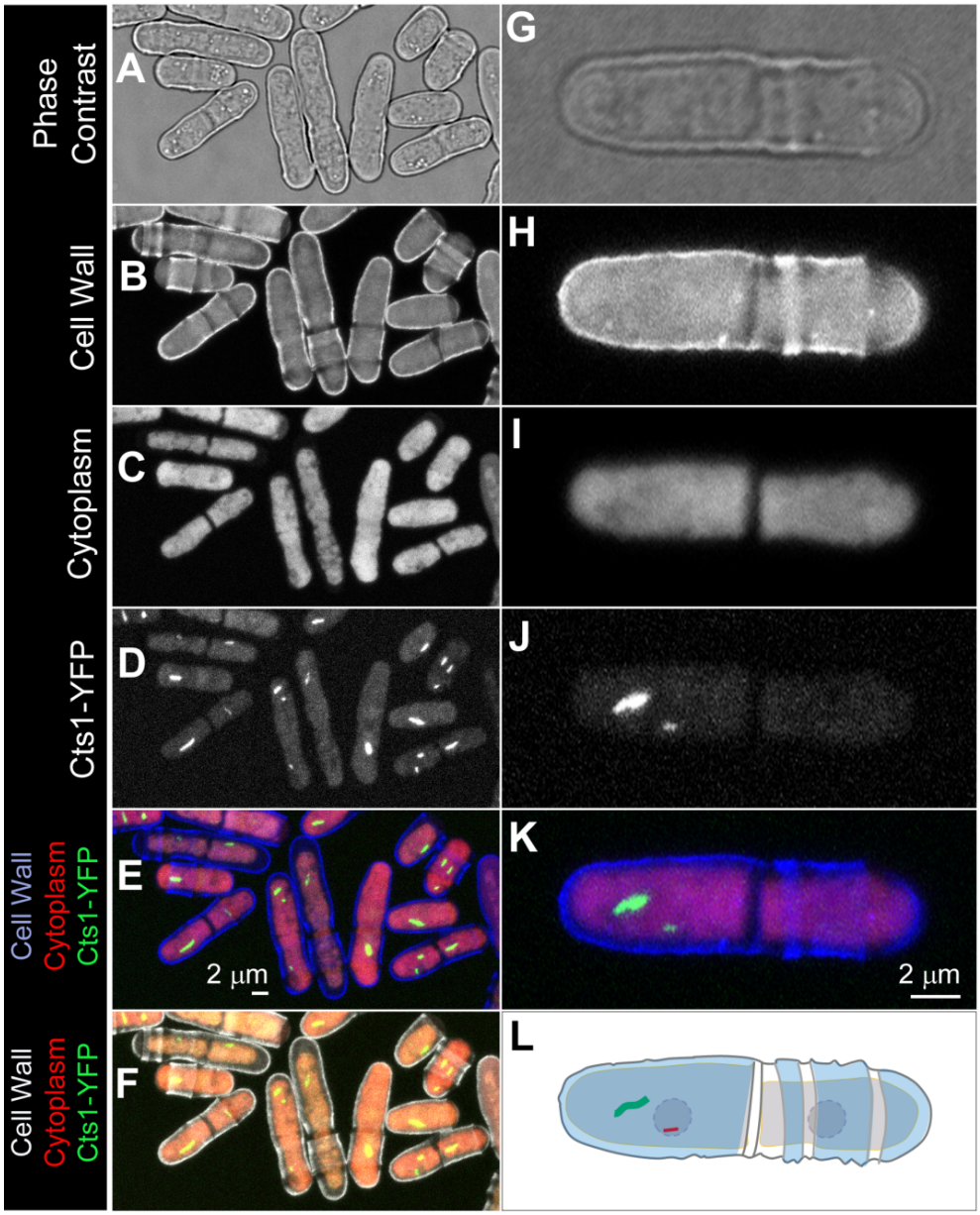


Figure 5.2.3 ***S. pombe*** **cells are morphologically asymmetric and polarized. (A-F)** A group of *S. pombe* cells at different phases of cell cycle. The difference between the two poles can be appreciated when the cell wall (**B**) and the cytoplasm (**C**) are outlined by Calcofluro white staining. **(G-L)** A pair of *S. pombe* cells. These two cells are morphologically distinct from one another. In these panels, the cell on the left inherited the old end ('Smooth cell') also inherited the cytoplasmic and N-cytoophidia, while the other cell ('Rough cell') inherited neither cytoophidium.

5.2.3 Both C-cytoophidia and N-cytoophidia are highly dynamic

For a better understanding of the cytoophidium behaviour, live imaging experiments were performed with CTPS-YFP expressed *S. pombe* cells and recorded every 5 seconds. The film showed that both N-cytoophidia and C-cytoophidia were highly dynamic (**Figure 5.2.4; Videos 4 and 5**). The traces of N-cytoophidia were merged together and showed that N-cytoophidia prefer to stay at the periphery of the nucleus, suggesting that N-cytoophidia are possibly tethered to the nuclear envelope (**Figure 5.2.4 B-E)**. Merged C-cytoophidia traces displayed their dynamic movement within a restricted region in cytoplasm between nucleus and the cell end, indicating a barrier role the nucleus may serve to prevent the C-cytoophidia moving from one cell tip towards another cell tip (**Figure 5.2.4 A-E; Videos 4 and 5**).

To examine the effect of C-cytoophidia confinement during cell division, another live imaging experiment was performed. The *S. pombe* cells were recorded from G2 phase to cytokinesis, cytokinesis and abscission. Unsurprisingly, the differential inheritance of cytoophidia correlated with the location of cytoophidia during G2 phase. Cytoophidia were separated and distributed into two daughter cells by the septum generated in the middle of the mother cell, and the location of cytoophidia was the major factor affecting their inheritance (**Figure 5.2.4 F and G; Video 6**).

In many cases, only one daughter cell inherited the C-cytoophidium during cytokinesis, while the other daughter cell had no C-cytoophidium. Since more than 90% of *S. pombe* cells contained C-cytoophidia in the visual fields under microscopy, it was speculated that after cell division, C-cytoophidia would be *de novo* generated in the daughter cell that had not inherited the C-cytoophidium. The speculation was confirmed by long-period time lapse studies (**Figure 5.2.5; Video 7**).

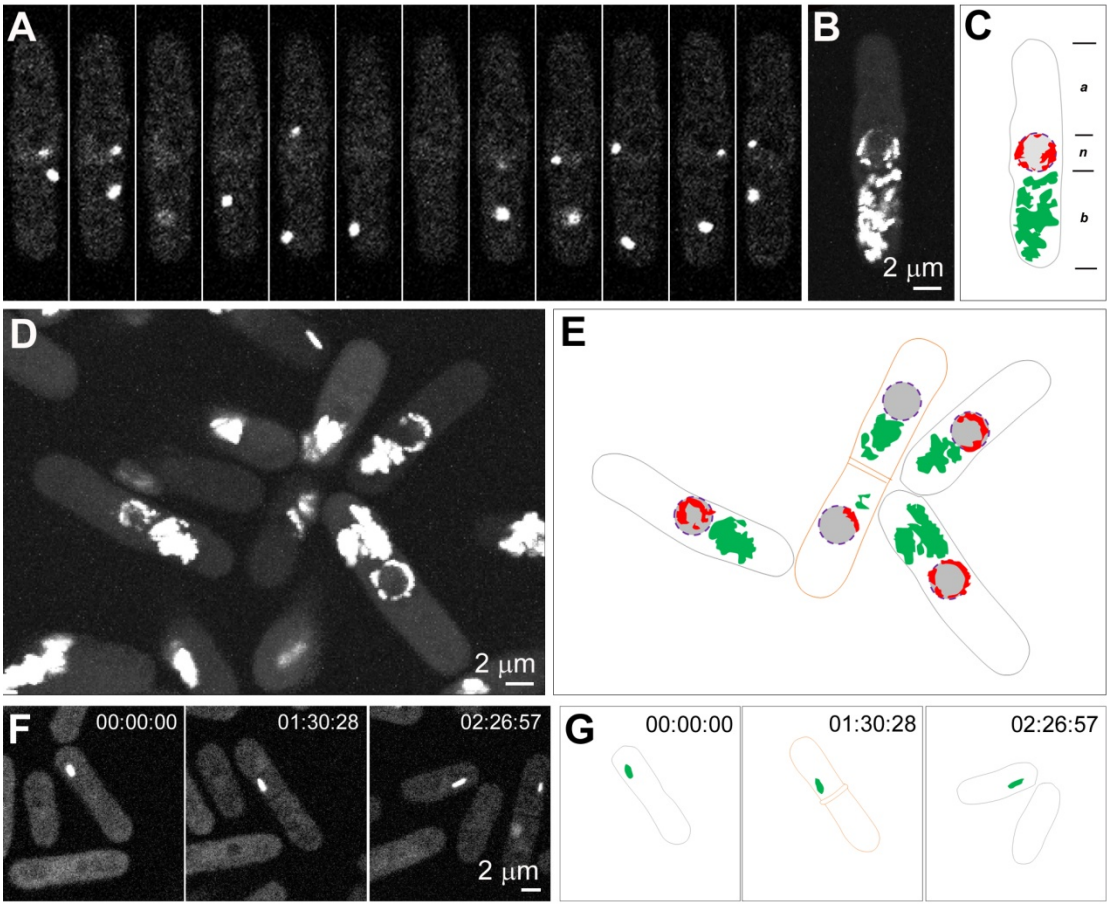


Figure 5.2.4 Cytoophidia are dynamic and constrained in *S. pombe*. The YFP tagged CTPS was expressed using the endogenous *cts1* promoter at the endogenous *cts1* locus. (A-E) Live imaging of *S. pombe* cells are recorded every 5 seconds for 10 minutes. (A) 12 snapshots of a cell. (B-E) 118 frames were recorded in 10 minutes for a cell (B) or a group of cells (D). (C-E) The merged traces of C-cytoophidia (green) displayed the constrained movement area between nucleus and cell end. The merged traces of N-cytoophidia (red) displayed its preference of localisation at the nuclear periphery. (F-G) Live imaging snapshots of dividing cells.

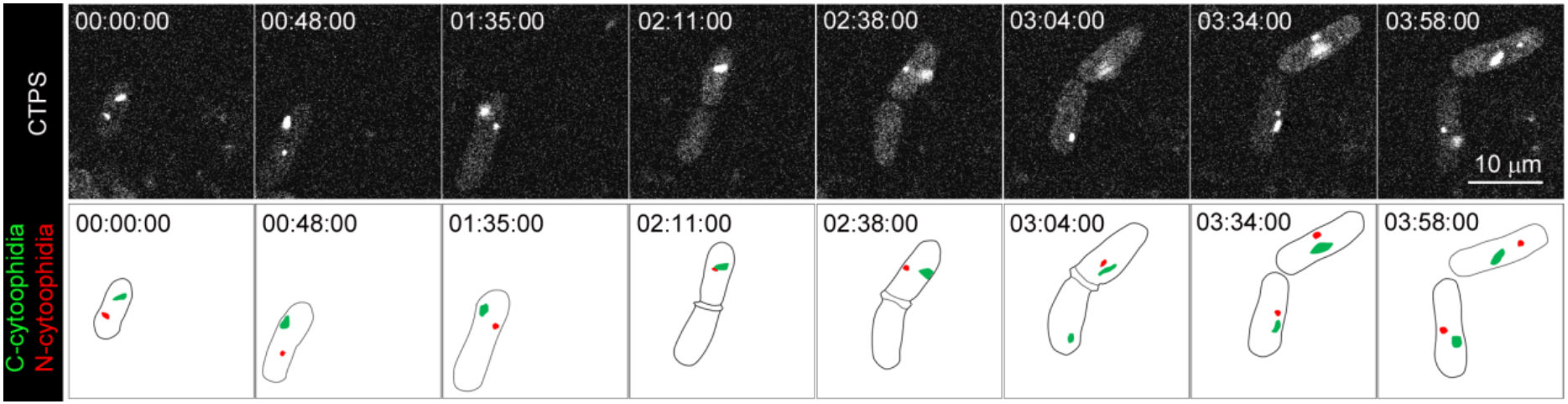
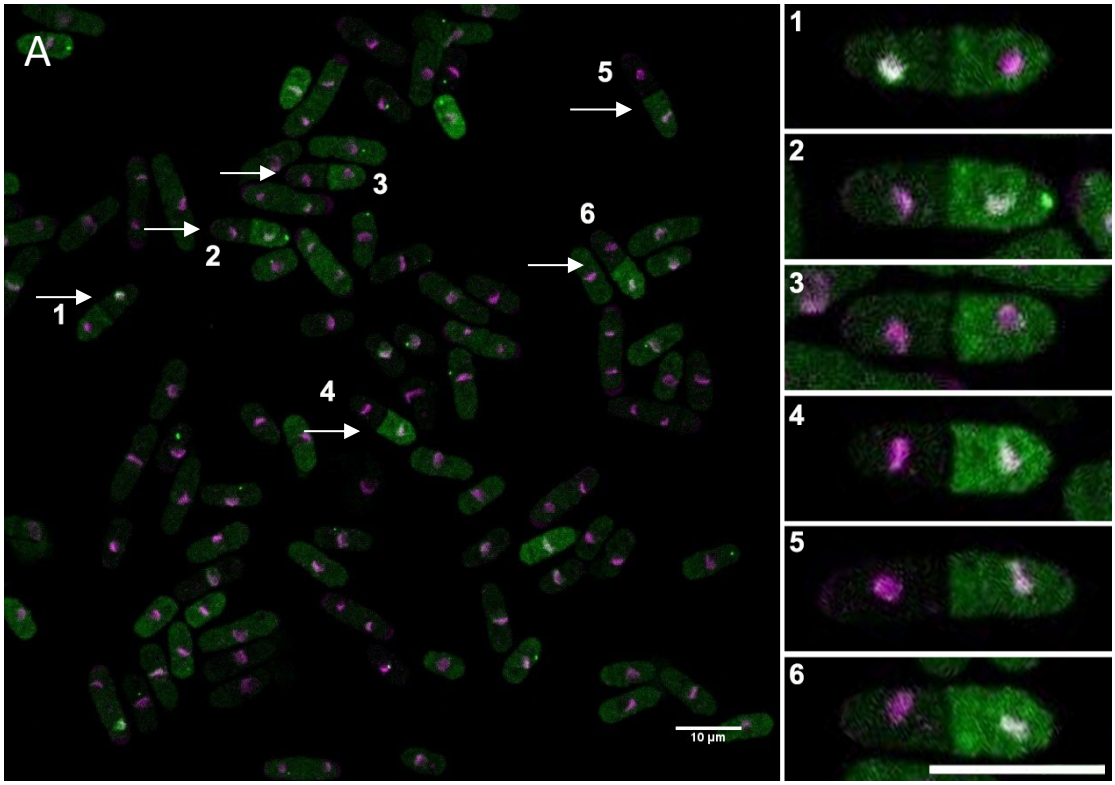


Figure 5.2.5 *de novo* formation of cytoophidia in *S. pombe*. Snapshots of a 4-hour live imaging recording a cell undergoing cell division. The YFP tagged CTPS was expressed using the endogenous *cts1* promoter at the endogenous *cts1* locus. At the starting time point (00:00:00), one C-cytoophidium (green in the cartoons) and one N-cytoophidium (red in the cartoons) can be clearly detected in the cell. During cell division (02:11:00), only one daughter cell inherited the cytoophidium, while the other had no cytoophidium, until a new C-cytoophidium *de novo* generated after about 50 minutes (03:04:00) and the formation of a new N-cytoophidium after about 80 minutes (03:34:00). Both the newly generated C-cytoophidium and N-cytoophidium grew longer and thicker along with the cell growth.

5.2.4 Asymmetric inheritance of cytoophidia during cytokinesis leads to different CTPS levels between the two daughter cells after cell division

The asymmetric inheritance of cytoophidia during cytokinesis led to unevenly distributed cytoophidia between two daughter cells. How about the CTPS protein level? Does the cellular level of CTPS also have an asymmetric distribution during cytokinesis? To answer this question, cytoophidia in *S. pombe* cells should be disassembled with CTPS released in cytoplasm. The disassembly of cytoophidia under cold stress has already been described in Chapter 4. Utilising this feature, the *S. pombe* cells were incubated at 0˚C to get CTPS released and paired cells were analysed. The asymmetric distribution of CTPS could be easily observed in many paired cells after cold treatment (**Figure 5.2.6**), indicating the difference of CTPS cellular levels between two daughter cells. It is speculated that the differential distributions of CTPS between two daughter cells were achieved via asymmetric inheritance of cytoophidia.


A
1
2
3
4
5
6
10 µm

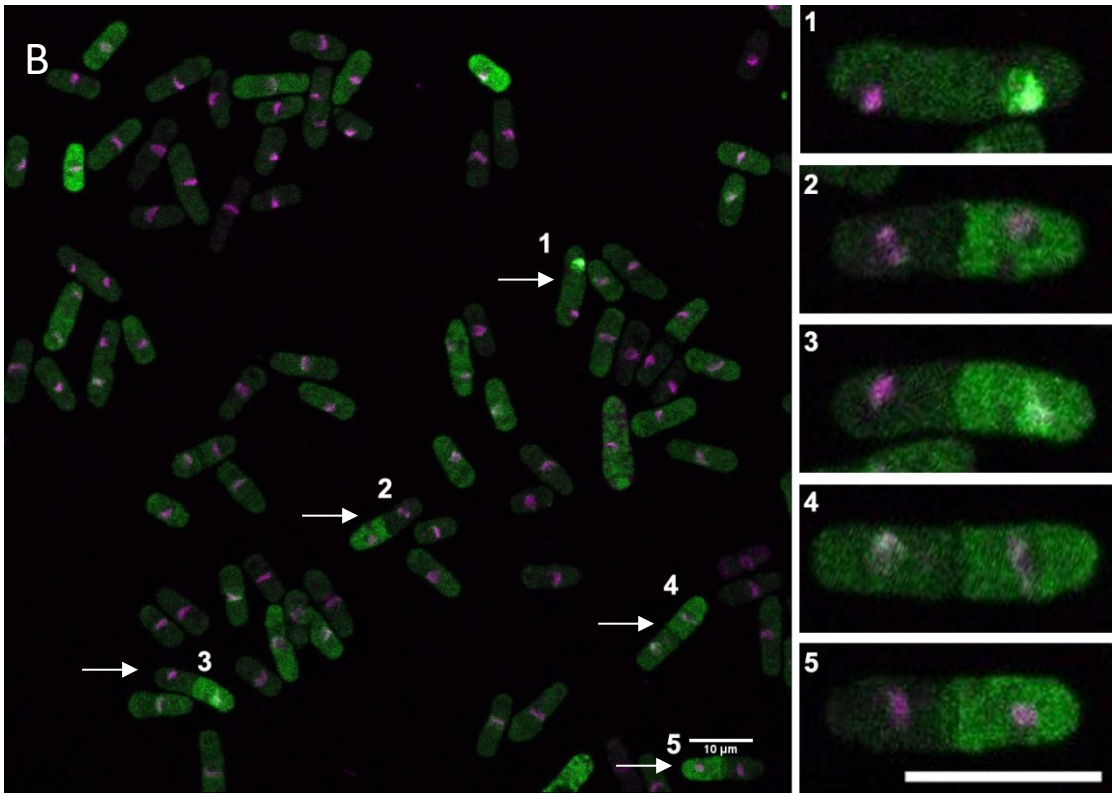

B
1
2
3
4
5
10 µm

Figure 5.2.6 Asymmetric distribution of CTPS between paired cells under cold stress. CTPS is tagged with YFP at C-terminus (green) and DNA is stained with Hoechst (magenta). The arrows point to the pairs of daughter cells with CTPS asymmetric inheritance pattern. (A) Cold shock for 30 minutes. (B) Cold shock for 2 hours. Scale bars: 10μm.

5.3 Discussion

5.3.1 Cytoplasmic and nuclear cytoophidia

It has been demonstrated that CTPS can form both cytoplasmic (C-cytoophidia) and nuclear cytoophidia (N-cytoophidia) in mammalian cells (Gou, K. M., et al., 2014), which parallels our study in that both N-cytoophidia and C-cytoophidia can be observed in *S. pombe* (Chapter 3). The corresponding of the experiment results on two different organisms suggests that the compartmentalisation of both C-cytoophidia and N-cytoophidia are evolutionarily conserved.

The live imaging results suggested that N-cytoophidia are mostly localised at the periphery of the nuclei in *S. pombe*. This raised the question of whether N-cytoophidia are tethered at the inner side of the nuclear envelope. Although there was no direct evidence to answer this question, speculations can still be made for N-cytoophidia's potential functions. A previously published paper demonstrated that the CTPS functionally interacts with the intermediate filament crescentin in curved bacteria *Caulobacter crescentus* to regulate cell curvature, showing the structural role CTPS played in *Caulobacter crescentus* cells (Ingerson-Mahar, M., et al., 2010). It is possible that in *S. pombe* N-cytoophidia potentially have a structural role related to the nuclear envelope. It is worth seeing if N-cytoophidia are functionally linked to the nuclear lamina, which is composed of intermediate filaments and membrane-associated

proteins. Since C-cytoophidia can help regulate cytoplasmic CTPS activity as a CTPS storage depot (Noree, C., et al., 2014; Barry, R. M., et al., 2014; Aughey, G. N., 2014; Aughey, G. N. & Liu, J. L., 2015; Liu, J. L., 2016), it is also possible that N-cytoophidia are taking similar roles in nuclei. Another interesting question is whether there is communication between N-cytoophidia and C-cytoophidia. It is expected to see a relationship between C-cytoophidia and N- cytoophidia, and they may work together to organise and balance the enzymes' metabolic regulation within cells. The speculation still needs to be confirmed by further experiments.

5.3.2 Dynamics of cytoophidia

Cytoophidia are highly dynamic according to several criteria. Firstly, both C-cytoophidia and N-cytoophidia underwent continuous micro-movement in *S. pombe* cells. In addition, the *de novo* formation of new cytoophidia was observed in *S. pombe* cells after cytokinesis. The growing process of cytoophidia from small and short foci into long and thick filaments was also demonstrated, which is similar to what was observed in *C. crescentus*: cytoophidia assembly starts from small foci and later grow into 500-nm long filaments in stalked cells (Ingerson-Mahar, M., et al., 2010). Finally, previous studies in mammalian cells have shown that smaller cytoophidia can fuse together into larger cytoophidia (Gou, K. M., et al., 2014), corresponding to our live imaging result in *S. pombe* (cells recovered from cold stress; **Video 3**); and a large cytoophidium can also separate into smaller cytoophidia (Azide treatment cells; **Figure 4.2.3**).

Multifaceted dynamics of cytoophidia reflect the complexity of their biogenesis. The micro-movement of cytoophidia may be independent from, or act together with, other organelles. It remains to be confirmed whether cytoophidia movement in the cytoplasm is based on microtubules and/or actin filaments. It can be speculated that that movement of cytoophidia serves as an efficient way to transport concentrated enzymes to certain areas. The formation and growth of cytoophidia provides a quick response to environmental stresses. Equilibrium between small and large cytoophidia through fusion-fission process might add another layer of regulations for metabolic pathways.

5.3.3 Asymmetries of *S. pombe* cells

S. pombe cells have been considered asymmetric but unpolarised (Macara, I. G. & Mili, S., 2008). Under phase contrast microscopy, *S. pombe* cells can be seen as rod-shaped and measured typically 3-4μm in diameter and 7-14μm in length (**Figure 5.2.5**). Although the two ends of *S. pombe* cell were assumed to be identical, multiple lines of evidence shows that this might not be the truth. First of all, each *S. pombe* cell has one 'old' end, which was inherited from the mother cell, and one 'new' end arising from cell division. Secondly, the two ends have a different growth time point and growth rate. After cytokinesis, the growth initiates immediately at the 'old' end while the 'new' end only grow when cells reach a critical size threshold and enter a certain time point in G2 phase known

as the New End Take Off (NETO) (Castagnetti et al., 2007; May, J. W. & Mitchison, J. M., 1995; Mitchison, J. M., and Nurse, P., 1985). Thirdly, the cell wall associated with the two ends has a different pattern, especially with birth scars, which mark the position of a previous septum forming site (Mitchison, J. M., and Nurse, P., 1985). Birth scars are not only obvious under scanning electron microscopy and transmit electron microscopy. They are easily detected by Calcofluor White staining, a fluorescent dye that specifically stains cell walls in *S. pombe*. Fourthly, spindle pole bodies have been found to be non-equivalent during cytokinesis and remain so throughout interphase (Cerutti, L. & Simanis, V., 1999).

In this chapter, we have demonstrated that cytoophidia are also distributed asymmetrically during cell division. The inheritance of cytoophidia may be a random process, since there seems to be no preference for the two daughter cells. Although no other metabolic enzymes have been reported as undergoing a similar asymmetric inheritance pattern as *S. pombe*, it would be interesting to check whether there are additional cellular components and whether fate determinants are asymmetrically inherited in *S. pombe*. IMPDH and Ack are good candidates, since they colocalise with CTPS in other organisms (Keppeke, G. D., et al., 2015; Strochlic, T. I., et al., 2014). Further studies are required to investigate whether CTPS asymmetric inheritance is linked with the spindle pole bodies. However, it remains to be determined whether there is a

mechanism that regulates asymmetric segregation of cytoophidia, since there is normally only one of each C-cytoophidium and N-cytoophidium in *S. pombe* cells and the septum possibly segregates them randomly without any additional mechanism.

Asymmetric cell division has been suggested as a widespread strategy for many cellular organisms to restrict senescence in one daughter cell, leaving another daughter cell clean (Macara, I. G. & Mili, S., 2008). Asymmetric segregation of large aggregates has been observed under stress and is speculated to link to cell aging in *S. pombe* (Coelho, M., et al., 2013; Coelho, M., et al., 2014) and *S. cerevisiae* (Denoth Lippuner, A., et al., 2014). Whether and how the asymmetric inheritance of cytoophidia and other cellular components contribute to fitness and aging is left for future studies.

Chapter 6 Truncated deletions and point mutations of CTPS

6.1 Introduction

6.1.1 CTPS N-terminal is conserved and critical for cytoophidia formation in *Drosophila*

There are 3 isoforms of CTPS that have been observed in *Drosophila melanogaster*: isoforms A, B and C (**Figure 6.1.1**, www.flybase.org). Although isoform A and C have different 5' untranslated regions in their mRNA sequences, the final product of them is identical. Here we consider isoforms C and isoform A as the same protein and discuss only isoform A. The only difference between isoforms B and A is the N-terminal 56 amino acids of isoform A (52 amino acids of isoform B), yet only isoform A is able to form cytoophidia (Azzam, G., et al., 2013), indicating that the N-terminus of CTPS is critical for its polymerisation. Neither the truncated isoform A without N-terminal 56 amino acids, nor the 56 amino acids N- terminus fragment alone can form into filaments, indicating that the N-terminus of CTPS is necessary but not sufficient to form cytoophidia (Azzam, G., et al., 2013).

Previous studies of the CTPS crystal structure showed that the N-terminus of CTPS is more conserved than the C-terminus (Endrizzi, J. A., et al., 2004). In **figure 6.1.2**, BLAST results showed that CTPS is conserved evolutionarily in

different species. *Drosophila* CTPS isoform A has 80.4% identity with *S. pombe* CTPS within N-terminal first 56 amino acids (**Figure 6.1.2 D**).

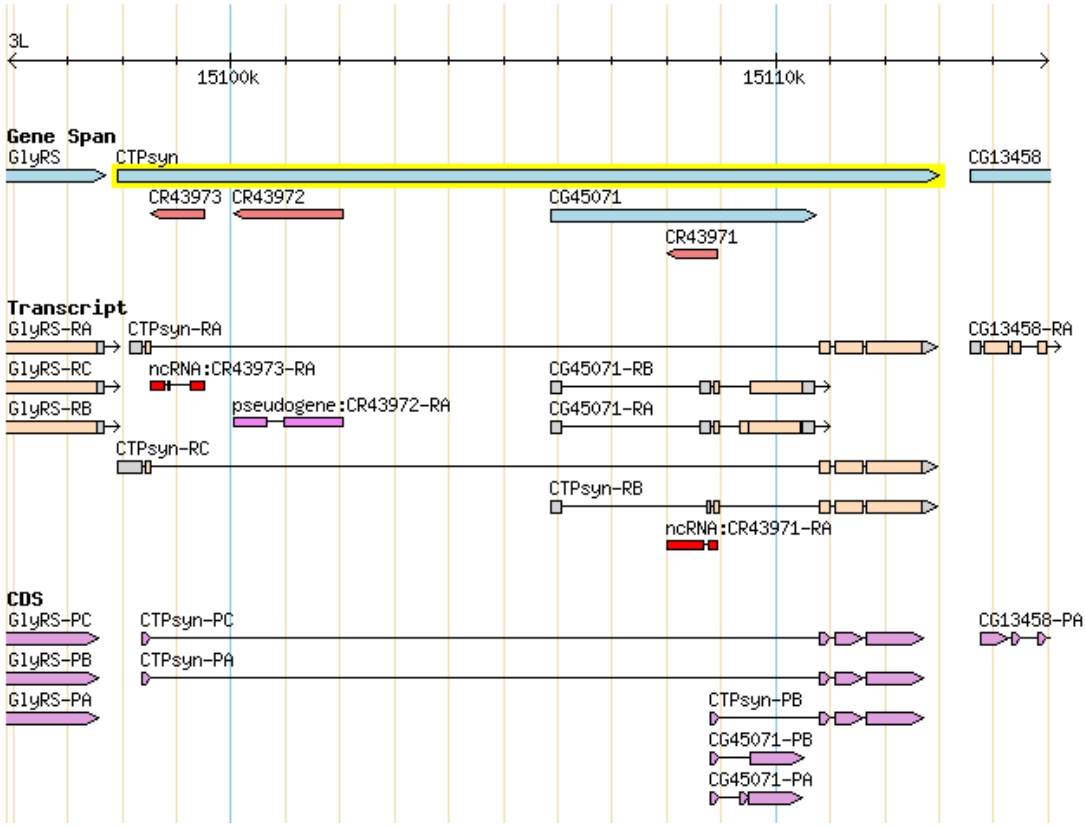


Figure 6.1.1 The *Drosophila* CTPS gene locus encodes three isoforms. Isoform A and isoform C are identical. Isoform B and isoform C are only different at the N-terminus.

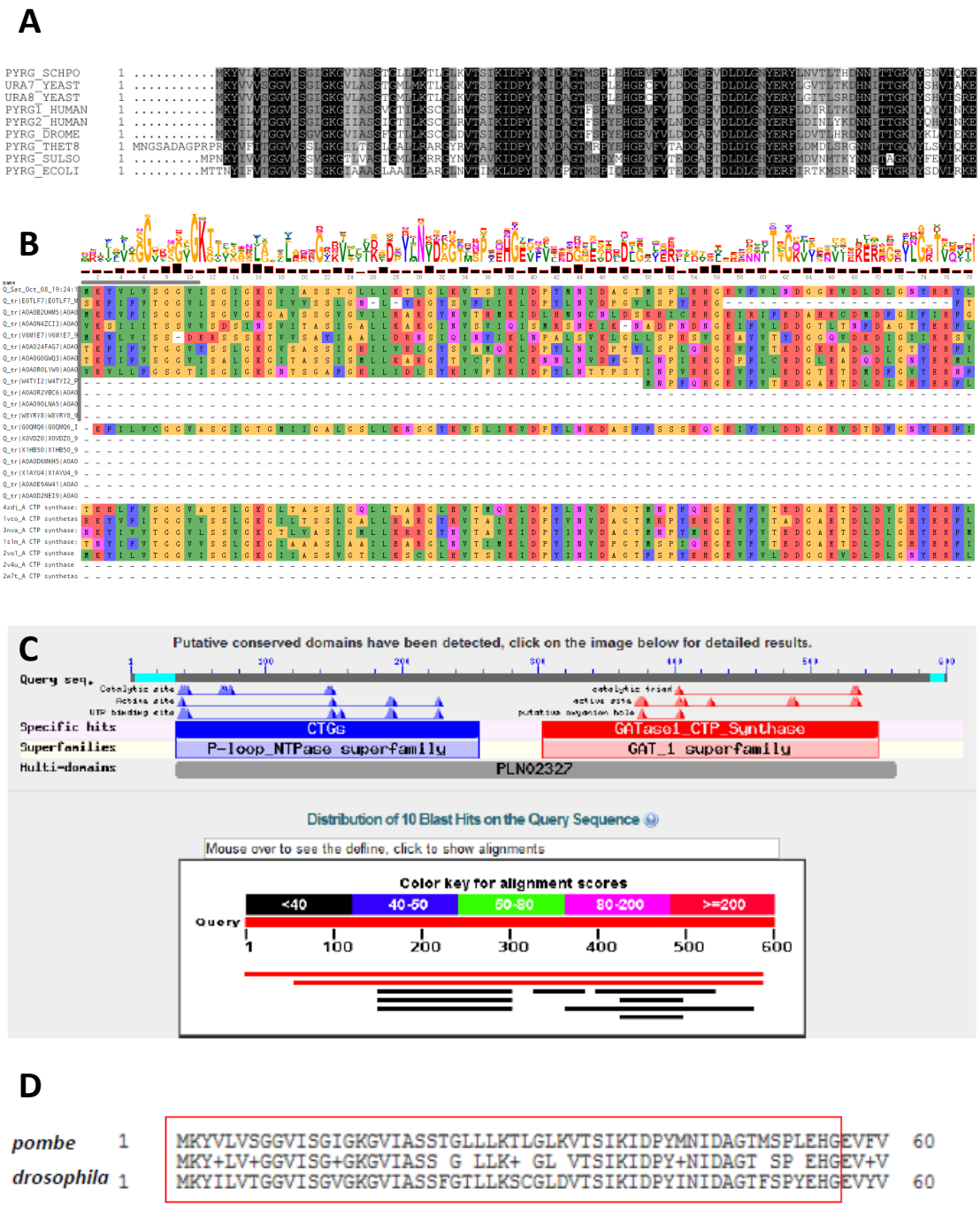


Figure 6.1.2 BLAST result of CTPS among different species. (A-B) CTPS N-terminus are evolutionarily conserved among several species. (C) CTPS are highly conserved in *Drosophila* and *S. pombe*. (D) *Drosophila* CTPS isoform A has 80.4% identity with *S. pombe* CTPS within N-terminal first 56 amino acids (in red box).

6.1.2 CTPS structure models in *Escherichia coli, Thermus thermophiles* and *Sulfolobus solfataricus*

Previous research has established the structure model of CTPS in *Escherichia coli* (Endrizzi, J.A., et al., 2004), *Thermus thermophiles* (Goto, M., et al., 2004) and *Sulfolobus solfataricus* (Lauritsen, I, et al., 2011) and revealed that the dimeric and tetrameric interfaces are highly conserved and almost identical among these three species (Lauritsen, I., et al., 2011). Active CTPS has been revealed as the homotetrameric form and is actually the dimer of a dimer. CTPS has two major domains, the N-terminal synthase domain and the C-terminal amidotransferase domain. The process of tetramerisation involves only the synthase domain: two CTPS interact through the dimer interface and then two dimer interact through the dimer-dimer interface. The four amidotransferase domains are located far away from the interact interfaces and are thus not involved in the oligomeric process. The ATP-binding site and CTP/UTP binding site are located at the tetramer interface, which explains why ATP and CTP/UTP binding can stabilise the tetramerisation. The sequence alignment of CTP synthases from *S. solfataricus, E. coli* and *T. thermophilus* and the details of their CTPS oligomeric interact interfaces are shown in **Figure 6.1.3** (Lauritsen, I., et al., 2011).

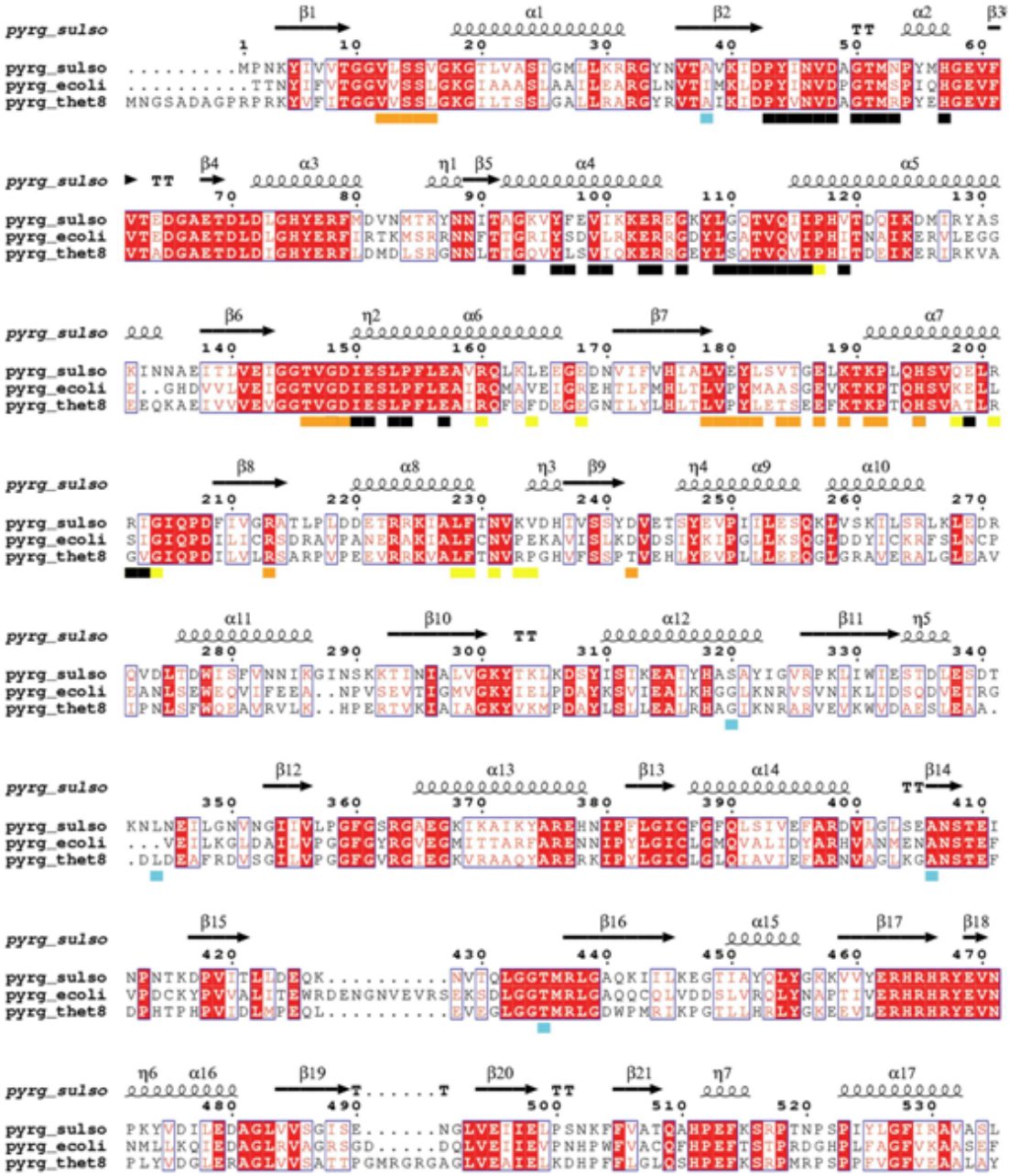


Figure 6.1.3 The sequence alignment of CTP synthases from *S. solfataricus*, *E. coli* and *T. thermophilus* (Lauritsen, I., et al., 2011). Black bars indicate dimer interfaces, orange bars indicate one dimer–dimer (tetramer) interface, yellow bars indicate the other tetramer interface and cyan bars indicate phosphorylation sites that have been established to influence tetramerisation in yeast enzymes (Park, T. S., et al., 1999; Park, T. S., et al., 2003).

6.1.3 Chapter aims

In this chapter, to have a better understanding of the structure and protein-protein interaction of CTPS, the 3D reconstruction model of *S. pombe* CTPS was made. The model was used to help design point mutations and truncated deletion strains. Although the construction of point mutation strains was faced with difficulty and unsuccessful, the truncated deletion strains were successfully made and applied to experiments. With the data generated by the truncated CTPS experiments, we managed to narrow down the range of critical amino acids affecting cytoophidia compartmentalisation. We also provided ideas and designed several applicable screenings that can be useful for the future cytoophidium research.

6.2 Results

6.2.1 Prediction of CTPS 3D reconstruction in *S. pombe*

To help design point mutation and truncated deletion *S. pombe* strains, the HHpred (Homology detection & structure prediction by HMM-HMM comparison) (https://toolkit.tuebingen.mpg.de/hhpred) and the software PyMOL were used to predict the 3D structure view of *S. pombe* CTPS. The results are presented in **Figures 6.2.1** and **6.2.2**. We can clearly see in **Figure 6.2.1 (A)** that CTPS has two domains, the CTP synthase domain (magenta) and the amidotransferase domain (blue), responsible for the different functions respectively.

Previously published papers have demonstrated the crystal structure of *Escherichia coli* CTPS and revealed the details of ATP binding, UTP binding, L-glutamine binding and the CTPS dimer interface and the tetramer interface (Endrizzi, J. A., et al., 2004; Goto, M., et al., 2004; Lauritsen, I., et al., 2011). Using the already established model (**Figure 6.1.3**, Lauritsen, I., et al., 2011), the predicted *S. pombe* CTPS structure was constructed as in **Figure 6.2.1 (B)**, showing the dimer interface (coloured red) and tetramer interface (coloured orange and yellow). The potential phosphorylation sites were also presented (coloured cyan).

As already mentioned in Section 6.1.1, the N-terminus of CTPS is critical for cytoophidia compartmentalisation. According to the reconstructed 3D model in **Figure 6.2.2**, the 1-56 amino acids (coloured in red) contains a α-helix and two β-sheets which are in the interior of the protein, indicating that the N-terminal 56 amino acids are important for the maintenance of CTPS conformational structure. The N-terminal 1-56 amino acids are also involved in the ATP binding and UTP binding actions (Goto, M., et al., 2004), indicating that the N-terminus of CTPS is also important for its functional maintenance.

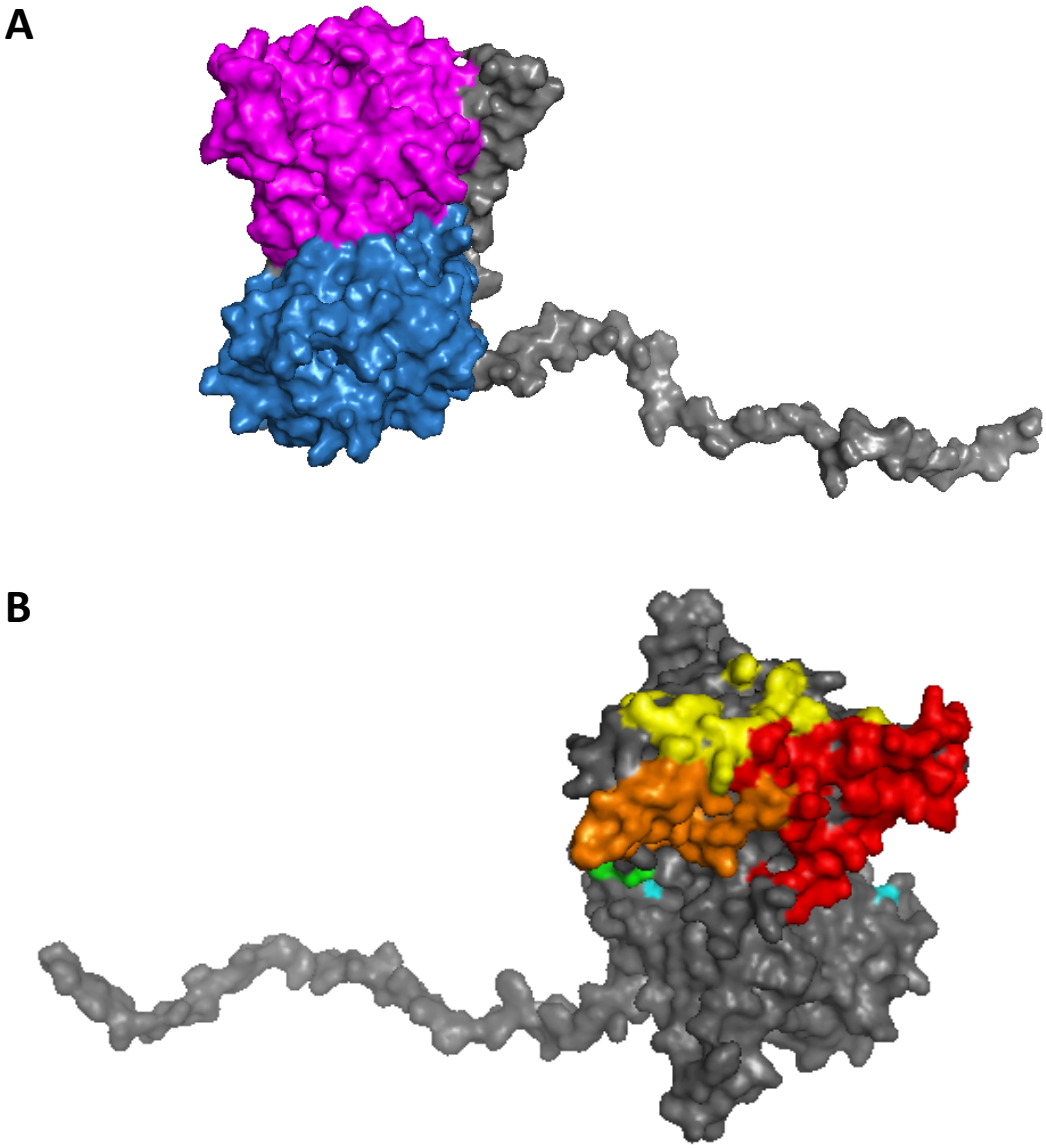


Figure 6.2.1 3D reconstruction of *S. pombe* CTPS. (A) Magenta part (2-270 amino acids) represents the CTP synthase domain. Blue part (315-550 amino acids) represents the Glutamine amidotransferase domain. (B) The red part represents the dimer interface. The yellow and orange part represents the tetramer interface. The cyan part represents the predicted phosphorylation sites. Both ATP-binding site and CTP-binding site are located in the synthase domain as well as in the tetramer interface, making ATP and UTP the prerequisite for CTPS tetramerisation. The amidotransferase domain is located away from the tetramer interface and therefore not affected by the polymeric process or state.



Figure 6.2.2 The 1-56 amino acids of *S. pombe* CTPS (coloured red) are in the interior of the protein. The *S. pombe* CTPS structure was predicted and its 3D model was reconstructed using software PyMOL. In this model, we can see the N-terminus of CTPS contains a α-helix and two β-sheets. We can also see that N-terminus of CTPS is embedded inside the whole protein.

6.2.2 Truncated CTPS show different cluster patterns in *S. pombe*

With the 3D reconstruction of the CTPS N-terminus (**Figure 6.2.3 K**), we were able to design the truncated deletions. In this experiment, the *S. pombe* strains are induced with truncated CTPS-YFP proteins while at the same time having the endogenous wild-type CTPS as the strains background. Depending on the secondary structures and the functionary groups, the CTPS N-terminus was divided into several sections (**Figure 6.2.3. A-J**).

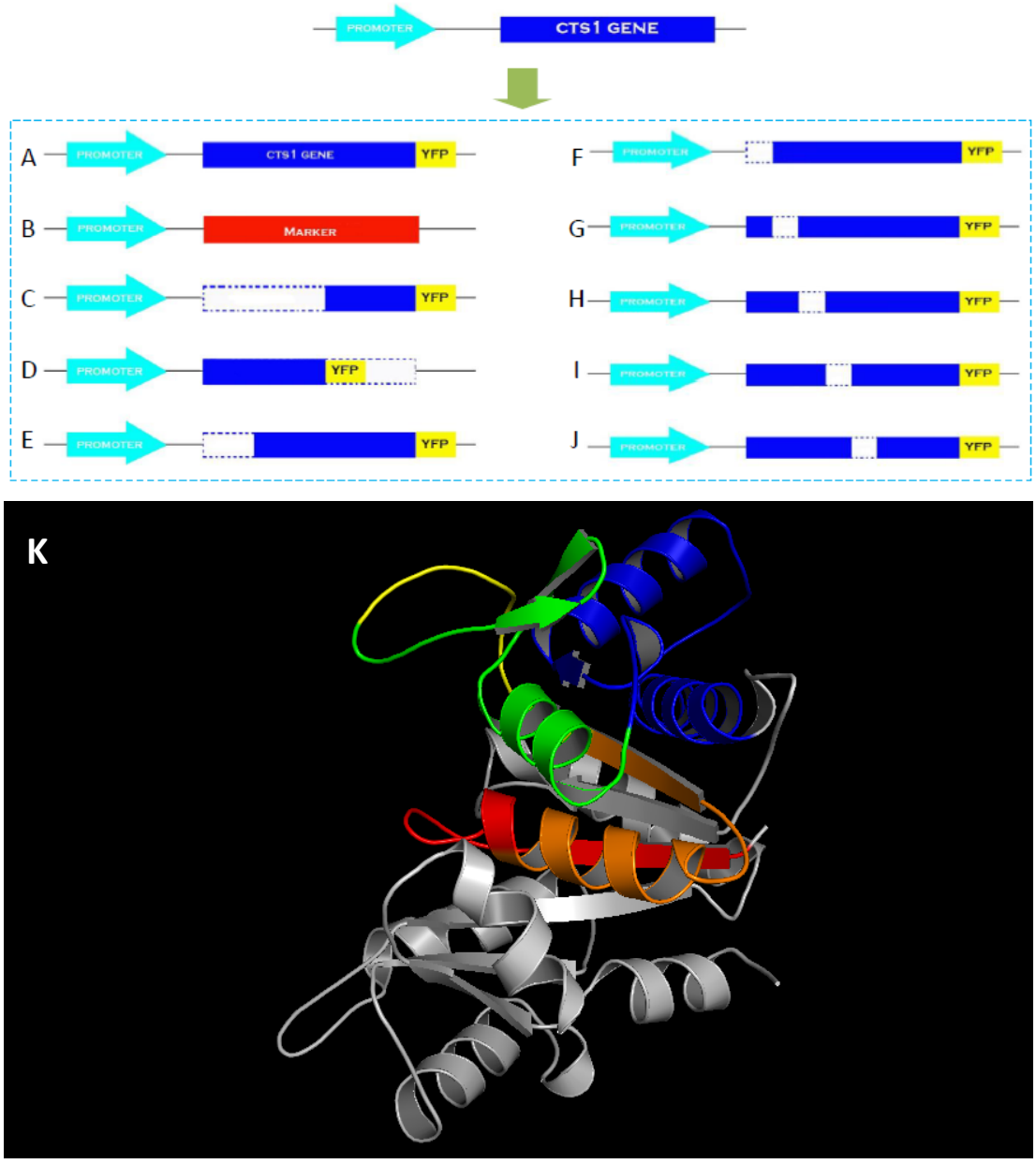


Figure 6.2.3 Design of truncate deletion strains. (A) Control group with whole CTPS YFP tagged; (B) Whole *cts1* gene deleted; (C) Amidotransferase domain YFP tagged; (D) Synthase domain YFP tagged; (E) 2-56 amino acid deleted and YFP tagged; (F-J) 2-18, 19-37, 38-52, 53-79 and 80-129 amino acid deleted and YFP tagged; (K) 3D model showing different sections in CTPS (only synthase domain is shown). 2-18, 19-37, 38-52, 53-79 and 80-129 amino acids are coloured red, orange, yellow, green and blue respectively.

With all 8 different truncated CTPS strains, only 38-52 amino acids deleted cell showed a similar phenotype with control group cells (**Figure 6.2.4**). From **Figure 6.2.4** we can see, in 2-56 and 53-79 amino acids deleted strains, no filaments or foci can be observed and all truncated CTPS are dispersed in cytoplasm and nucleoplasm. In 19-37, 53-79 and 80-129 amino acid deleted strains, the clustered or punctate fluorescence signals can be observed, indicating that the assembly of truncated CTPS is affected. Neither the CTPS synthase domain nor the amidotransferase domain deleted cells showed the observation of cytoophidia (**Figure 6.2.4 H, I**), indicating that both domains are involved in cytoophidia compartmentalisation. The whole CTPS deleted cells showed a lethal phenotype, which is reasonable because *cts1* (CTPS gene in *S. pombe*) has only a single copy in *S. pombe* and CTPS is a central metabolic enzyme involved in cell activities. The truncated CTPS-YFP without endogenous CTPS strains were also constructed and showed lethal phenotype, which is also reasonable since the truncated domains are all critical part for the protein function (**Figure 6.1.3**).

Several point mutations then have been designed (**Table 6.3.1),** since we have narrowed down the region where are the critical amino acids for CTPS compartamentation. Unforturnately, none of these point mutation strains showed survival phenotype.

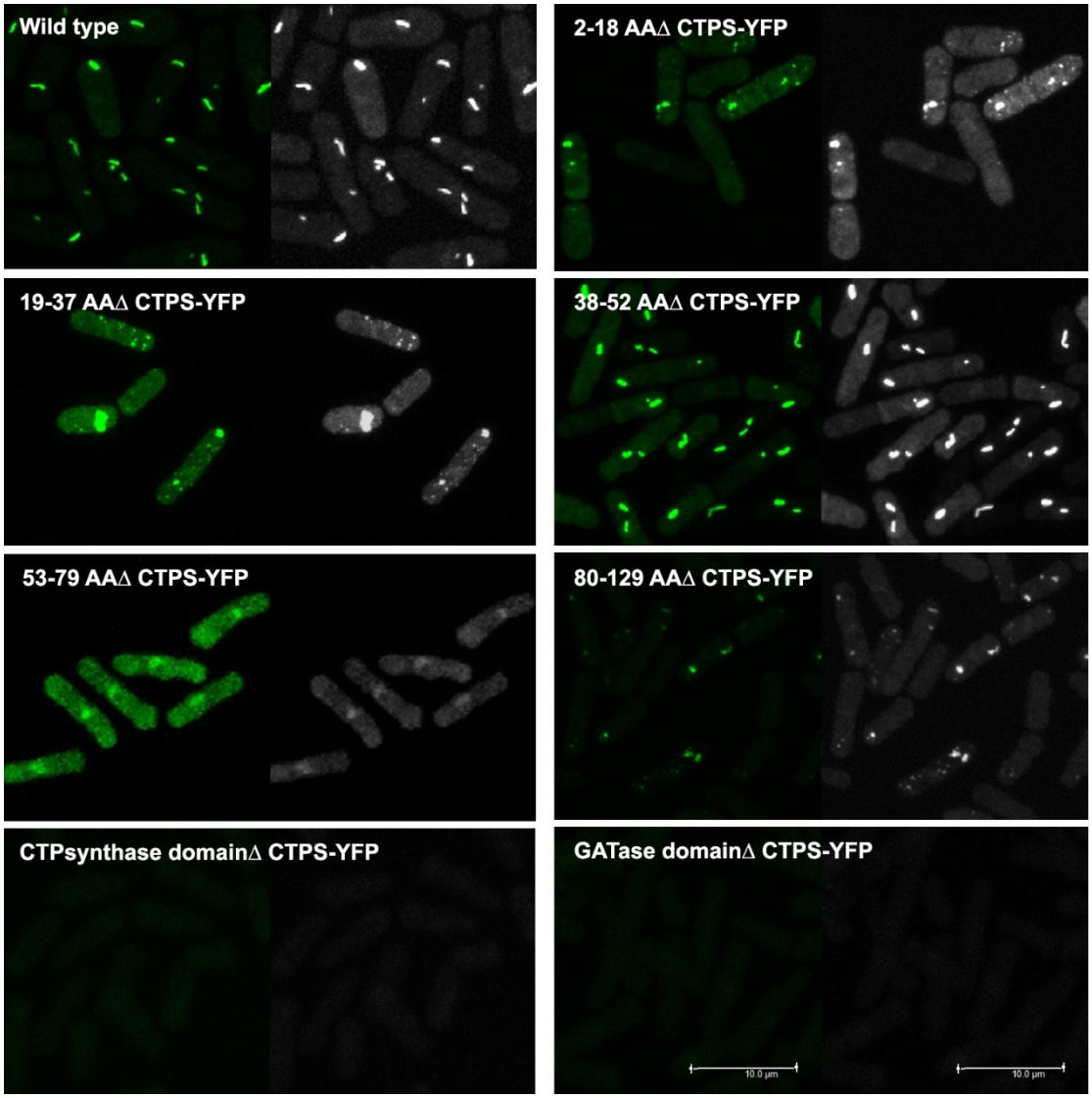


Figure 6.2.4 Truncated CTPS-YFP proteins show different compartmentalisation patterns in *S. pombe*. Wild type CTPS tagged with YFP is used as control. 2-18, 19-37, 38-52, 53-79 and 80-129 amino acid deleted CTPS-YFP shows different patterns of protein expression. 2-18, 19-37 and 80-129 amino acids deleted CTPS-YFP presented as clumps or a punctuated pattern. 38-52 amino acids deleted CTPS-YFP can be observed with filaments having similar patterns to the wild type cytoophidia. CTPS synthase domain and glutamine amidotransferase domain deleted strains have low level of protein expression and no filament was detected. Scale bar: 10μm.

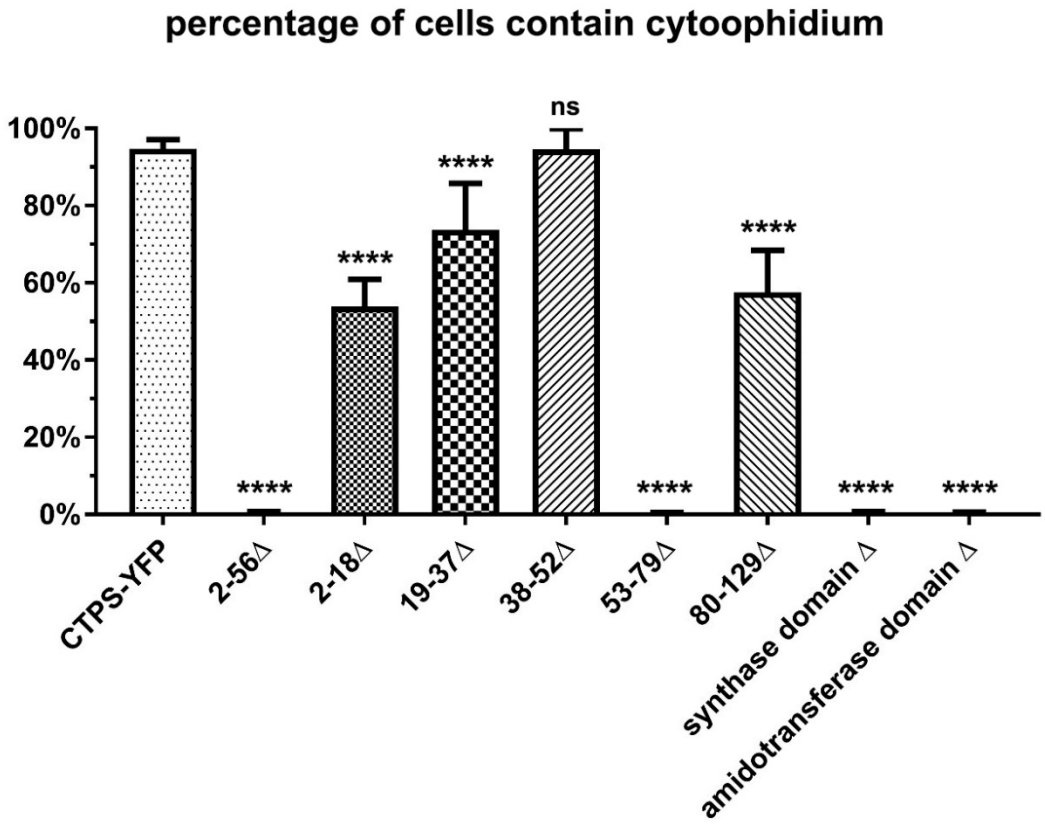


Figure 6.2.5 Analysis of CTPS truncated deletion strains in *S. pombe*: percentage of cells that contain CTPS foci or filaments. 2-56, 53-79 amino acid deleted strains and synthase domain, and amidotransferase domain deleted strains showed no filament or foci. 2-18, 19-37 and 80-129 amino acids deleted strains can be detected with clumps or punctuate fluorescence signals and show significantly different cell counting results in comparison with the control group. Only 38-52 amino acids deleted cells showed a similar pattern to the control group. Error bar: Mean±SEM.

Results fo the T test analysis:

Percentage of cells contain cytoophidia

Dunnett's multiple comparisons test	Mean Diff. (%)	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
w.t. vs. 2-56Δ	94.49	83.01 to 106	Yes	****	<0.0001
w.t. vs. 2-18Δ	40.88	28.3 to 53.46	Yes	****	<0.0001
w.t. vs. 19-37Δ	21.01	11.11 to 30.91	Yes	****	<0.0001
w.t. vs. 38-52Δ	0.1396	-11.35 to 11.62	No	ns	>0.9999
w.t. vs. 53-79Δ	94.58	84.3 to 104.8	Yes	****	<0.0001
w.t. vs. 80-129Δ	37.19	24.61 to 49.77	Yes	****	<0.0001
w.t. vs. synthase domainΔ	94.49	83.01 to 106	Yes	****	<0.0001
w.t. vs. amidotransferase domainΔ	94.54	83.77 to 105.3	Yes	****	<0.0001

6.3 Discussion

6.3.1 Truncated CTPS and the cytoophidium

The *cts1* (gene of CTPS in *S. pombe*) is a single copy gene in *S. pombe* and has no duplicated gene. The CTPS is a central enzyme involved in cell metabolism. We expectated that the whole CTPS deleted strain whould show a lethal phenotype. The endogenous *cts1* gene was thus maintained in *S. pombe* cells among these CTPS truncated strains to guarantee the cell metabolic functions. From **Figures 6.2.4** and **6.2.5**, we can see that among 8 different CTPS truncated strain, only 38-52 amino acid deleted cells showed a similar phenotype with control group cells. It is possible to speculate that the truncated CTPS has an altered structure and affected its dimer or tetramer interface, and therefore interfered in cytoophidia assembly. It is already known that only the synthase domain of CTPS is involved in the tetramerisation process, while the amidotransferase domain is located away from the dimer and tetramer interface (Endrizzi, J. A., et al., 2004; Endrizzi, J. A., et al., 2005; Lauritsen, I., et al., 2011). However, in the *S. pombe* cells deleted with the CTPS amidotransferase domain, no filament can be detected, indicating that cytoophidia compartmentalisation is also related to the amidotransferase domain (**Figure 6.2.4**). Since previous data have already mentioned that some specific phosphorylation sites in the amidotransferase domain is involved in CTPS tetramerisation in budding yeast (Pappas, A., et al., 1998; Park, T. S., et al., 2003), there is no firm conclusion regarding whether the failure of phosphorylation affected CTPS tetramerisation and therefore affected

the cytoophidium formation, or whether the amidotransferase domain has other interfaces involved in CTPS compartmentalisation. Further experiments need to be undertaken to confirm the cytoophidium compartmentalisation mechanism.

6.3.2 Future work

With the narrowed-down range of CTPS N-terminal amino acids affecting cytoophidia assembly, we can now design site-directed point mutations and find the critical amino acids for CTPS compartmentalisation. Aside from the N-terminal regions, there exist other potential candidates. For example, previously published papers show that CTPS activity is regulated by feedback inhibition (Endrizzi, J. A., et al., 2005). In addition, yeast and human CTPS are regulated by phosphorylation by protein kinases A and C (Chang & Carman, 2008; Chang *et al.*, 2007). The oligomeric process of the CTPS has also been demonstrated to be influenced by phosphorylation in budding yeast (Pappas, A., et al., 1998) and a glycine residue (Gly 142) in UTP binding site of *E. coli* CTPS (Lunn, F. A., et al., 2008). The treatment with alkaline phosphatase can induce the dissociation of tetramers into dimers even with the presence of ATP and UTP in budding yeast (Pappas, A., *et al.*, 1998). Furthermore, we already know that DON can covalently bind to glutamine binding sites and enhance cytoophidia formation. Based on this information, we designed point mutations at several sites potentially affecting cytoophidia

compartmentalisation (**Table 6.3.1**), including the CTP feedback inhibition binding site, the UTP binding sites, the glutamine binding sites, the phosphorylation sites, and the tetramer interface amino acids.

Several screens were also designed (**Figure 6.3.1**). The *cts1* gene is planned as the template to generate the random point mutant library by using error-prone PCR, containing around 5000 point mutants. The library will then be applied to four different screens, detecting respectively of cytoophidia formation using microscopy analyse, nucleotides and deoxynucleotides level using HPLC, CTPS expression level using a fluorescence detector, and CTPS catalytic activities using indirect ADP measurement (**Figure 6.3.1**). All these screens except the HPLC can theoretically and technically be applied to 96 well plates. If successfully finished, the four screens will provide a huge amount of information and establish a solid basis for the future cytoophidium research.

Function region	Site-directed mutations
N-terminal amino acids	S22A
	L27A
	G31A
	V34A
Glutamine binding sites	C403G
	C403S
	C403A
	H532A
	E534A
CTP feedback inhibition binding site	E161A
Potential phosphorylation sites	S36A
	S353A
	T451A
	T459A
	S468A
	S573A
Tetramerisation sites	G148A
	G149A
	G152A

Table 6.3.1 Site-directed mutation design

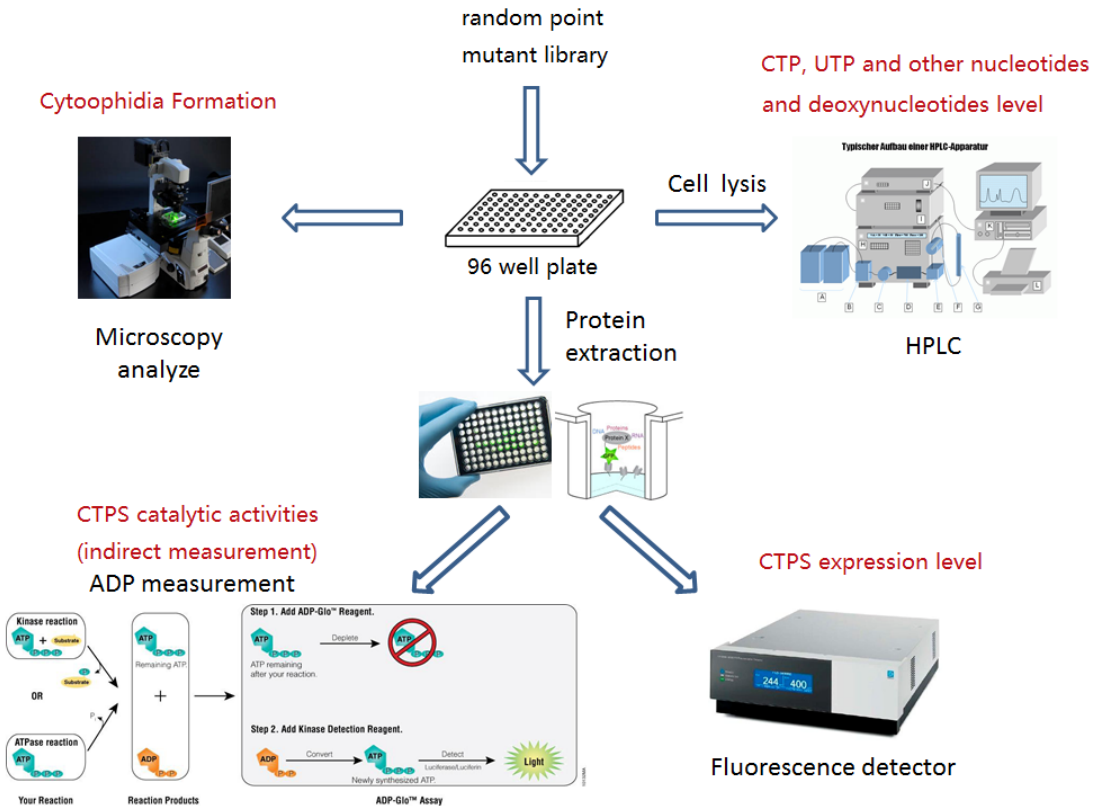


Figure 6.3.1 Designed high-throughput screen process and detail

Chapter 7 Discussion and conclusion

7.1 Overview

An increasing number of metabolic enzymes have revently been revealed with the novel feature of self-assembly and filament forming (Noree, C., et al., 2010; Liu, J. L., 2010; Ingerson-Mahar, M. et al., 2010; Barry, R. M. & Gitai, Z., 2011; Ingerson-Mahar, M. & Gitai, Z., 2012). The assembly mechanism, functions and regulations of these filaments are still only vaguely understood. In this thesis, we have presented a detailed study of cytoophidia, the CTPS filaments, in *S. pombe*. Previously published papers have demonstrated cytoophidia properties in the cells of humans, rats, fruit flies, budding yeast and bacteria (Chen, K., et al., 2011; Noree, C., et al.,2010; Liu, J. L., 2010; Ingerson-Mahar, M., et al., 2010), but no research has yet been carried out in fission yeast. The data we have presented in this thesis makes a good contribution to the cytoophidium research field.

In Chapter 1, we demonstrated that abundant cytoophidia can be observed during the exponential phase in *S. pombe*. Both cytoplasm and nucleus contain cytoophidia. We also presented that cytoophidia are regulated through the cell growing stage, and that the stationary phase induced cytoophidia dispersal is reversible. This result partially contrasts with the previous data collected on budding yeast, that the stationary phase promotes cytoophidia assembly while

the exponential stage leads to cytoophidia dispersal (Noree, C., et al., 2010). This suggests the regulation mechanism of the cytoophidium diverge between budding yeast and fission yeast. Notably, overexpression of CTPS did not enhance CTPS compartmentalisation in *S. pombe*. This result differs from previously observed phenomenon in *Drosophila* and human cells, where overexpression of CTPS enhances cytoophidia assembly (Azzam, G. & Liu, J. L., 2013; Aughey, G. N., et al., 2014), indicating that the regulating mechanism of cytoophidia in *S. pombe* might be different from that of *Drosophila* and human cells.

In *S. pombe*, cytoophidia assembly and disruption are reversible. CTPS can show a segregated or aggregated pattern depending on the cell growth conditions and the environmental conditions. This can be explained by the hypothesis that polymerised CTPS are altered with their catalytic activities, and thus adjust the CTPS-related metabolic rate through compartmentalisation or dispersal. This is supported by the previous research showing that, in *Drosophila*, budding yeast, and the *in vitro* condition of *E. coli* CTPS, filamentous form CTPS are inactive or low in catalytic activity, while segregated CTPS are active, having a high efficiency of CTP synthesis (Barry, R. M., et al, 2014; Noree, C., et al., 2014; Aughey, G. N., et al., 2014). Biological synthesis takes a series of time- and energy-consuming steps, making it unworthy of the degradation once enzymes finished their synthetic jobs. Having them stored and reutilised

when they are needed is a widely-used cellular strategy; hence cytoophidia are speculated to be the storage depot and to regulate activities of metabolic enzymes.

In *S. pombe*, most stress responses are mainly depending on the sty1 MAPK pathway (Sanso, M., et al., 2100). Cytoophidia respond to several stresses in *S. pombe* according to the data shown in this thesis. Although there is no direct evidence that cytoophidia are regulated through the MAPK pathway, we can still speculate that the mechanism of cytoophidia regulating is related to stress response. Interestingly, live imaging experiments reveal that cytoophidia are highly dynamic, and remain filamentous form and undergo an asymmetrically distribution during cytokinesis. This is unlike most other proteins in *S. pombe*, and makes it a newly-discovered distribution pattern for metabolic enzymes during cytokinesis in *S. pombe*.

7.2 *S. pombe* as a tool for studying the cytoophidium

S. pombe is a single-cell eukaryotic organism. It is environment-friendly, not infective, and easy to grow and manipulate in the lab. Its generation time is as short as 2 hours, which means researchers take less time for genetics experiments compared with the use of other animal models. *S. pombe* contains only three chromosomes, making it one of the smallest known eukaryotic genomes. Its whole genome was fully sequenced and published in 2002, with

about 4,979 genes containing around 14Mb of DNA. Among all these genes, about 43% of them contain introns, only 5% of which have duplicated genes (Wood, V., et al., 2002). More introns make it possible for alternative splicing, and therefore give the opportunity to produce an increase range of protein types. This is very different from *S. cerevisiae*, but is very similar to human cells. Besides, *S. pombe* have much longer chromosomes, about 100-300 times longer than those of *S. cerevisiae*. Having more condensed chromosomes is another characteristic resembling human cells (Wood, V., et al., 2002). *S. pombe* is often used for cell division and cellular organisation because most of its genes relating to cell division and cell growth are highly conserved in human cells, including heterochromatin proteins, large origins of replication, large centromeres, conserved cellular checkpoints, telomere function, gene splicing, and many other cellular processes (Hoffman, C. S., et al., 2015).

The simplicity of the genome, its similarities with the mammalian genome, its easily manipulable and grown features, its capability of being used for drug analysis—all these properties make *S. pombe* a proper model system for genetic analysis, as well as for biomedical and cellular biology research. There are several reasons why we chose *S. pombe* as the platform for studying the cytoophidium. Firstly, CTPS is highly conserved among *S. pombe*, *Drosophila* and human cells. Secondly, it takes less time and has higher efficiency to undergo gene knock-out or knock-in compared with *Drosophila* and human

cells. Thirdly, it is possible to apply the drug treatments into a high-throughput system using single cell organisms. Finally, no one has reported detecting the cytoophidium before in *S. pombe.*

7.3 Cytoophidia and cytoskeletons

Several reviews have discussed the evolution of cytoskeletal proteins and speculated that they may have evolved from a common cytoskeletal ancestor. It has also been proposed that dual-role filaments formed by self-assembly metabolic enzymes may be an origin of cytoskeletal proteins (Barry, R. M. & Gitai, Z., 2011; Ingerson-Mahar, M. & Gitai, Z., 2012). Crystallin is the major component of animal eyes. It is a dual-role protein that possesses enzymatic activity while at the same time having structural function when assembled into filaments (Zigler & Rao, 1991). Eukaryote actin and bacteria MreB are both cellular structural function proteins. Interestingly, these two proteins share a structurally similar domain with sugar kinases such as hexokinase (Bork et al., 1992), suggesting that the modern actin may originally evolved from a self-assembly metabolic enzyme. In bacteria *C. crescentus*, CTPS filaments are identified to participate in the regulation of cell shape independently of its enzymatic activity and regulate cell curvature through crescentin (a curvature regulator in *C. crescentus*). CTPS and crescentin functionally interact with each other and colocalise to the inner curvature of *Caulobacter* cell. In addition, *E. coli* CTPS compartmentalisation happens both *in vivo* and *in vitro*, resembling many

cytoskeletal proteins. *E. coli* CTPS can complement both the metabolic and cytoskeletal functions of *C. caulobacter* CTPS (Ingerson-Mahar, M., et al., 2010). All these data raise the possibility that CTPS acquired the ability of filament formation first and then evolved a structural function and served as the dual-role protein in *C. Caulobacter* (Ingerson-Mahar, M., et al., 2010). Another hypothesis is that the enzymes might have initially been dual-role proteins, and that some of them lost their catalytic or structural function later in the evolutionary process, resulting in separate functionary proteins. Both these theories can explain the structural similarity shared by actin and MreB with sugar kinase like hexokinase and Hsp70 family chaperones (Bork, P., et al., 1992; Barry, R. M. & Gitai, Z., 2011).

7.4 Possible advantages of filaments formation

As one of the critical and central metabolic enzymes, CTPS is already tightly controlled by so many different regulatory strategies, including allosteric control, competitive inhibition, transcriptional and post-translational modification, and small-scale oligomerisation (Barry, R. M., et al., 2014). Why do we need another additional regulating mechanism? CTPS is so critical in nucleotide metabolism, and it directly interacts with and is regulated by all four ribonucleotides (ATP, CTP, GTP and UTP) in a way that necessitates the essential cellular metabolism and cell proliferation to have extremely strict control of CTPS. Adding of an extra regulatory strategy thus means the adding

to the assurance that cells will be protected from dysregulated nucleotide levels (Barry, R. M., et al., 2014).

Aside from the already demonstrated functions of regulating CTPS activity and storing temporarily unused CTPS, cytoophidia can be proposed with other advantages. No clear evidence has, however, been published that cytoophidia are the multicomponent structures. Several central metabolic enzymes have been identified to form filaments and colocalise with cytoophidia, including IMPDH and Ack (Carcamo, W. C., et al., 2011; Calise, S.J., et al., 2014; Strochlic, T. I., et al., 2014), indicating that cytoophidia may contain components other than CTPS. The sequestration of different critical metabolic enzymes into a common structure may be a strategy to increase the efficiency of cellular metabolism. CTPS and IMPDH are both important nucleotide biosynthesis enzymes. CTPS is involved in pyrimidine *de novo* synthesis and IMPDH is critical for purine *de novo* synthesis. The compartmentalisation of these two enzymes together can potentially maximise their utilities and regulate nucleotide metabolism more easily.

In addition, compartmentalisation may cause the conformational change of enzymes and thus hide or expose the reaction or binding sites on the protein surface, changing the enzyme activity or stability. It has been reported that filamentous CTPS has downregulated catalytic functions (Barry, R. M., et al.,

2014; Noree, C., et al., 2014), while IMPDH activity is upregulated after being compartmentalised into filaments (Chang, C. C., et al., 2015). Different protein arrangements may also help conceal or uncover modification sites (phosphorylation site, ubiquitin binding site, etc.) or even generate new binding sites, and consequently affect the enzyme activity or stability. For example, cytoophidia induced by DON are increased with CTPS stability (Liu, J. L., 2016). The assembly of CTPS may also help conceal the ubiquitin binding sites and prevent protein degradation, prolonging the CTPS life.

Furthermore, cytoophidia may act as the enzyme storage pool as well as the metabolic buffer system to respond to environmental stress rapidly and effectively, and help cells to adapt to different conditions. For example, cytoophidia in *S. pombe* can react to cold stress and disassemble within 5 minutes. When cells shifted back to the normal growth temperature (32˚C), cytoophidia emerged again within as short a time as 5 minutes. Although the mechanism of this rapid reaction to cold stress is still not clear, we can speculate that cytoophidia may take some functional role during the cold stress response.

Cytoophidia may also benefit cell proliferation, especially in fast-growing cells like stem cells and cancer cells. Published papers described the observation of abundant cytoophidia in *Drosophila* neural stem cells (Tastan, O. Y. & Liu, J. L., 2015) and mouse embryonic stem cells (Carcamo, W. C., et al., 2011).

Interestingly, when the mouse embryonic stem cells finished differentiation, cytoophidia were observed to disperse, which supports our hypothesis that cytoophidia potentially benefits cell proliferation. Likewise, in *S. pombe*, the fast dividing exponential stage cells have plentiful cytoophidia. which are rarely observed in the stationary phase, during which stage cells stop dividing and step into quiescence. Although not proved, it can be speculated that cytoophidia may have functions relating to cell proliferation.

7.5 CTPS and the cytoophidium in the study of disease

Nucleotide metabolic enzymes have been the popular targets used in cancer therapeutic strategies for decades. Nucleotide and its analogues are considered promising targets for the treatment of cancer and viral infections due to their proven anti-proliferative effects in animal models and clinical trials (Jordheim, L. P., et al., 2013). In 2002, the complete genome of *S. pombe* was sequenced and published, revealing that 23 protein-coding genes and mutations are human cancer related (Wood, V., et al., 2002). *cts1* (the CTPS gene in *S. pombe*) is one of the best-studied genes among these 23 genes. It has been observed that the CTPS activity in human and rat tumors (Williams, J. C., et al., 1978; Kizaki, H., et al., 1980) as well as the intracellular CTP concentration (Meuth, M., 1981; Meuth, M., 1984) were upregulated, and the inhibition by the anticancer agent cyclopentenyl cytosine has also been reported (Kang, G. J., et al., 1989; Verschuur, A. C., et al., 2000 [a & b]), showing close links between CTPS and

various tumours. In addition, CTPS is directly bound and regulated by all four ribonucleotides, making it the critical factor for keeping the cellular nucleotide pool balanced (Meuth, M., 1984). CTPS thus becomes the attractive target in the development of antitumour and antiviral agents (Kensler, T. W. & Cooney, D. A., 1981). Since CTPS enzymatic activity is regulated via oligomerisation and downregulated after forming into filaments, cytoophidia also become potential targets for cancer chemotherapy as well. Interestingly, although no datum have demonstrated a direct connection between cytoophidia and tumour generation, it has been revealed that cytoophidia are regulated by the oncogene Myc and required for Myc-mediated cell size control (Aughey, G. N., et al., 2016). The observation of abundant cytoophidia in *Drosophila* neural stem cells (Tastan, O. Y. & Liu, J. L., 2015) and mouse embryonic stem cells (Carcamo, W. C., et al., 2011) has also been demonstrated. It seems that cytoophidia prefer to be highly expressed in fast-growing cells like cancer cells and stem cells, leading to a speculation that cytoophidia assembly may be a strategy for conferring a selective advantage for cell proliferation. Furthermore, cytoophidia could potentially be used as future biomarkers for disease.

7.6 Future work

7.6.1 CTPS structure and point mutation design

CTPS is the majority component and backbone constituent of cytoophidia.

Although many researchers have applied CTPS as a cytoophidia marker protein, few studies have been devoted to its filament structure and assembly mechanism. Recently published data demonstrate that cytoophidia are possibly multi-component filaments, containing not only CTPS but also IMDPH and perhaps other enzymes. It is also demonstrated that the cytoophidia are composed of CTPS inactive dimers in *Drosophila* and human HeLa cells (Noree, C., et al., 2014; Aughey, G. N., et al., 2014) and 'readily activatable' tetramers in (Barry, R. M., et al., 2014). How protein-protein reactions takes place, and which domain takes the central role of assembly, is still unknown. To answer these questions, we firstly utilised a genetic approach and designed different point mutations of CTPS, expecting to see diverse phenotypes of cytoophidia as assembly or disassembly, thus allowing us to identify which amino acid is important for CTPS compartmentalisation. However, we encountered difficulties generating mutant cells, since the *cts1* gene only has a single copy in the *S. pombe* genome, and the CTPS enzymatic activity and filament-forming ability are closely interrelated, leading to the easily-caused lethal phenotype of the designed mutant cells. The separation of the catalytic function and the filament formation thus becomes essential before the point mutation exploration. If we can consequently obtain mutants that have a disrupted filament-forming property but intact enzymatic activity, or retaining the ability of assembly while having lost enzymatic activity, this will be beneficial for future investigation of cytoophidia functions. With the

phenotypic of filament-forming function and enzymatic activity separated mutants, we can then inspect the functions of CTPS assembly on cell cycle, cell size, organelle morphology, and other cellular metabolism processes.

7.6.2 The MAPK pathway and related work

The mitogen-activated protein kinases signalling pathway is the central regulation pathway for stress response in *S. pombe*. Several genes are involved in the MAPK pathway in *S. pombe*, including spy1, Msc4, Wak1 and Win1, Wis1, Sty1, *Atf1* and Pcr1. *Pyp1* and *pyp2* are also related to the MAPK pathway. The deficiency of the MAPK pathway may result in the uncontrolled cell growth, and may consequently lead to cancer. In this thesis, I demonstrate a decrease of cytoophidia numbers in deletion strains *pyp2*Δ and *atf1*Δ, indicating that both *atf1* and *pyp2* potentially affect cytoophidia. It is worthwhile continuing to construct other gene deletion strains to test if cytoophidia are regulated through the MAPK pathway.

7.6.3 The TOR pathway and related work

The TOR pathway, target of rapamycin, is a structurally and functionally conserved pathway from yeast to human. In *S. pombe*, the TOR pathway functions in two distinct multi-protein complexes, TORC 1 and TORC 2. TORC 1 promotes cell growth and regulates cell size and the cell cycle. It is also related to stress responses, nutrient uptake and intermediary metabolism. TORC2 is

responsible for the regulation of spatial growth, such as cell polarity. Previous studies in fruit flies and mammalian cell lines have suggested that aberrant TORC 1 signalling leads to various pathologies including metabolic diseases, hyper-proliferative disorders and cancers. Accordingly, the research of TOR signalling and study of rapamycin and related compounds are being evaluated in clinical trials. In this thesis, we have demonstrated that cytoophidia are regulated through cell growth and respond to nitrogen deprivation. It is possible that cytoophidia regulation is related to the TOR pathway. To answer the question of whether cytoophidia are regulated by the cell cycle, and to discover the specific mechanism of cytoophidia regulation, further detailed researche is worth pursuing.

7.7 Concluding remarks

CTPS is one of the central enzymes involved in pyrimidine biosynthesis. Its structure and regulating mechanisms have been studied for decades, but there are still unknown fields about CTPS. A new property of CTPS that has recently been discovered in addition to its catalytic functions shows that CTPS is also able to compartment into filaments, termed cytoophidia. This self-assembly property is not restricted to CTPS, and can also be observed on other central enzymes, like ACC, IMPDH, Cdc42 kinase, glutamine synthase and glutamate synthase (Gregolin, C., et al., 1966; Carcamo, W. C., et al., 2011; Strochlic, T. I., et al., 2014; Petrovska, I., et al., 2014; Noree, C., et al., 2010). With the increasing

number of enzymes with filament-forming ability that have been uncovered, the study of cytoophidia becomes more and more fascinating. In this thesis, we have used *S. pombe* as the model organism for studying cytoophidia to get an insight of its biological function and regulation mechanism. We discovered that cytoophidia in *S. pombe* are sensitive to stress and that its regulation is related with cell growth condition. Published data also show that cytoophidia are related with environmental stresses (nutrient deprivation in budding yeast and *Drosophila*), neurodevelopment (Optic Lobe Homeostasis in *Drosophila*), and cell shape regulation (cell curvature of *Caulobacter crescentus*). More research, however, needs to be done to fully unmask the mystery of cytoophidia.

References

De-Serres, Frederick, J. (1985). Genetic consequences of nucleotide pool imbalance. Basic Life Sci 31, 1-523.

Abenza, J.F., Chessel, A., Raynaud, W.G., and Carazo-Salas, R.E. (2014). Dynamics of cell shape inheritance in fission yeast. PLoS One 9, e106959.

Ahn, J., Won, M., Choi, J.H., Kyun, M.L., Cho, H.S., Park, H.M., Kang, C.M., and Chung, K.S. (2012). Small heat-shock protein Hsp9 has dual functions in stress adaptation and stress-induced G2-M checkpoint regulation via Cdc25 inactivation in Schizosaccharomyces pombe. Biochem Biophys Res Commun 417, 613-618.

Alao, J.P., and Sunnerhagen, P. (2008). Rad3 and Sty1 function in Schizosaccharomyces pombe: an integrated response to DNA damage and environmental stress? Mol Microbiol 68, 246-254.

An, S., Kumar, R., Sheets, E.D., and Benkovic, S.J. (2008). Reversible compartmentalization of de novo purine biosynthetic complexes in living cells. Science 320, 103-106.

Aughey, G.N., Grice, S.J., and Liu, J.L. (2016). The Interplay between Myc and CTP Synthase in Drosophila. PLoS Genet 12, e1005867.

Aughey, G.N., Grice, S.J., Shen, Q.J., Xu, Y., Chang, C.C., Azzam, G., Wang, P.Y., Freeman-Mills, L., Pai, L.M., Sung, L.Y., et al. (2014). Nucleotide synthesis is regulated by cytoophidium formation during neurodevelopment and adaptive metabolism. Biol Open 3, 1045-1056.

Aughey, G.N., and Liu, J.L. (2015). Metabolic regulation via enzyme filamentation. Crit Rev Biochem Mol Biol 51, 282-293.

Ausmees, N. (2006). Intermediate filament-like cytoskeleton of Caulobacter crescentus. J Mol Microbiol Biotechnol 11, 152-158.

Ausmees, N., Kuhn, J.R., and Jacobs-Wagner, C. (2003). The bacterial cytoskeleton: an intermediate filament-like function in cell shape. Cell 115, 705-713.

Azzam, G., and Liu, J.L. (2013). Only one isoform of Drosophila melanogaster CTP synthase forms the cytoophidium. PLoS Genet 9, e1003256.

Bagchi, S., Tomenius, H., Belova, L.M., and Ausmees, N. (2008). Intermediate filament-like proteins in bacteria and a cytoskeletal function in Streptomyces. Mol Microbiol 70, 1037-1050.

Bahler, J. (2005). Cell-cycle control of gene expression in budding and fission yeast. Annu Rev Genet 39, 69-94.

Bakthisaran, R., Tangirala, R., and Rao Ch, M. (2015). Small heat shock proteins: Role in cellular functions and pathology. Biochim Biophys Acta 1854, 291-319.

Banh, S., and Hales, B.F. (2013). Hydroxyurea exposure triggers tissue-specific activation of p38 mitogen-activated protein kinase signaling and the DNA damage response in organogenesis-stage mouse embryos. Toxicol Sci 133, 298-308.

Barnes, C.A., MacKenzie, M.M., Johnston, G.C., and Singer, R.A. (1995). Efficient translation of an SSA1-derived heat-shock mRNA in yeast cells limited for cap-binding protein and eIF-4F. Mol Gen Genet 246, 619-627.

Barry, R.M., Bitbol, A.F., Lorestani, A., Charles, E.J., Habrian, C.H., Hansen, J.M., Li, H.J., Baldwin, E.P., Wingreen, N.S., Kollman, J.M., et al. (2014). Large-scale filament formation inhibits the activity of CTP synthetase. Elife 3, e03638.

Barry, R.M., and Gitai, Z. (2011). Self-assembling enzymes and the origins of the cytoskeleton. Curr Opin Microbiol 14, 704-711.

Baserga, R. (1986). Molecular biology of the cell cycle. International journal of radiation biology and related studies in physics, chemistry, and medicine 49, 219-226.

Bearne, S.L., Hekmat, O., and Macdonnell, J.E. (2001). Inhibition of Escherichia coli CTP synthase by glutamate gamma-semialdehyde and the role of the allosteric effector GTP in glutamine hydrolysis. Biochem J 356, 223-232.

Beaty, N.B., and Lane, M.D. (1983). Kinetics of activation of acetyl-CoA carboxylase by citrate. Relationship to the rate of polymerization of the enzyme. J Biol Chem 258, 13043-13050.

Beaty, N.B., and Lane, M.D. (1983). The polymerization of acetyl-CoA carboxylase. J Biol Chem 258, 13051-13055.

Beckley, J.R., Chen, J.S., Yang, Y., Peng, J., and Gould, K.L. (2015). A Degenerate Cohort of Yeast Membrane Trafficking DUBs Mediates Cell Polarity and Survival. Mol Cell Proteomics 14, 3132-3141.

Benko, Z., Liang, D., Agbottah, E., Hou, J., Taricani, L., Young, P.G., Bukrinsky,

M., and Zhao, R.Y. (2007). Antagonistic interaction of HIV-1 Vpr with Hsf-mediated cellular heat shock response and Hsp16 in fission yeast (Schizosaccharomyces pombe). Retrovirology 4, 16.

Berman, J.J. (1989). Amsacrine-induced cytoplasmic rods. J Submicrosc Cytol Pathol 21, 201-204.

Bezanilla, M., Gladfelter, A.S., Kovar, D.R., and Lee, W.L. (2015). Cytoskeletal dynamics: a view from the membrane. J Cell Biol 209, 329-337.

Bi, E., and Park, H.O. (2012). Cell polarization and cytokinesis in budding yeast. Genetics 191, 347-387.

Bork, P., Sander, C., and Valencia, A. (1992). An ATPase domain common to prokaryotic cell cycle proteins, sugar kinases, actin, and hsp70 heat shock proteins. Proc Natl Acad Sci U S A 89, 7290-7294.

Bowne, S.J., Sullivan, L.S., Blanton, S.H., Cepko, C.L., Blackshaw, S., Birch, D.G., Hughbanks-Wheaton, D., Heckenlively, J.R., and Daiger, S.P. (2002). Mutations in the inosine monophosphate dehydrogenase 1 gene (IMPDH1) cause the RP10 form of autosomal dominant retinitis pigmentosa. Hum Mol Genet 11, 559-568.

Bowne, S.J., Sullivan, L.S., Mortimer, S.E., Hedstrom, L., Zhu, J., Spellicy, C.J., Gire, A.I., Hughbanks-Wheaton, D., Birch, D.G., Lewis, R.A., et al. (2006). Spectrum and frequency of mutations in IMPDH1 associated with autosomal dominant retinitis pigmentosa and leber congenital amaurosis. Invest Ophthalmol Vis Sci 47, 34-42.

Brown, A.J., Budge, S., Kaloriti, D., Tillmann, A., Jacobsen, M.D., Yin, Z., Ene, I.V., Bohovych, I., Sandai, D., Kastora, S., et al. (2014). Stress adaptation in a pathogenic fungus. J Exp Biol 217, 144-155.

Budini, M., Buratti, E., Stuani, C., Guarnaccia, C., Romano, V., De Conti, L., and Baralle, F.E. (2012). Cellular model of TAR DNA-binding protein 43 (TDP-43) aggregation based on its C-terminal Gln/Asn-rich region. J Biol Chem 287, 7512-7525.

Calise, S.J., Carcamo, W.C., Krueger, C., Yin, J.D., Purich, D.L., and Chan, E.K. (2014). Glutamine deprivation initiates reversible assembly of mammalian rods and rings. Cell Mol Life Sci 71, 2963-2973.

Carcamo, W.C., Satoh, M., Kasahara, H., Terada, N., Hamazaki, T., Chan, J.Y., Yao, B., Tamayo, S., Covini, G., von Muhlen, C.A., et al. (2011). Induction of cytoplasmic rods and rings structures by inhibition of the CTP and GTP

synthetic pathway in mammalian cells. PLoS One 6, e29690.

Cargnello, M., and Roux, P.P. (2011). Activation and function of the MAPKs and their substrates, the MAPK-activated protein kinases. Microbiol Mol Biol Rev 75, 50-83.

Carman, G.M., and Han, G.S. (2007). Regulation of phospholipid synthesis in Saccharomyces cerevisiae by zinc depletion. Biochim Biophys Acta 1771, 322-330.

Carman, G.M., and Han, G.S. (2009). Regulation of phospholipid synthesis in yeast. J Lipid Res 50 Suppl, S69-73.

Carman, G.M., and Henry, S.A. (1999). Phospholipid biosynthesis in the yeast Saccharomyces cerevisiae and interrelationship with other metabolic processes. Prog Lipid Res 38, 361-399.

Carman, G.M., and Kersting, M.C. (2004). Phospholipid synthesis in yeast: regulation by phosphorylation. Biochem Cell Biol 82, 62-70.

Carpy, A., Krug, K., Graf, S., Koch, A., Popic, S., Hauf, S., and Macek, B. (2014). Absolute proteome and phosphoproteome dynamics during the cell cycle of Schizosaccharomyces pombe (Fission Yeast). Mol Cell Proteomics 13, 1925-1936.

Carr, A.M. (1995). DNA structure checkpoints in fission yeast. Semin Cell Biol 6, 65-72.

Carr, A.M. (1998). Analysis of fission yeast DNA structure checkpoints. Microbiology 144 (Pt 1), 5-11.

Catane, R., Von Hoff, D.D., Glaubiger, D.L., and Muggia, F.M. (1979). Azaserine, DON, and azotomycin: three diazo analogs of L-glutamine with clinical antitumor activity. Cancer Treat Rep 63, 1033-1038.

Cerutti, L., and Simanis, V. (1999). Asymmetry of the spindle pole bodies and spg1p GAP segregation during mitosis in fission yeast. J Cell Sci 112 (Pt 14), 2313-2321.

Chang, C.C., Lin, W.C., Pai, L.M., Lee, H.S., Wu, S.C., Ding, S.T., Liu, J.L., and Sung, L.Y. (2015). Cytoophidium assembly reflects upregulation of IMPDH activity. J Cell Sci 128, 3550-3555.

Chang, Y.F., and Carman, G.M. (2008). CTP synthetase and its role in phospholipid synthesis in the yeast Saccharomyces cerevisiae. Prog Lipid Res 47, 333-339.

Chang, Y.F., Martin, S.S., Baldwin, E.P., and Carman, G.M. (2007). Phosphorylation of human CTP synthetase 1 by protein kinase C: identification of Ser(462) and Thr(455) as major sites of phosphorylation. J Biol Chem 282, 17613-17622.

Chen, B., Piel, W.H., Gui, L., Bruford, E., and Monteiro, A. (2005). The HSP90 family of genes in the human genome: insights into their divergence and evolution. Genomics 86, 627-637.

Chen, B., Zhong, D., and Monteiro, A. (2006). Comparative genomics and evolution of the HSP90 family of genes across all kingdoms of organisms. BMC Genomics 7, 156.

Chen, D., Toone, W.M., Mata, J., Lyne, R., Burns, G., Kivinen, K., Brazma, A., Jones, N., and Bahler, J. (2003). Global transcriptional responses of fission yeast to environmental stress. Mol Biol Cell 14, 214-229.

Chen, K., Zhang, J., Tastan, O.Y., Deussen, Z.A., Siswick, M.Y., and Liu, J.L. (2011). Glutamine analogs promote cytoophidium assembly in human and Drosophila cells. J Genet Genomics 38, 391-402.

Chen, L., Zhong, H., Ren, F., Guo, Q.Q., Hu, X.P., and Li, X.B. (2011). A novel cold-regulated gene, COR25, of Brassica napus is involved in plant response and tolerance to cold stress. Plant Cell Rep 30, 463-471.

Chittur, S.V., Klem, T.J., Shafer, C.M., and Davisson, V.J. (2001). Mechanism for acivicin inactivation of triad glutamine amidotransferases. Biochemistry 40, 876-887.

Choi, M.G., and Carman, G.M. (2007). Phosphorylation of human CTP synthetase 1 by protein kinase A: identification of Thr455 as a major site of phosphorylation. J Biol Chem 282, 5367-5377.

Choi, M.G., Park, T.S., and Carman, G.M. (2003). Phosphorylation of Saccharomyces cerevisiae CTP synthetase at Ser424 by protein kinases A and C regulates phosphatidylcholine synthesis by the CDP-choline pathway. J Biol Chem 278, 23610-23616.

Coelho, M., Dereli, A., Haese, A., Kuhn, S., Malinovska, L., DeSantis, M.E., Shorter, J., Alberti, S., Gross, T., and Tolic-Norrelykke, I.M. (2013). Fission yeast does not age under favorable conditions, but does so after stress. Curr Biol 23, 1844-1852.

Coelho, M., Lade, S.J., Alberti, S., Gross, T., and Tolic, I.M. (2014). Fusion of protein aggregates facilitates asymmetric damage segregation. PLoS Biol 12,

e1001886.

Coudreuse, D., and Nurse, P. (2010). Driving the cell cycle with a minimal CDK control network. Nature 468, 1074-1079.

Crotty, S., Cameron, C., and Andino, R. (2002). Ribavirin's antiviral mechanism of action: lethal mutagenesis? J Mol Med (Berl) 80, 86-95.

Csermely, P., Schnaider, T., Soti, C., Prohaszka, Z., and Nardai, G. (1998). The 90-kDa molecular chaperone family: structure, function, and clinical applications. A comprehensive review. Pharmacol Ther 79, 129-168.

Dann, S.G., and Thomas, G. (2006). The amino acid sensitive TOR pathway from yeast to mammals. FEBS Lett 580, 2821-2829.

De Virgilio, C., and Loewith, R. (2006). The TOR signalling network from yeast to man. Int J Biochem Cell Biol 38, 1476-1481.

Degols, G., Shiozaki, K., and Russell, P. (1996). Activation and regulation of the Spc1 stress-activated protein kinase in Schizosaccharomyces pombe. Mol Cell Biol 16, 2870-2877.

Denoth Lippuner, A., Julou, T., and Barral, Y. (2014). Budding yeast as a model organism to study the effects of age. FEMS Microbiol Rev 38, 300-325.

Devaney, E., Egan, A., Lewis, E., Warbrick, E.V., and Jecock, R.M. (1992). The expression of small heat shock proteins in the microfilaria of Brugia pahangi and their possible role in development. Mol Biochem Parasitol 56, 209-217.

Di, Y., Holmes, E.J., Butt, A., Dawson, K., Mironov, A., Kotiadis, V.N., Gourlay, C.W., Jones, N., and Wilkinson, C.R. (2012). H(2)O(2) stress-specific regulation of S. pombe MAPK Sty1 by mitochondrial protein phosphatase Ptc4. EMBO J 31, 563-575.

Drummond, D.R., and Cross, R.A. (2000). Dynamics of interphase microtubules in Schizosaccharomyces pombe. Curr Biol 10, 766-775.

Eliyahu, E., Lesnik, C., and Arava, Y. (2012). The protein chaperone Ssa1 affects mRNA localization to the mitochondria. FEBS Lett 586, 64-69.

Endrizzi, J.A., Kim, H., Anderson, P.M., and Baldwin, E.P. (2004). Crystal structure of Escherichia coli cytidine triphosphate synthetase, a nucleotide-regulated glutamine amidotransferase/ATP-dependent amidoligase fusion protein and homologue of anticancer and antiparasitic drug targets. Biochemistry 43, 6447-6463.

Endrizzi, J.A., Kim, H., Anderson, P.M., and Baldwin, E.P. (2005). Mechanisms of product feedback regulation and drug resistance in cytidine triphosphate synthetases from the structure of a CTP-inhibited complex. Biochemistry 44, 13491-13499.

Erickson, H.P. (2007). Evolution of the cytoskeleton. Bioessays 29, 668-677.

Ewing, R.M., Chu, P., Elisma, F., Li, H., Taylor, P., Climie, S., McBroom-Cerajewski, L., Robinson, M.D., O'Connor, L., Li, M., et al. (2007). Large-scale mapping of human protein-protein interactions by mass spectrometry. Mol Syst Biol 3, 89.

Fantes, P.A., and Hoffman, C.S. (2016). A Brief History of Schizosaccharomyces pombe Research: A Perspective Over the Past 70 Years. Genetics 203, 621-629.

Feng, W., Di Rienzi, S.C., Raghuraman, M.K., and Brewer, B.J. (2011). Replication stress-induced chromosome breakage is correlated with replication fork progression and is preceded by single-stranded DNA formation. G3 (Bethesda) 1, 327-335.

Forsburg, S.L. (2001). The art and design of genetic screens: yeast. Nat Rev Genet 2, 659-668.

Forsburg, S.L. (2005). The yeasts Saccharomyces cerevisiae and Schizosaccharomyces pombe: models for cell biology research. Gravit Space Biol Bull 18, 3-9.

Forsburg, S.L., and Rhind, N. (2006). Basic methods for fission yeast. Yeast 23, 173-183.

Fulton, B., and Markham, A. (1996). Mycophenolate mofetil. A review of its pharmacodynamic and pharmacokinetic properties and clinical efficacy in renal transplantation. Drugs 51, 278-298.

Furuya, K., and Carr, A.M. (2003). DNA checkpoints in fission yeast. J Cell Sci 116, 3847-3848.

Gaglio, D., Metallo, C.M., Gameiro, P.A., Hiller, K., Danna, L.S., Balestrieri, C., Alberghina, L., Stephanopoulos, G., and Chiaradonna, F. (2011). Oncogenic K-Ras decouples glucose and glutamine metabolism to support cancer cell growth. Mol Syst Biol 7, 523.

Gallo, G.J., Schuetz, T.J., and Kingston, R.E. (1991). Regulation of heat shock factor in Schizosaccharomyces pombe more closely resembles regulation in mammals than in Saccharomyces cerevisiae. Mol Cell Biol 11, 281-288.

Gao, W.Y., Johns, D.G., and Mitsuya, H. (2000). Potentiation of the anti-HIV activity of zalcitabine and lamivudine by a CTP synthase inhibitor, 3-deazauridine. Nucleosides Nucleotides Nucleic Acids 19, 371-377.

Goldstein, B.M., and Colby, T.D. (1999). IMP dehydrogenase : structural aspects of inhibitor binding. Curr Med Chem 6, 519-536.

Goto, M., Omi, R., Nakagawa, N., Miyahara, I., and Hirotsu, K. (2004). Crystal structures of CTP synthetase reveal ATP, UTP, and glutamine binding sites. Structure 12, 1413-1423.

Gou, K.M., Chang, C.C., Shen, Q.J., Sung, L.Y., and Liu, J.L. (2014). CTP synthase forms cytoophidium in the cytoplasm and nucleus. Exp Cell Res 323, 242-253.

Gregolin, C., Ryder, E., Kleinschmidt, A.K., Warner, R.C., and Lane, M.D. (1966). Molecular characteristics of liver acetyl CoA carboxylase. Proc Natl Acad Sci U S A 56, 148-155.

Gregolin, C., Ryder, E., Warner, R.C., Kleinschmidt, A.K., and Lane, M.D. (1966). Liver acetyl coa carboxylase: the dissociation-reassociation process and its relation to catalytic activity. Proc Natl Acad Sci U S A 56, 1751-1758.

Guerriero, C.J., Weiberth, K.F., and Brodsky, J.L. (2013). Hsp70 targets a cytoplasmic quality control substrate to the San1p ubiquitin ligase. J Biol Chem 288, 18506-18520.

Hall, M.N. (2010). Structure, function and regulation of TOR complexes from yeasts to mammals. Part A (Amsterdam: Elsevier/Academic Press).

Hall, M.N. (2010). Structure, function and regulation of TOR complexes from yeasts to mammals. Part B (Amsterdam ; London: Elsevier/Academic Press).

Han, G.S., Sreenivas, A., Choi, M.G., Chang, Y.F., Martin, S.S., Baldwin, E.P., and Carman, G.M. (2005). Expression of Human CTP synthetase in Saccharomyces cerevisiae reveals phosphorylation by protein kinase A. J Biol Chem 280, 38328-38336.

Hartwell, L.H., Culotti, J., Pringle, J.R., and Reid, B.J. (1974). Genetic control of the cell division cycle in yeast. Science 183, 46-51.

Harvey, J., Hardy, S.C., and Ashford, M.L. (1999). Dual actions of the metabolic inhibitor, sodium azide on K(ATP) channel currents in the rat CRI-G1 insulinoma cell line. Br J Pharmacol 126, 51-60.

Haschemeyer, R.H., and De Harven, E. (1974). Electron microscopy of enzymes.

Annu Rev Biochem 43, 279-301.

Hayles, J., and Nurse, P. (2001). A journey into space. Nat Rev Mol Cell Biol 2, 647-656.

Hayles, J., Wood, V., Jeffery, L., Hoe, K.L., Kim, D.U., Park, H.O., Salas-Pino, S., Heichinger, C., and Nurse, P. (2013). A genome-wide resource of cell cycle and cell shape genes of fission yeast. Open Biol 3, 130053.

Heckman, D.S., Geiser, D.M., Eidell, B.R., Stauffer, R.L., Kardos, N.L., and Hedges, S.B. (2001). Molecular evidence for the early colonization of land by fungi and plants. Science 293, 1129-1133.

Hedges, S.B. (2002). The origin and evolution of model organisms. Nat Rev Genet 3, 838-849.

Hedstrom, L. (2009). IMP dehydrogenase: structure, mechanism, and inhibition. Chem Rev 109, 2903-2928.

Higgins, M.J., Graves, P.R., and Graves, L.M. (2007). Regulation of human cytidine triphosphate synthetase 1 by glycogen synthase kinase 3. J Biol Chem 282, 29493-29503.

Higgins, M.J., Loiselle, D., Haystead, T.A., and Graves, L.M. (2008). Human cytidine triphosphate synthetase 1 interacting proteins. Nucleosides Nucleotides Nucleic Acids 27, 850-857.

Hirose, M., Tohda, H., Giga-Hama, Y., Tsushima, R., Zako, T., Iizuka, R., Pack, C., Kinjo, M., Ishii, N., and Yohda, M. (2005). Interaction of a small heat shock protein of the fission yeast, Schizosaccharomyces pombe, with a denatured protein at elevated temperature. J Biol Chem 280, 32586-32593.

Hofer, A., Steverding, D., Chabes, A., Brun, R., and Thelander, L. (2001). Trypanosoma brucei CTP synthetase: a target for the treatment of African sleeping sickness. Proc Natl Acad Sci U S A 98, 6412-6416.

Hoffman, C.S., Wood, V., and Fantes, P.A. (2015). An Ancient Yeast for Young Geneticists: A Primer on the Schizosaccharomyces pombe Model System. Genetics 201, 403-423.

Hohmann, S. (2002). Osmotic stress signaling and osmoadaptation in yeasts. Microbiol Mol Biol Rev 66, 300-372.

Holbrook, N.J., and Ikeyama, S. (2002). Age-related decline in cellular response to oxidative stress: links to growth factor signaling pathways with common defects. Biochem Pharmacol 64, 999-1005.

Homma, T., Iwahashi, H., and Komatsu, Y. (2003). Yeast gene expression during growth at low temperature. Cryobiology 46, 230-237.

Huberman, J.A. (1999). DNA damage and replication checkpoints in the fission yeast, Schizosaccharomyces pombe. Prog Nucleic Acid Res Mol Biol 62, 369-395.

Humphrey, T. (2000). DNA damage and cell cycle control in Schizosaccharomyces pombe. Mutat Res 451, 211-226.

Ikner, A., and Shiozaki, K. (2005). Yeast signaling pathways in the oxidative stress response. Mutat Res 569, 13-27.

Ingerson-Mahar, M., Briegel, A., Werner, J.N., Jensen, G.J., and Gitai, Z. (2010). The metabolic enzyme CTP synthase forms cytoskeletal filaments. Nat Cell Biol 12, 739-746.

Ingerson-Mahar, M., and Gitai, Z. (2012). A growing family: the expanding universe of the bacterial cytoskeleton. FEMS Microbiol Rev 36, 256-266.

Iyengar, A., and Bearne, S.L. (2003). Aspartate-107 and leucine-109 facilitate efficient coupling of glutamine hydrolysis to CTP synthesis by Escherichia coli CTP synthase. Biochem J 369, 497-507.

Jakopec, V., Walla, E., and Fleig, U. (2011). Versatile use of Schizosaccharomyces pombe plasmids in Saccharomyces cerevisiae. FEMS Yeast Res 11, 653-655.

Jarolim, S., Ayer, A., Pillay, B., Gee, A.C., Phrakaysone, A., Perrone, G.G., Breitenbach, M., and Dawes, I.W. (2013). Saccharomyces cerevisiae genes involved in survival of heat shock. G3 (Bethesda) 3, 2321-2333.

Jaspersen, S.L., and Winey, M. (2004). The budding yeast spindle pole body: structure, duplication, and function. Annu Rev Cell Dev Biol 20, 1-28.

Jekely, G. (2014). Origin and evolution of the self-organizing cytoskeleton in the network of eukaryotic organelles. Cold Spring Harb Perspect Biol 6, a016030.

Jordheim, L.P., Durantel, D., Zoulim, F., and Dumontet, C. (2013). Advances in the development of nucleoside and nucleotide analogues for cancer and viral diseases. Nat Rev Drug Discov 12, 447-464.

Jorgensen, C.M., Hammer, K., and Martinussen, J. (2003). CTP limitation increases expression of CTP synthase in Lactococcus lactis. J Bacteriol 185, 6562-6574.

Juanes, M.A., Twyman, H., Tunnacliffe, E., Guo, Z., ten Hoopen, R., and Segal,

M. (2013). Spindle pole body history intrinsically links pole identity with asymmetric fate in budding yeast. Curr Biol 23, 1310-1319.

Juda, P., Smigova, J., Kovacik, L., Bartova, E., and Raska, I. (2014). Ultrastructure of cytoplasmic and nuclear inosine-5'-monophosphate dehydrogenase 2 "rods and rings" inclusions. J Histochem Cytochem 62, 739-750.

Kabeche, R., Baldissard, S., Hammond, J., Howard, L., and Moseley, J.B. (2011). The filament-forming protein Pil1 assembles linear eisosomes in fission yeast. Mol Biol Cell 22, 4059-4067.

Kang, G.J., Cooney, D.A., Moyer, J.D., Kelley, J.A., Kim, H.Y., Marquez, V.E., and Johns, D.G. (1989). Cyclopentenylcytosine triphosphate. Formation and inhibition of CTP synthetase. J Biol Chem 264, 713-718.

Kassel, K.M., Au da, R., Higgins, M.J., Hines, M., and Graves, L.M. (2010). Regulation of human cytidine triphosphate synthetase 2 by phosphorylation. J Biol Chem 285, 33727-33736.

Kaul, S.C., Obuchi, K., and Komatsu, Y. (1992). Cold shock response of yeast cells: induction of a 33 kDa protein and protection against freezing injury. Cell Mol Biol (Noisy-le-grand) 38, 553-559.

Kensler, T.W., and Cooney, D.A. (1981). Chemotherapeutic inhibitors of the enzymes of the de novo pyrimidine pathway. Adv Pharmacol Chemother 18, 273-352.

Kent, C., Carman, G.M., Spence, M.W., and Dowhan, W. (1991). Regulation of eukaryotic phospholipid metabolism. FASEB J 5, 2258-2266.

Keppeke, G.D., Calise, S.J., Chan, E.K., and Andrade, L.E. (2015). Assembly of IMPDH2-based, CTPS-based, and mixed rod/ring structures is dependent on cell type and conditions of induction. J Genet Genomics 42, 287-299.

Kim, C.W., Moon, Y.A., Park, S.W., Cheng, D., Kwon, H.J., and Horton, J.D. (2010). Induced polymerization of mammalian acetyl-CoA carboxylase by MIG12 provides a tertiary level of regulation of fatty acid synthesis. Proc Natl Acad Sci U S A 107, 9626-9631.

Kim, D.U., Hayles, J., Kim, D., Wood, V., Park, H.O., Won, M., Yoo, H.S., Duhig, T., Nam, M., Palmer, G., et al. (2010). Analysis of a genome-wide set of gene deletions in the fission yeast Schizosaccharomyces pombe. Nat Biotechnol 28, 617-623.

Kim, S.Y., Kim, Y.W., Hegerl, R., Cyrklaff, M., and Kim, I.S. (2005). Novel type of enzyme multimerization enhances substrate affinity of oat beta-glucosidase. J Struct Biol 150, 1-10.

Kirbach, B.B., and Golenhofen, N. (2011). Differential expression and induction of small heat shock proteins in rat brain and cultured hippocampal neurons. J Neurosci Res 89, 162-175.

Kitchin, J.E., Pomeranz, M.K., Pak, G., Washenik, K., and Shupack, J.L. (1997). Rediscovering mycophenolic acid: a review of its mechanism, side effects, and potential uses. J Am Acad Dermatol 37, 445-449.

Kizaki, H., Ohsaka, F., and Sakurada, T. (1982). Role of GTP in CTP synthetase from Ehrlich ascites tumor cells. Biochem Biophys Res Commun 108, 286-291.

Kizaki, H., Williams, J.C., Morris, H.P., and Weber, G. (1980). Increased cytidine 5'-triphosphate synthetase activity in rat and human tumors. Cancer Res 40, 3921-3927.

Klar, A.J. (1987). Differentiated parental DNA strands confer developmental asymmetry on daughter cells in fission yeast. Nature 326, 466-470.

Kleinschmidt, A.K., Moss, J., and Lane, D.M. (1969). Acetyl coenzyme A carboxylase: filamentous nature of the animal enzymes. Science 166, 1276-1278.

Knop, M., Pereira, G., and Schiebel, E. (1999). Microtubule organization by the budding yeast spindle pole body. Biol Cell 91, 291-304.

Knutsen, J.H., Rein, I.D., Rothe, C., Stokke, T., Grallert, B., and Boye, E. (2011). Cell-cycle analysis of fission yeast cells by flow cytometry. PLoS One 6, e17175.

Kondo, K., Kowalski, L.R., and Inouye, M. (1992). Cold shock induction of yeast NSR1 protein and its role in pre-rRNA processing. J Biol Chem 267, 16259-16265.

Kouranti, I., McLean, J.R., Feoktistova, A., Liang, P., Johnson, A.E., Roberts-Galbraith, R.H., and Gould, K.L. (2010). A global census of fission yeast deubiquitinating enzyme localization and interaction networks reveals distinct compartmentalization profiles and overlapping functions in endocytosis and polarity. PLoS Biol 8.

Kramhoft, B., and Zeuthen, E. (1971). Synchronization of cell divisions in the fission yeast, Schizosaccharomyces pombe, using heat shocks. C R Trav Lab Carlsberg 38, 351-368.

Kramhoft, B., and Zeuthen, E. (1975). Synchronization of the fission yeast Schizosaccharomyces pombe using heat shocks. Methods Cell Biol 12, 373-380.

Kucuk, Z.Y., Zhang, K., Filipovich, L., and Bleesing, J.J. (2016). CTP Synthase 1 Deficiency in Successfully Transplanted Siblings with Combined Immune Deficiency and Chronic Active EBV Infection. J Clin Immunol.

Kursula, P., Flodin, S., Ehn, M., Hammarstrom, M., Schuler, H., Nordlund, P., and Stenmark, P. (2006). Structure of the synthetase domain of human CTP synthetase, a target for anticancer therapy. Acta Crystallogr Sect F Struct Biol Cryst Commun 62, 613-617.

Lauritsen, I., Willemoes, M., Jensen, K.F., Johansson, E., and Harris, P. (2011). Structure of the dimeric form of CTP synthase from Sulfolobus solfataricus. Acta Crystallogr Sect F Struct Biol Cryst Commun 67, 201-208.

Lebedeva, Z.I., Kabanova, E.A., and Berezov, T.T. (1986). 6-diazo-5-oxo-L-norleucine and azaserine as affinity inhibitors of glutamin(asparagin)ase. Biochem Int 12, 413-420.

Lee, J., Kim, J.H., Biter, A.B., Sielaff, B., Lee, S., and Tsai, F.T. (2013). Heat shock protein (Hsp) 70 is an activator of the Hsp104 motor. Proc Natl Acad Sci U S A 110, 8513-8518.

Lehninger, A.L., Nelson, D.L., and Cox, M.M. (2013). Lehninger principles of biochemistry, 6th edn (New York: W.H. Freeman).

Li, H., Huang, Y., Wang, P.Y., Ye, F., and Liu, J.L. (2016). Data on dynamic study of cytoophidium in Saccharomyces cerevisiae. Data Brief 8, 40-44.

Li, S., Deng, Z., Fu, J., Xu, C., Xin, G., Wu, Z., Luo, J., Wang, G., Zhang, S., Zhang, B., et al. (2015). Spatial Compartmentalization Specializes the Function of Aurora A and Aurora B. J Biol Chem 290, 17546-17558.

Li, W., Li, X., Huang, T., Teng, Q., Crnovcic, I., Rader, C., and Shen, B. (2016). Engineered production of cancer targeting peptide (CTP)-containing C-1027 in Streptomyces globisporus and biological evaluation. Bioorg Med Chem 24, 3887-3892.

Li, X.W., Feng, Z.G., Yang, H.M., Zhu, X.P., Liu, J., and Yuan, H.Y. (2010). A novel cold-regulated gene from Camellia sinensis, CsCOR1, enhances salt- and dehydration-tolerance in tobacco. Biochem Biophys Res Commun 394, 354-359.

Li, Z., and Srivastava, P. (2004). Heat-shock proteins. Curr Protoc Immunol Appendix 1, Appendix 1T.

Lim, C.J., Cho, Y.W., Hong, S.M., Lim, H.W., and Park, E.H. (2003). The thioltransferase (glutaredoxin) 1 gene of fission yeast is regulated by *Atf1* and

Pap1. Mol Cells 16, 123-127.

Liu, J.L. (2010). Intracellular compartmentalisation of CTP synthase in Drosophila. J Genet Genomics 37, 281-296.

Liu, J.L. (2011). The enigmatic cytoophidium: compartmentalisation of CTP synthase via filament formation. Bioessays 33, 159-164.

Liu, J.L. (2016). The Cytoophidium and Its Kind: Filamentation and Compartmentalisation of Metabolic Enzymes. Annu Rev Cell Dev Biol.

Liu, S., Wang, X., Fan, Z., Pang, Y., Sun, X., Wang, X., and Tanga, K. (2004). Molecular cloning and characterization of a novel cold-regulated gene from Capsella bursa-pastoris. DNA Seq 15, 262-268.

Liu, Y.C., Li, F., Handler, J., Huang, C.R., Xiang, Y., Neretti, N., Sedivy, J.M., Zeller, K.I., and Dang, C.V. (2008). Global regulation of nucleotide biosynthetic genes by c-Myc. PLoS One 3, e2722.

Loewith, R., and Hall, M.N. (2011). Target of rapamycin (TOR) in nutrient signaling and growth control. Genetics 189, 1177-1201.

Long, C.W., and Pardee, A.B. (1967). Cytidine triphosphate synthetase of Escherichia coli B. I. Purification and kinetics. J Biol Chem 242, 4715-4721.

Lunn, F.A., MacDonnell, J.E., and Bearne, S.L. (2008). Structural requirements for the activation of Escherichia coli CTP synthase by the allosteric effector GTP are stringent, but requirements for inhibition are lax. J Biol Chem 283, 2010-2020.

Lunn, F.A., Macleod, T.J., and Bearne, S.L. (2008). Mutational analysis of conserved glycine residues 142, 143 and 146 reveals Gly(142) is critical for tetramerization of CTP synthase from Escherichia coli. Biochem J 412, 113-121.

Macara, I.G., and Mili, S. (2008). Polarity and differential inheritance--universal attributes of life? Cell 135, 801-812.

MacDonnell, J.E., Lunn, F.A., and Bearne, S.L. (2004). Inhibition of E. coli CTP synthase by the "positive" allosteric effector GTP. Biochim Biophys Acta 1699, 213-220.

MacLeod, T.J., Lunn, F.A., and Bearne, S.L. (2006). The role of lysine residues 297 and 306 in nucleoside triphosphate regulation of E. coli CTP synthase: inactivation by 2',3'-dialdehyde ATP and mutational analyses. Biochim Biophys Acta 1764, 199-210.

Madrid, M., Soto, T., Franco, A., Paredes, V., Vicente, J., Hidalgo, E., Gacto, M., and Cansado, J. (2004). A cooperative role for *Atf1* and Pap1 in the detoxification of the oxidative stress induced by glucose deprivation in Schizosaccharomyces pombe. J Biol Chem 279, 41594-41602.

Mahony, T.J., and Miller, D.J. (1998). Linkage of genes encoding enolase (eno) and CTP synthase (pyrG) in the beta-subdivision proteobacterium Nitrosomonas europaea. FEMS Microbiol Lett 165, 153-157.

Marguerat, S., Schmidt, A., Codlin, S., Chen, W., Aebersold, R., and Bahler, J. (2012). Quantitative analysis of fission yeast transcriptomes and proteomes in proliferating and quiescent cells. Cell 151, 671-683.

Marianayagam, N.J., Sunde, M., and Matthews, J.M. (2004). The power of two: protein dimerization in biology. Trends Biochem Sci 29, 618-625.

Martin, E., Palmic, N., Sanquer, S., Lenoir, C., Hauck, F., Mongellaz, C., Fabrega, S., Nitschke, P., Esposti, M.D., Schwartzentruber, J., et al. (2014). CTP synthase 1 deficiency in humans reveals its central role in lymphocyte proliferation. Nature 510, 288-292.

Matsuyama, A., Arai, R., Yashiroda, Y., Shirai, A., Kamata, A., Sekido, S., Kobayashi, Y., Hashimoto, A., Hamamoto, M., Hiraoka, Y., et al. (2006). ORFeome cloning and global analysis of protein localization in the fission yeast Schizosaccharomyces pombe. Nat Biotechnol 24, 841-847.

May, J.W., and Mitchison, J.M. (1995). Pattern of polar extension of the cell wall in the fission yeast Schizosaccharomyces pombe. Can J Microbiol 41, 273-277.

Mc Cluskey, G.D., Mohamady, S., Taylor, S.D., and Bearne, S.L. (2016). Exploring the Potent Inhibition of CTP Synthase by Gemcitabine-5'-triphosphate. Chembiochem.

McLaren, J.D., and Chu, E.H. (1984). The kinetics and feedback inhibition of cytidine 5'-triphosphate synthetase in wild-type and mutant Chinese hamster cells. Biochem Genet 22, 717-727.

Meng, Q., Turnbough, C.L., Jr., and Switzer, R.L. (2004). Attenuation control of pyrG expression in Bacillus subtilis is mediated by CTP-sensitive reiterative transcription. Proc Natl Acad Sci U S A 101, 10943-10948.

Meuth, M. (1981). Sensitivity of a mutator gene in Chinese hamster ovary cell to deoxynucleoside triphosphate pool alterations. Mol Cell Biol 1, 652-660.

Meuth, M., L'Heureux-Huard, N., and Trudel, M. (1979). Characterization of a

mutator gene in Chinese hamster ovary cells. Proc Natl Acad Sci U S A 76, 6505-6509.

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

Mitchison, J.M. (1958). The growth of single cells. II. Saccharomyces cerevisiae. Exp Cell Res 15, 214-221.

Mitchison, J.M., and Nurse, P. (1985). Growth in cell length in the fission yeast Schizosaccharomyces pombe. J Cell Sci 75, 357-376.

Moller-Jensen, J., and Lowe, J. (2005). Increasing complexity of the bacterial cytoskeleton. Curr Opin Cell Biol 17, 75-81.

Mostowy, S., and Cossart, P. (2012). Septins: the fourth component of the cytoskeleton. Nat Rev Mol Cell Biol 13, 183-194.

Nakagawa, C.W., Yamada, K., and Mutoh, N. (1998). Two distinct upstream regions are involved in expression of the catalase gene in Schizosaccharomyces pombe in response to oxidative stress. J Biochem 123, 1048-1054.

Nakagawa, C.W., Yamada, K., and Mutoh, N. (2000). Role of *Atf1* and Pap1 in the induction of the catalase gene of fission yeast schizosaccharomyces pombe. J Biochem 127, 233-238.

Narayanaswamy, R., Levy, M., Tsechansky, M., Stovall, G.M., O'Connell, J.D., Mirrielees, J., Ellington, A.D., and Marcotte, E.M. (2009). Widespread reorganization of metabolic enzymes into reversible assemblies upon nutrient starvation. Proc Natl Acad Sci U S A 106, 10147-10152.

Noree, C., Monfort, E., Shiau, A.K., and Wilhelm, J.E. (2014). Common regulatory control of CTP synthase enzyme activity and filament formation. Mol Biol Cell 25, 2282-2290.

Noree, C., Sato, B.K., Broyer, R.M., and Wilhelm, J.E. (2010). Identification of novel filament-forming proteins in Saccharomyces cerevisiae and Drosophila melanogaster. J Cell Biol 190, 541-551.

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

Oberti, D., Biasini, A., Kirschmann, M.A., Genoud, C., Stunnenberg, R., Shimada, Y., and Buhler, M. (2015). Dicer and Hsp104 function in a negative feedback loop to confer robustness to environmental stress. Cell Rep 10, 47-61.

Oburoglu, L., Tardito, S., Fritz, V., de Barros, S.C., Merida, P., Craveiro, M., Mamede, J., Cretenet, G., Mongellaz, C., An, X., et al. (2014). Glucose and glutamine metabolism regulate human hematopoietic stem cell lineage specification. Cell Stem Cell 15, 169-184.

O'Connell, J.D., Zhao, A., Ellington, A.D., and Marcotte, E.M. (2012). Dynamic Reorganization of Metabolic Enzymes into Intracellular Bodies. Annu Rev Cell Dev Bi 28, 89-111.

Ortlund, E., Lacount, M.W., Lewinski, K., and Lebioda, L. (2000). Reactions of Pseudomonas 7A glutaminase-asparaginase with diazo analogues of glutamine and asparagine result in unexpected covalent inhibitions and suggests an unusual catalytic triad Thr-Tyr-Glu. Biochemistry 39, 1199-1204.

Ostrander, D.B., O'Brien, D.J., Gorman, J.A., and Carman, G.M. (1998). Effect of CTP synthetase regulation by CTP on phospholipid synthesis in Saccharomyces cerevisiae. J Biol Chem 273, 18992-19001.

Ovadi, J., and Saks, V. (2004). On the origin of intracellular compartmentalisation and organized metabolic systems. Mol Cell Biochem 256-257, 5-12.

Ovejera, A.A., Houchens, D.P., Catane, R., Sheridan, M.A., and Muggia, F.M. (1979). Efficacy of 6-diazo-5-oxo-L-norleucine and N-[N-gamma-glutamyl-6-diazo-5-oxo-norleucinyl]-6-diazo-5-oxo-norleucine against experimental tumors in conventional and nude mice. Cancer Res 39, 3220-3224.

Pai, L.M., Wang, P.Y., Lin, W.C., Chakraborty, A., Yeh, C.T., and Lin, Y.H. (2016). Ubiquitination and filamentous structure of cytidine triphosphate synthase. Fly 10, 108-114.

Palabiyik, B., Kig, C., Pekmez, M., Dalyan, L., Arda, N., and Temizkan, G. (2012). Investigation of the relationship between oxidative stress and glucose signaling in Schizosaccharomyces pombe. Biochem Genet 50, 336-349.

Papadakis, M.A., and Workman, C.T. (2015). Oxidative stress response pathways: Fission yeast as archetype. Crit Rev Microbiol 41, 520-535.

Pappas, A., Yang, W.L., Park, T.S., and Carman, G.M. (1998). Nucleotide-dependent tetramerization of CTP synthetase from Saccharomyces cerevisiae. J Biol Chem 273, 15954-15960.

Park, T.S., O'Brien, D.J., and Carman, G.M. (2003). Phosphorylation of CTP synthetase on Ser36, Ser330, Ser354, and Ser454 regulates the levels of CTP and phosphatidylcholine synthesis in Saccharomyces cerevisiae. J Biol Chem 278,

20785-20794.

Park, T.S., Ostrander, D.B., Pappas, A., and Carman, G.M. (1999). Identification of Ser424 as the protein kinase A phosphorylation site in CTP synthetase from Saccharomyces cerevisiae. Biochemistry 38, 8839-8848.

Pearson, G., Robinson, F., Beers Gibson, T., Xu, B.E., Karandikar, M., Berman, K., and Cobb, M.H. (2001). Mitogen-activated protein (MAP) kinase pathways: regulation and physiological functions. Endocr Rev 22, 153-183.

Pemberton, L.F. (2014). Preparation of yeast cells for live-cell imaging and indirect immunofluorescence. Methods Mol Biol 1205, 79-90.

Perez, P., and Cansado, J. (2010). Cell integrity signaling and response to stress in fission yeast. Curr Protein Pept Sci 11, 680-692.

Peters, L.Z., Hazan, R., Breker, M., Schuldiner, M., and Ben-Aroya, S. (2013). Formation and dissociation of proteasome storage granules are regulated by cytosolic pH. J Cell Biol 201, 663-671.

Petrovska, I., Nuske, E., Munder, M.C., Kulasegaran, G., Malinovska, L., Kroschwald, S., Richter, D., Fahmy, K., Gibson, K., Verbavatz, J.M., et al. (2014). Filament formation by metabolic enzymes is a specific adaptation to an advanced state of cellular starvation. Elife.

Pinkus, L.M. (1977). Glutamine binding sites. Methods Enzymol 46, 414-427.

Raboy, B., Sharon, G., Parag, H.A., Shochat, Y., and Kulka, R.G. (1991). Effect of stress on protein degradation: role of the ubiquitin system. Acta Biol Hung 42, 3-20.

Ransom, J.T. (1995). Mechanism of action of mycophenolate mofetil. Ther Drug Monit 17, 681-684.

Rines, D.R., Thomann, D., Dorn, J.F., Goodwin, P., and Sorger, P.K. (2011). Live cell imaging of yeast. Cold Spring Harb Protoc 2011.

Robertson, J.G. (1995). Determination of subunit dissociation constants in native and inactivated CTP synthetase by sedimentation equilibrium. Biochemistry 34, 7533-7541.

Rose, A.H., Harrison, J.S., and Wheals, A.E. (1987). The yeasts, 2nd edn (London: Academic Press).

Rout, M.P., and Kilmartin, J.V. (1990). Components of the yeast spindle and spindle pole body. J Cell Biol 111, 1913-1927.

Rout, M.P., and Kilmartin, J.V. (1991). Yeast spindle pole body components. Cold Spring Harb Symp Quant Biol 56, 687-692.

Roy, A.C., Lunn, F.A., and Bearne, S.L. (2010). Inhibition of CTP synthase from Escherichia coli by xanthines and uric acids. Bioorg Med Chem Lett 20, 141-144.

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

Sabatinos, S.A., Mastro, T.L., Green, M.D., and Forsburg, S.L. (2013). A mammalian-like DNA damage response of fission yeast to nucleoside analogs. Genetics 193, 143-157.

Sadatmousavi, P., Soltani, M., Nazarian, R., Jafari, M., and Chen, P. (2011). Self-assembling peptides: potential role in tumor targeting. Curr Pharm Biotechnol 12, 1089-1100.

Sajiki, K., Hatanaka, M., Nakamura, T., Takeda, K., Shimanuki, M., Yoshida, T., Hanyu, Y., Hayashi, T., Nakaseko, Y., and Yanagida, M. (2009). Genetic control of cellular quiescence in S. pombe. J Cell Sci 122, 1418-1429.

Sajiki, K., Pluskal, T., Shimanuki, M., and Yanagida, M. (2013). Metabolomic analysis of fission yeast at the onset of nitrogen starvation. Metabolites 3, 1118-1129.

Sakono, M., Utsumi, A., Zako, T., Abe, T., Yohda, M., and Maeda, M. (2013). Formation of non-toxic Abeta fibrils by small heat shock protein under heat-stress conditions. Biochem Biophys Res Commun 430, 1259-1264.

Sanso, M., Vargas-Perez, I., Garcia, P., Ayte, J., and Hidalgo, E. (2011). Nuclear roles and regulation of chromatin structure by the stress-dependent MAP kinase Sty1 of Schizosaccharomyces pombe. Mol Microbiol 82, 542-554.

Senechal, P., Arseneault, G., Leroux, A., Lindquist, S., and Rokeach, L.A. (2009). The Schizosaccharomyces pombe Hsp104 disaggregase is unable to propagate the [PSI] prion. PLoS One 4, e6939.

Shen, Q.J., Kassim, H., Huang, Y., Li, H., Zhang, J., Li, G., Wang, P.Y., Yan, J., Ye, F., and Liu, J.L. (2016). Filamentation of Metabolic Enzymes in Saccharomyces cerevisiae. J Genet Genomics 43, 393-404.

Shiber, A., Breuer, W., Brandeis, M., and Ravid, T. (2013). Ubiquitin conjugation triggers misfolded protein sequestration into quality control foci when Hsp70 chaperone levels are limiting. Mol Biol Cell 24, 2076-2087.

Shiozaki, K., and Russell, P. (1996). Conjugation, meiosis, and the osmotic stress response are regulated by Spc1 kinase through *Atf1* transcription factor in fission yeast. Genes Dev 10, 2276-2288.

Siam, R., Dolan, W.P., and Forsburg, S.L. (2004). Choosing and using Schizosaccharomyces pombe plasmids. Methods 33, 189-198.

Simard, D., Hewitt, K.A., Lunn, F., Iyengar, A., and Bearne, S.L. (2003). Limited proteolysis of Escherichia coli cytidine 5'-triphosphate synthase. Identification of residues required for CTP formation and GTP-dependent activation of glutamine hydrolysis. Eur J Biochem 270, 2195-2206.

Sintchak, M.D., Fleming, M.A., Futer, O., Raybuck, S.A., Chambers, S.P., Caron, P.R., Murcko, M.A., and Wilson, K.P. (1996). Structure and mechanism of inosine monophosphate dehydrogenase in complex with the immunosuppressant mycophenolic acid. Cell 85, 921-930.

Soto, T., Beltran, F.F., Paredes, V., Madrid, M., Millar, J.B., Vicente-Soler, J., Cansado, J., and Gacto, M. (2002). Cold induces stress-activated protein kinase-mediated response in the fission yeast Schizosaccharomyces pombe. Eur J Biochem 269, 5056-5065.

Souba, W.W. (1993). Glutamine and cancer. Ann Surg 218, 715-728.

Steeves, C.H., and Bearne, S.L. (2011). Activation and inhibition of CTP synthase from Trypanosoma brucei, the causative agent of African sleeping sickness. Bioorg Med Chem Lett 21, 5188-5190.

Streiblova, E., and Girbardt, M. (1980). Microfilaments and cytoplasmic microtubules in cell division cycle mutants of Schizosaccharomyces pombe. Can J Microbiol 26, 250-254.

Strochlic, T.I., Stavrides, K.P., Thomas, S.V., Nicolas, E., O'Reilly, A.M., and Peterson, J.R. (2014). Ack kinase regulates CTP synthase filaments during Drosophila oogenesis. EMBO Rep 15, 1184-1191.

Su, S.S., Tanaka, Y., Samejima, I., Tanaka, K., and Yanagida, M. (1996). A nitrogen starvation-induced dormant G0 state in fission yeast: the establishment from uncommitted G1 state and its delay for return to proliferation. J Cell Sci 109 (Pt 6), 1347-1357.

Sun, Y., and MacRae, T.H. (2005). The small heat shock proteins and their role in human disease. FEBS J 272, 2613-2627.

Suslu, K.G., Palabiyik, B., and Temizkan, G. (2011). Genes involved in glucose

repression and oxidative stress response in the fission yeast Schizosaccharomyces pombe. Genet Mol Res 10, 4041-4047.

Tan, Z., Chang, X., Puga, A., and Xia, Y. (2002). Activation of mitogen-activated protein kinases (MAPKs) by aromatic hydrocarbons: role in the regulation of aryl hydrocarbon receptor (AHR) function. Biochem Pharmacol 64, 771-780.

Tang, R., Cui, X.B., Wang, J.N., and Chen, S.Y. (2013). CTP synthase 1, a smooth muscle-sensitive therapeutic target for effective vascular repair. Arterioscler Thromb Vasc Biol 33, 2336-2344.

Taricani, L., Feilotter, H.E., Weaver, C., and Young, P.G. (2001). Expression of hsp16 in response to nucleotide depletion is regulated via the spc1 MAPK pathway in Schizosaccharomyces pombe. Nucleic Acids Res 29, 3030-3040.

Tastan, O.Y., and Liu, J.L. (2015). CTP Synthase Is Required for Optic Lobe Homeostasis in Drosophila. J Genet Genomics 42, 261-274.

Tastan, O.Y., and Liu, J.L. (2015). Visualizing Cytoophidium Expression in Drosophila Follicle Cells via Immunohistochemistry. Methods Mol Biol 1328, 179-189.

Tormos-Perez, M., Perez-Hidalgo, L., and Moreno, S. (2016). Fission Yeast Cell Cycle Synchronization Methods. Methods Mol Biol 1369, 293-308.

Tronchoni, J., Medina, V., Guillamon, J.M., Querol, A., and Perez-Torrado, R. (2014). Transcriptomics of cryophilic Saccharomyces kudriavzevii reveals the key role of gene translation efficiency in cold stress adaptations. BMC Genomics 15, 432.

Truck, J., Kelly, D.F., Taylor, J.M., Kienzler, A.K., Lester, T., Seller, A., Pollard, A.J., and Patel, S.Y. (2016). Variable phenotype and discrete alterations of immune phenotypes in CTP synthase 1 deficiency: Report of 2 siblings. J Allergy Clin Immunol.

Tsubaki, M. (1993). Fourier-transform infrared study of azide binding to the Fea3-CuB binuclear site of bovine heart cytochrome c oxidase: new evidence for a redox-linked conformational change at the binuclear site. Biochemistry 32, 174-182.

van den Berg, A.A., van Lenthe, H., Kipp, J.B., de Korte, D., van Kuilenburg, A.B., and van Gennip, A.H. (1995). Cytidine triphosphate (CTP) synthetase activity during cell cycle progression in normal and malignant T-lymphocytic cells. Eur J Cancer 31A, 108-112.

van den Ent, F., Amos, L.A., and Lowe, J. (2001). Prokaryotic origin of the actin cytoskeleton. Nature 413, 39-44.

Verschuur, A.C., Van Gennip, A.H., Leen, R., Meinsma, R., Voute, P.A., and van Kuilenburg, A.B. (2000). In vitro inhibition of cytidine triphosphate synthetase activity by cyclopentenyl cytosine in paediatric acute lymphocytic leukaemia. Br J Haematol 110, 161-169.

Verschuur, A.C., Van Gennip, A.H., Leen, R., Muller, E.J., Elzinga, L., Voute, P.A., and Van Kuilenburg, A.B. (2000). Cyclopentenyl cytosine inhibits cytidine triphosphate synthetase in paediatric acute non-lymphocytic leukaemia: a promising target for chemotherapy. Eur J Cancer 36, 627-635.

Vivancos, A.P., Jara, M., Zuin, A., Sanso, M., and Hidalgo, E. (2006). Oxidative stress in Schizosaccharomyces pombe: different H2O2 levels, different response pathways. Mol Genet Genomics 276, 495-502.

Vivekananda, J., Smith, D., and King, R.J. (2001). Sphingomyelin metabolites inhibit sphingomyelin synthase and CTP:phosphocholine cytidylyltransferase. Am J Physiol Lung Cell Mol Physiol 281, L98-L107.

Wadskov-Hansen, S.L., Willemoes, M., Martinussen, J., Hammer, K., Neuhard, J., and Larsen, S. (2001). Cloning and verification of the Lactococcus lactis pyrG gene and characterization of the gene product, CTP synthase. J Biol Chem 276, 38002-38009.

Wang, P.Y., Lin, W.C., Tsai, Y.C., Cheng, M.L., Lin, Y.H., Tseng, S.H., Chakraborty, A., and Pai, L.M. (2015). Regulation of CTP Synthase Filament Formation During DNA Endoreplication in Drosophila. Genetics 201, 1511-1523.

Wang, X., Martindale, J.L., Liu, Y., and Holbrook, N.J. (1998). The cellular response to oxidative stress: influences of mitogen-activated protein kinase signalling pathways on cell survival. Biochem J 333 (Pt 2), 291-300.

Wang, Y., Gibney, P.A., West, J.D., and Morano, K.A. (2012). The yeast Hsp70 Ssa1 is a sensor for activation of the heat shock response by thiol-reactive compounds. Mol Biol Cell 23, 3290-3298.

Weisman, R. (2010). Fission Yeast TOR and Rapamycin. In The Enzymes: Structure, Function and Regulation of TOR Complexes from Yeasts to Mammals, M. Hall, and F. Tamanoi, eds. (Elsevier Academic Press, Burlington), pp. 251-269.

Weng, M.L., and Zalkin, H. (1987). Structural role for a conserved region in the

CTP synthetase glutamine amide transfer domain. J Bacteriol 169, 3023-3028.

Whelan, J., Phear, G., Yamauchi, M., and Meuth, M. (1993). Clustered base substitutions in CTP synthetase conferring drug resistance in Chinese hamster ovary cells. Nat Genet 3, 317-322.

Whelan, J., Smith, T., Phear, G., Rohatiner, A., Lister, A., and Meuth, M. (1994). Resistance to cytosine arabinoside in acute leukemia: the significance of mutations in CTP synthetase. Leukemia 8, 264-265.

Wickstead, B., and Gull, K. (2011). The evolution of the cytoskeleton. J Cell Biol 194, 513-525.

Willemoes, M. (2004). Competition between ammonia derived from internal glutamine hydrolysis and hydroxylamine present in the solution for incorporation into UTP as catalysed by Lactococcus lactis CTP synthase. Arch Biochem Biophys 424, 105-111.

Willemoes, M., Molgaard, A., Johansson, E., and Martinussen, J. (2005). Lid L11 of the glutamine amidotransferase domain of CTP synthase mediates allosteric GTP activation of glutaminase activity. FEBS J 272, 856-864.

Willemoes, M., and Sigurskjold, B.W. (2002). Steady-state kinetics of the glutaminase reaction of CTP synthase from Lactococcus lactis. The role of the allosteric activator GTP incoupling between glutamine hydrolysis and CTP synthesis. Eur J Biochem 269, 4772-4779.

Williams, J.C., Kizaki, H., Weber, G., and Morris, H.P. (1978). Increased CTP synthetase activity in cancer cells. Nature 271, 71-73.

Willingham, M.C., Richert, N.D., and Rutherford, A.V. (1987). A novel fibrillar structure in cultured cells detected by a monoclonal antibody. Exp Cell Res 171, 284-295.

Winey, M., and Byers, B. (1993). Assembly and functions of the spindle pole body in budding yeast. Trends Genet 9, 300-304.

Wise, D.R., and Thompson, C.B. (2010). Glutamine addiction: a new therapeutic target in cancer. Trends Biochem Sci 35, 427-433.

Wood, V., Gwilliam, R., Rajandream, M.A., Lyne, M., Lyne, R., Stewart, A., Sgouros, J., Peat, N., Hayles, J., Baker, S., et al. (2002). The genome sequence of Schizosaccharomyces pombe. Nature 415, 871-880.

Wu, C. (1995). Heat shock transcription factors: structure and regulation. Annu Rev Cell Dev Biol 11, 441-469.

Wu, C.H., Sahoo, D., Arvanitis, C., Bradon, N., Dill, D.L., and Felsher, D.W. (2008). Combined analysis of murine and human microarrays and ChIP analysis reveals genes associated with the ability of MYC to maintain tumorigenesis. PLoS Genet 4, e1000090.

Wu, H., Ng, B.S., and Thibault, G. (2014). Endoplasmic reticulum stress response in yeast and humans. Biosci Rep 34.

Yamauchi, M., Yamauchi, N., Phear, G., Spurr, N.K., Martinsson, T., Weith, A., and Meuth, M. (1991). Genomic organization and chromosomal localization of the human CTP synthetase gene (CTPS). Genomics 11, 1088-1096.

Yang, W.L., Bruno, M.E., and Carman, G.M. (1996). Regulation of yeast CTP synthetase activity by protein kinase C. J Biol Chem 271, 11113-11119.

Yang, W.L., and Carman, G.M. (1995). Phosphorylation of CTP synthetase from Saccharomyces cerevisiae by protein kinase C. J Biol Chem 270, 14983-14988.

Yang, W.L., and Carman, G.M. (1996). Phosphorylation and regulation of CTP synthetase from Saccharomyces cerevisiae by protein kinase A. J Biol Chem 271, 28777-28783.

Yoshida, J., and Tani, T. (2005). Hsp16p is required for thermotolerance in nuclear mRNA export in fission yeast Schizosaccharomyces pombe. Cell Struct Funct 29, 125-138.

Yoshida, T., Nasu, H., Namba, E., Ubukata, O., and Yamashita, M. (2012). Discovery of a compound that acts as a bacterial PyrG (CTP synthase) inhibitor. J Med Microbiol 61, 1280-1285.

Zhang, J., Hulme, L., and Liu, J.L. (2014). Asymmetric inheritance of cytoophidium in Schizosaccharomyces pombe. Biol Open 3, 1092-1097.

Zigler, J.S., Jr., and Rao, P.V. (1991). Enzyme/crystallins and extremely high pyridine nucleotide levels in the eye lens. FASEB J 5, 223-225.

Zuin, A., Castellano-Esteve, D., Ayte, J., and Hidalgo, E. (2010). Living on the edge: stress and activation of stress responses promote lifespan extension. Aging (Albany NY) 2, 231-237.

Appendix 1: Oligo library

number	name	sequence
001	*XhoI cts1* rv	tttctcgag ACTGATGGTGACGACAGTGGCT
002	*BamHI*-YFP rv	tttggatcc GTCGACCTTGTACAG
003	*ApaI-cts1*-upstream fw	tttgggccc CCGTAAATAGTCTGGACTTAGGGGA
004	1-18 del fw	ATTGCCTCTTCCACTGGATTACTG
005	1-18 del rv	agtggaagaggcaat CATATTGGCTGTCCCCTGATG
006	19-37 del fw	AAGATTGACCCTTACATGAATATTGATGC
007	19-37 del rv	gtaagggtcaatctt TACACCTTTTCCAATACCACTAATAACAC
008	38-52 del fw	CTTGAACATGGGGAAGTTTTTGTTTTG
009	38-52 del rv	ttccccatgttcaag GATGCTAGTAACTTTCAATCCTAGGG
010	53-79 del fw	GTTACATTAACCCATGATAACAATATTACAACTGG
011	53-79 del rv	atgggttaatgtaac GGGAGACATAGTGCCAGCAT
012	80-129 del fw	CGAATTCCAGTTGATCAAAGTGGTG
013	80-129 del rv	atcaactggaattcg ATTAAGATAGCGCTCATAGTTTCCAAG
014	53-79 del fw	GTTACATTAACCCATGATAACAATATTACAACTGG
015	Vector Primer XL39 (reverse seq primer)	5′ ATTAGGACAAGGCTGGTGGG 3′
016	Vector Primer v1.5 (forward seq primer)	5′ GGACTTTCCAAAATGTCG 3'
017	Vector Primer XL39(reverse seq primer)	5′ ATTAGGACAAGGCTGGTGGG 3′
018	Vector Primer v1.5 (forward seq primer)	5′ GGACTTTCCAAAATGTCG 3'
019	*cts1* upstream *BamHI* rv	tttggatcc GGCTGTCCCCTGATGTTTGC
020	*cts1* downstream *NcoI* fw	tttccatgg TGGGATTCCGGGAATTAGGA
021	*cts1* downstream *ApaI* rv	tttgggccc TTTGCAAGTATGCTTGTACACTTGAC
022	(sequencing) *cts1* up fw	CAAACATCAGGGGACAGCCAAT

023	1-56 del fw	GAAGTTTTTGTTTTGAATGATGGCGGT
024	1-56 del rv	caaaacaaaaacttc CATATTGGCTGTCCCCTGATG
025	1-56 insert fw	AAGTACGTTTTAGTATCTGGAGGTGTTA
026	1-56 insert rv	tactaaaacgtactt CCCATGTTCAAGGGGAGACATAG
027	5′ CFP rv	GCGGTGATATAGACGTTGTGGCTGA
028	3' ATG CYFP rv	ACAGCTCCTCGCCCTTGCTCACCAT
029	5′ *nmt1* fw	TCACTTTCTGACTTATAGTCGC
030	CFP fw	GGCCCACCCTCGTGACCACCCTGAC
031	*ApaI gua1 fw*	tttgggccc CGCCGAGGGAATCAGTTTGGA
032	*XhoI gua1 rv*	tttctcgag GTA AAG ACG CTT TTCGTA TGT AGC AAA AC
033	*ApaI cts1 fw*	tttgggccc CTGTCATGTTGGTCCCGAACAG
034	*PacI-cts1 fw*	tttttaattaa ATGAAGTACGTTTTAGTATCTGGAGGTG
035	Vector Primer v1.5 (forward seq primer)	5′ GGACTTTCCAAAATGTCG 3'
036	Vector Primer XL39 (reverse seq primer)	5′ ATTAGGACAAGGCTGGTGGG 3′
037	v1.5 fw seq	5′ ACGGGACTTTCCAAAATGTCGTAA 3'
038	(sequencing)pSMUY fw	CGAGTCAGTGAGCGAGGAAG
039	(sequencing)pSMUY rv	ACGACGTTGTAAAACGACGGC
040	GATase_del rv	AAATTCATCCAACAAGCCAGCACT AGTGTATTTTCCTACGAGGACGATGG
041	GATase_del fw	AGTGCTGGCTTGTTGGATGAA
042	CTP_synth_del fw	GCTCTCGCAGGCGAGAATATG
043	CTP_synth_del rv	TCCACATATTCTCGCCTGCGAGAGC CATATTGGCTGTCCCCTGATGTTT
044	1-18 del rv	GCAGTAATCCAGTGGAAGAGGCAAT CATATTGGCTGTCCCCTGATG
045	19-37 del rv	CAATATTCATGTAAGGGTCAATCTT TACACCTTTTCCAATACCACTAATAACAC

046	38-52 del rv	AAACAAAAACTTCCCCATGTTCAAG GATGCTAGTAACTTTCAATCCTAGGG
047	53-79 del rv	GTAATATTGTTATCATGGGTTAATGTAAC GGGAGACATAGTGCCAGCAT
048	80-129 del rv	CCACTTTGATCAACTGGAATTCG ATTAAGATAGCGCTCATAGTTTCCAAG
049	1-56 del rv	CGCCATCATTCAAAACAAAAACTTC CATATTGGCTGTCCCCTGATG
050	1-56 insert rv	CACCTCCAGATACTAAAACGTACTT CCCATGTTCAAGGGGAGACATAG
051	*cts1* upstream *XhoI rv*	tttctcgag GGCTGTCCCCTGATGTTTGC
052	*kanMX6_rv*	5'-CCGCGATTAAATTCCAACATGGATG-3'
053	*kanMX6_fw*	5'-TTAGAAAAACTCATCGAGCATCAAATGAAACTG-3'
054	*AmpR_fw*	5'-TTCAAATATGTATCCGCTCATGAGACAAT-3'
055	*AmpR_rv*	5'-TTACCAATGCTTAATCAGTGAGGCAC-3'
056	*leu1_fw*	5'-ATGTGTGCAAAGAAAATCGTCGTCT-3'
057	*leu1_rv*	5'-TTCAAGTTCTTCAACAACAGCCTTAGT-3'
058	CTP_synth_del rv	5'-TCCACATATTCTCGCCTGCGAGAGCCATGGGCCCGGTACCAG-3'
059	*ura4_rv*	GCT GAG AAA GTC TTT GCT GAT ATG CC
060	*ura4_fw*	GGA TGC TAG AGT ATT TCA AAG CTA TTC AGC
061	3HA_rv	ttttt GCACTGAGCAGCGTAATCTGG
062	3HA_fw	ttttt AACATCTTTTACCCATACGATGTTCCTG
063	GFP_fw	ttttt AAAGGAGAAGAACTTTTCACTGGAGTTG
064	GFP_rv	ttttt TGTATAGTTCATCCATGCCATGTGTAATC
065	*cts1*_up_rv	ATTGGCTGTCCCCTGATGTTTG
066	*cts1* 5'_fw	GATGAACGTGGTGAGCGTATGG
067	YFP 5'_fw	TCGAGGGCGACACCCTG

068	*cts1* (intro) _fw	GTGCATTTTCTGAAGAATTCGATAAGGAATG
069	*cts1* (intro)_rv	TTATCGAATTCTTCAGAAAATGCAC CTTCAATTCCACAAACACTTCTCGC
070	130-146 del_fw	CTCGGTGGTACGGTTGGC
071	147-175 del_fw	GTATCAATCCATGTTTCTCTTGTCCCTG
072	176-216 del_fw	AAACAACCTCTTGAGAAATCTGTGATTG
073	217-246 del_fw	AGTACATATCACGTTCCTCAACTGCT
074	247-267 del_fw	GCACTGGATAAGATAAGTGTATCTCGC
075	268-286 del_fw	AGCTCGTGGAAACATTTAACACAGG
076	287-297 del_fw	CATCTTTTCAAGAAGGTCACCATCGTC
077	298-318 del_fw	ATATCAGTCATCAAAGCATTGGAGCAC
078	319-347 del_fw	GCTAGTACAAATACTAGTGACCCACTTAG
079	CTP_synth_del rv	TCCACATATTCTCGCCTGCGAGAGC CATATTGGCTGTCCCCTGATGTTT
080	130-146 del_rv	CGATATCGCCAACCGTACCACCGAG AGCAACACGCTCCACCC
081	147-175 del_rv	GGACAAGAGAAACATGGATTGATAC TTCAACAATACAAACATCTGGTTCTTCACC
082	176-216 del_rv	TCACAGATTTCTCAAGAGGTTGTTT AAAGTTTTCATGACCCACACGAAATTG
083	217-246 del_rv	GCAGTTGAGGAACGTGATATGTACT ACAGCGGCAAGCAATCAAGTC
084	247-267 del_rv	GAGATACACTTATCTTATCCAGTGC AGAGACATCATGCACAGCTAGCA
085	268-286 del_rv	CCTGTGTTAAATGTTTCCACGAGCT GAAACGAATTTTCAAATATTCCAATAGCTTGTC
086	287-297 del_rv	CGATGGTGACCTTCTTGAAAAGATG CCACATATTCTCGCCTGCGAG

087	298-318 del_rv	GCTCCAATGCTTTGATGACTGATAT ATCGTATCCCTGTGTTAAATGTTTCCAC
088	319-347 del_rv	GTGGGTCACTAGTATTTGTACTAGC TATAGGAATCTTGAAGATGAGTGTATTTTCCTAC
089	(#085) 268-286 del_rv	CCTGTGTTAAATGTTTCCACGAGCT GAAACGAATTTTCAAATATTCCAATAGCTTGTC
090	*cts1* C403S _fw	CCCTACTTGGGCATTAGCCTTGGTATGCAAG
091	*cts1* C403S _rv	CTTGCATACCAAGGCTAATGCCCAAGTAGGG
092	*cts1*_C403S_QC_fw	CACCCTACTTGGGCATTAGCCTTGGTATGCAAGTTG
093	*cts1*_C403S_QC_rv	CAACTTGCATACCAAGGCTAATGCCCAAGTAGGGTG
094	*cts1*_C403G_QC_fw	CACCCTACTTGGGCATTGGCCTTGGTATGCAAGTTG
095	*cts1*_C403G_QC_rv	CAACTTGCATACCAAGGCCAATGCCCAAGTAGGGTG
096	*cts1*_C403 del_QC_fw	CACCCTACTTGGGCATTCTTGGTATGCAAGTTG
097	*cts1*_C403 del_QC_rv	CAACTTGCATACCAAGAATGCCCAAGTAGGGTG
098	K2A_QC_fw	*
099	K2A_QC_rv	*
100	G8A_QC_fw	*
101	G8A_QC_rv	*
102	G9A_QC_fw	*
103	G9A_QC_rv	*
104	V10A_QC_fw	*
105	V10A_QC_rv	*
106	S12A_QC_fw	*
107	S12A_QC_rv	*
108	G15A_QC_fw	*
109	G15A_QC_rv	*

110	K16A_QC_fw	*
111	K16A_QC_rv	*
112	G17A_QC_fw	*
113	G17A_QC_rv	*
114	S22A_QC_fw	*
115	S22A_QC_rv	*
116	*nmt1* 3' fw	AGACAGAATAAGTCATCAGCGGTT
117	*nmt1* 5' rv	GCGACTATAAGTCAGAAAGTGAGAATGAG
118	YFP *BamHI* rv	tttggatcc GTCGACCTTGTACAG
119	*cts1* down *NdeI* fw	tttcatatg TGGGATTCCGGGAATTAGGA
120	(sequencing) pFA6a-kanMX6 fw	GTCGGGGCTGGCTTAACTATG
121	(sequencing) pFA6a-kanMX6 rv	GGGGACGAGGCAAGCTAAAC
122	*cts1*_down_IF_fw	TGAGAGTGCACCATA TGTGGGATTCCGGGAATTAGGAAA
123	*cts1*_down_IF_rv	TTACGGGGGCCCTTT GCAAGTATGCTTGTACACTTGACAT
124	*cts1*_up_IF_fw	AAAGGGCCC CCGTAAATAGTCTGGACTTAGGGGA
125	YFP_IF_rv	ATTAACCCGGGGATC CGTCGACCTTGTACAGCTCGT
126	*cts1*_S22A_QC_fw	GGTGTAATTGCCTCTGCCACTGGATTACTGC
127	*cts1*_S22A_QC_rv	GCAGTAATCCAGTGGCAGAGGCAATTACACC
128	*cts1*_L27A_QC_fw	GCCTCTTCCACTGGATTACTGGCAAAAACCCTAGGATTGAAAG
129	*cts1*_L27A_QC_rv	CTTTCAATCCTAGGGTTTTTGCCAGTAATCCAGTGGAAGAGGC
130	*cts1*_G31A_QC_fw	GGATTACTGCTAAAAACCCTAGCATTGAAAGTTACTAGCATCAAG
131	*cts1*_G31A_QC_rv	CTTGATGCTAGTAACTTTCAATGCTAGGGTTTTTAGCAGTAATCC
132	*cts1*_V34A_QC_fw	CTAAAAACCCTAGGATTGAAAGCTACTAGCATCAAGATTGACCC
133	*cts1*_V34A_QC_rv	GGGTCAATCTTGATGCTAGTAGCTTTCAATCCTAGGGTTTTTAG
134	*cts1*_C403A_QC_fw	CACCCTACTTGGGCATTGCCCTTGGTATGCAAGTTGC
135	*cts1*_C403A_QC_rv	GCAACTTGCATACCAAGGGCAATGCCCAAGTAGGGTG

136	T459A_QC_fw	CGTTTGGGATTGAGACCTGCCTTTTTCCAACCCAATT
137	T459A_QC_fw	GAATTGGGTTGGAAAAAGGCAGGTCTCAATCCCAAACG
138	S573A_QC_fw	GAAGTCGAATGGAGCAACTTTGCTCATTTTAACGCTGAATCTG
139	S573A_QC_rv	CAGATTCAGCGTTAAAATGAGCAAAGTTGCTCCATTCGACTTC
140	S468A_QC_fw	CAACCCAATTCCGAATGGGCTAAACTTCGTAAACTTC
141	S468A_QC_rv	GAAGTTTACGAAGTTTAGCCCATTCGGAATTGGGTTG

*Oligos sequences from 98 to 115 are not recorded in this library because the primers are not successful synthetized.

Appendix 2 yeast plasmids library

number	name
001	pSMUY2+*cts1*
002	pSMUC2+*cts1*
003	pSMRC2+*gua1*
004	pFA6a-kanMX-*p3nmt1-cts1*-YFP
005	pCMV6-AC-GFP-CTPS
006	pCMV6-AC-GFP-CTPS
007	pCMV6-AC-GFP-CTPSII (variant 1)
008	pCMV6-AC-GFP-CTPSII (variant 1)
009	pSMUY2+*cts1*(C-Terminal)
010	pSMUC2+*cts1*(C-Terminal)
011	pSMUC2+*cts1*(C-Terminal)
012	pSMRC2+gua1(C-Terminal)
013	pSMRC2+gua1(C-Terminal)
014	pFA6a-kanMX6-*3nmt1-cts1*-YFP
015	pFA6a-kanMX6-*3nmt1-cts1*-YFP
016	pCMV6-AC-GFP
017	pFA6a-kanMX6(AmpicilinR) -*NcoI-cts1*downstream-*ApaI-cts1* upstream-*BamHI* (*cts1* whole gene deletion)
018	pFA6a-kanMX6(AmpicilinR) -*NcoI-cts1*downstream-*ApaI-cts1* upstream-*BamHI* (*cts1* whole gene deletion)
019	pSMUY2+ *cts1* upstream-*cts1*
020	pSMUY2+ *ApaI-cts1* upstream-*cts1* GATase del-YFP-*BamHI*
021	pSMUY2+ *ApaI-cts1* upstream-*cts1* GATase del-YFP-*BamHI*
022	pSMUY2+ *ApaI-cts1* upstream-*cts1* GATase del-YFP-*BamHI*

023	pSMUY2+ *ApaI-cts1* upstream-*cts1* GATase del-YFP-*BamHI*
024	pSMRG2+
025	pSMRG2+
026	pSMRG2+
027	pSMRG2+
028	pFA6a-kanMX6(AmpicilinR) -*NcoI-cts1*downstream-*ApaI-cts1* upstream-*BamHI*-YFP (*cts1* whole gene deletion)
029	pFA6a-kanMX6(AmpicilinR) -*NcoI-cts1*downstream-*ApaI-cts1* upstream-*BamHI*-YFP (*cts1* whole gene deletion)
030	pFA6a-kanMX6(AmpicilinR) -*NcoI-cts1*downstream-*ApaI-cts1* upstream-*BamHI*-YFP (*cts1* whole gene deletion)
031	pSMUY2+ *ApaI-cts1* upstream-*cts1*(2-18aa del)-YFP-*BamHI*
032	pSMUY2+ *ApaI-cts1* upstream-*cts1*(19-37aa del)-YFP-*BamHI*
033	pSMUY2+ *ApaI-cts1* upstream-*cts1*(38-52aa del)-YFP-*BamHI*
034	pSMUY2+ *ApaI-cts1* upstream-*cts1*(53-79aa del)-YFP-*BamHI*
035	pSMUY2+ *ApaI-cts1* upstream-*cts1*(80-129aa del)-YFP-*BamHI*
036	pSMUY2+ *ApaI-cts1* upstream-*cts1*(2-56aa del)-YFP-*BamHI*
037	pSMUY2+ *ApaI-cts1* upstream-*cts1* CTPsynth del-YFP-*BamHI*
038	pFA6a-kanMX6-*cts1* downstream-*cts1* upstream-*cts1*-YFP
039	pFA6a-kanMX6-*cts1* downstream-*cts1* upstream-*cts1* 2-56 aa del-YFP
040	pFA6a-kanMX6
041	pFA6a-GFP(S65T)-kanMX6
042	pFA6a-3HA-kanMX6
043	pjk148 (*leu1*)
044	pSMUC2+
045	pSMUY2+
046	pSMRY2+
047	pSMUG-
048	pFA6a-kanMX6-*p41nmt*-3HA

049	pFA6a-kanMX6-*p41nmt*-GFP
050	pSMUG2
051	pFA6a-kanMX6(AmpicilinR)-*cts1*downstream-*cts1* upstream-*cts1*-YFP
052	pFA6a-kanMX6(AmpicilinR)-*cts1*downstream-*cts1* upstream-*cts1*(19-37 aa del)-YFP
053	pFA6a-kanMX6(AmpicilinR)-*cts1*downstream-*cts1* upstream-*cts1*(53-79 aa del)-YFP
054	pFA6a-kanMX6(AmpicilinR)-*cts1*downstream-*cts1* upstream-*cts1*(80-129 aa del)-YFP
055	pFA6A-VC155-KanMX6
056	pFA6A-VN173-kanMX6
057	REP 3X
058	REP 41X
059	PER 81X
060	pFA6a-kanMX6(AmpicilinR)-*cts1*downstream-*cts1* upstream-*cts1*(S22A)-YFP
061	pFA6a-kanMX6(AmpicilinR)-*cts1*downstream-*cts1* upstream-*cts1*(L27A)-YFP
062	pFA6a-kanMX6(AmpicilinR)-*cts1*downstream-*cts1* upstream-*cts1*(L27A)-YFP
063	pFA6a-kanMX6(AmpicilinR)-*cts1*downstream-*cts1* upstream-*cts1*(G31A)-YFP
064	pFA6a-kanMX6(AmpicilinR)-*cts1*downstream-*cts1* upstream-*cts1*(V34A)-YFP
065	pFA6a-kanMX6(AmpicilinR)-*cts1*downstream-*cts1* upstream-*cts1*(C403A)-YFP
066	pFA6a-kanMX6(AmpicilinR)-*cts1*downstream-*cts1* upstream-*cts1*(C403S)-YFP
067	pFA6a-kanMX6(AmpicilinR)-*cts1*downstream-*cts1* upstream-*cts1*(C403G)-YFP
068	pFA6a-kanMX6(AmpicilinR)-*cts1*downstream-*cts1* upstream-*cts1*(C403del)-YFP
069	pFA6a-kanMX6(AmpicilinR)-*cts1*downstream-*cts1* upstream-*cts1*(E161A)-YFP
070	pFA6a-kanMX6(AmpicilinR)-*cts1*downstream-*cts1* upstream-*cts1*(S468A)-YFP
071	pFA6a-kanMX6(AmpicilinR)-*cts1*downstream-*cts1* upstream-*cts1*(T459A)-YFP
072	pFA6a-kanMX6(AmpicilinR)-*cts1*downstream-*cts1* upstream-*cts1*(S573A)-YFP
073	pjk148
074	Pjk210

075	pFA6a-GFP(S65T)-kanMX6
076	pFA6a-3HA-kanMX6
077	pFA6a-kanMX6-*p3nmt1*-3HA
078	pFA6a-kanMX6-*p3nmt1*-GFP
079	pFA6a-kanMX6-*p41nmt1*-3FLAG

*The plasmids of 024-027, 040-050, 055-059 and 073-079 are kindly gifted by Professor Stephen Kearsey.

Full sequence of plasmid pSMUY2+:

```
   1 gcacttttcg gggaaatgtg cgcggaaccc ctatttgttt atttttctaa atacattcaa
  61 atatgtatcc gctcatgaga caataaccct gataaatgct tcaataatat tgaaaaagga
 121 agagtatgag tattcaacat ttccgtgtcg cccttattcc ctttttgcg gcattttgcc
 181 ttcctgtttt tgctcaccca gaaacgctgg tgaaagtaaa agatgctgaa gatcagttgg
 241 gtgcacgagt gggttacatc gaactggatc tcaacagcgg taagatcctt gagagtttc
 301 gccccgaaga acgtttcca atgatgagca cttttaaagt tctgctatgt ggcgcggtat
 361 tatcccgtat tgacgccggg caagagcaac tcggtcgccg catacactat tctcagaatg
 421 acttggttga gtactcacca gtcacagaaa agcatcttac ggatggcatg acagtaagag
 481 aattatgcag tgctgccata accatgagtg ataacactgc ggccaactta cttctgacaa
 541 cgatcggagg accgaaggag ctaaccgctt ttttgcacaa catgggggat catgtaactc
 601 gccttgatcg ttgggaaccg gagctgaatg aagccatacc aaacgacgag cgtgacacca
 661 cgatgcctgt agcaatggca acaacgttgc gcaaactatt aactggcgaa ctacttactc
 721 tagcttcccg gcaacaatta atagactgga tggaggcgga taaagttgca ggaccacttc
 781 tgcgctcggc ccttccggct ggctggttta ttgctgataa atctggagcc ggtgagcgtg
 841 ggtctcgcgg tatcattgca gcactggggc cagatggtaa gccctcccgt atcgtagtta
 901 tctacacgac ggggagtcag gcaactatgg atgaacgaaa tagacagatc gctgagatag
 961 gtgcctcact gattaagcat tggtaactgt cagaccaagt ttactcatat atactttaga
1021 ttgatttaaa acttcatttt taatttaaaa ggatctaggt gaagatcctt tttgataatc
1081 tcatgaccaa aatcccttaa cgtgagtttt cgttccactg agcgtcagac cccgtagaaa
```

1141 agatcaaagg atcttcttga gatccttttt ttctgcgcgt aatctgctgc ttgcaaacaa
1201 aaaaaccacc gctaccagcg gtggtttgtt tgccggatca agagctacca actcttttc
1261 cgaaggtaac tggcttcagc agagcgcaga taccaaatac tgtccttcta gtgtagccgt
1321 agttaggcca ccacttcaag aactctgtag caccgcctac atacctcgct ctgctaatcc
1381 tgttaccagt ggctgctgcc agtggcgata agtcgtgtct taccgggttg gactcaagac
1441 gatagttacc ggataaggcg cagcggtcgg gctgaacggg gggttcgtgc acacagccca
1501 gcttggagcg aacgacctac accgaactga gatacctaca gcgtgagcta tgagaaagcg
1561 ccacgcttcc cgaagggaga aaggcggaca ggtatccggt aagcggcagg gtcggaacag
1621 gagagcgcac gagggagctt ccagggggaa acgcctggta tctttatagt cctgtcgggt
1681 ttcgccacct ctgacttgag cgtcgatttt tgtgatgctc gtcaggggggg cggagcctat
1741 ggaaaaacgc cagcaacgcg gccttttac ggttcctggc cttttgctgg ccttttgctc
1801 acatgttctt tcctgcgtta tcccctgatt ctgtggataa ccgtattacc gcctttgagt
1861 gagctgatac cgctcgccgc agccgaacga ccgagcgcag cgagtcagtg agcgaggaag
1921 cggaagagcg cccaatacgc aaaccgcctc tccccgcgcg ttggccgatt cattaatgca
1981 gctggcacga caggtttccc gactggaaag cgggcagtga gcgcaacgca attaatgtga
2041 gttagctcac tcattaggca ccccaggctt tacactttat gcttccggct cgtatgttgt
2101 gtggaattgt gagcggataa caatttcaca caggaaacag ctatgaccat gattacgcca
2161 agctcgaaat taaccctcac taaagggaac aaaagctggt accgggccct tatgcatggt
2221 ctcgagggta gatctggtgc ccggggtggt gctggtgccg gagccggtgc tggtgctgaa
2281 gctttaatgg tgagcaaggg cgaggagctg ttcaccgggg tggtgcccat cctggtcgag
2341 ctggacggcg acgtaaacgg ccacaagttc agcgtgtccg gcgagggcga gggcgatgcc
2401 acctacggca agctgaccct gaagttcatc tgcaccaccg gcaagctgcc cgtgccctgg
2461 cccaccctcg tgaccacctt cggctacggc ctgcagtgct tcgcccgcta ccccgaccac
2521 atgaagcagc acgacttctt caagtccgcc atgcccgaag gctacgtcca ggagcgcacc
2581 atcttcttca aggacgacgg caactacaag acccgcgccg aggtgaagtt cgagggcgac
2641 accctggtga accgcatcga gctgaagggc atcgacttca aggaggacgg caacatcctg
2701 gggcacaagc tggagtacaa ctacaacagc cacaacgtct atatcatggc cgacaagcag

2761 aagaacggca tcaaggtgaa cttcaagatc cgccacaaca tcgaggacgg cagcgtgcag
2821 ctcgccgacc actaccagca gaacaccccc atcggcgacg gccccgtgct gctgcccgac
2881 aaccactacc tgagctacca gtccgccctg agcaaagacc ccaacgagaa gcgcgatcac
2941 atggtcctgc tggagttcgt gaccgccgcc gggatcactc tcggcatgga cgagctgtac
3001 aaggtcgacg gatcctgagc tccaattcgc cctatagtga gtcgtattac aattcactgg
3061 ccgtcgtttt acaacgtcgt gactgggaaa accctggcgt tacccaactt aatcgccttg
3121 cagcacatcc ccctttcgcc agctggcgta atagcgaaga ggcccgcacc gatcgccctt
3181 cccaacagtt gcgcagcctg aatggcgaat gggacgcgcc ctgtagcggc gcattaagcg
3241 cggcgggtgt ggtggttacg cgcagcgtga ccgctacact tgccagcgcc ctagcgcccg
3301 ctcctttcgc tttcttccct tcctttctcg ccacgttcgc cggcttagct acaaatccca
3361 ctggctatat gtatgcattt gtgttaaaaa agtttgtata gattatttaa tctactcagc
3421 attctttctc taaataggaa tttgttactt aatggagaaa aaaatgtttc gatttaccta
3481 gtgtatttgt ttgtatactc acgtttaatt tcaaacatcc attctatctt gtgtaatttt
3541 tggcatggtg aaaaagataa tcagccttat aatctttaca aaagtaagaa attctgtaaa
3601 taagccttaa tgcccttgct ttaaattaaa atggttcttt ttcatgataa tgtttgcact
3661 ttgtgaatat attttagata gttctgtgag gtataattaa gatgttttag agacttatac
3721 aattttgtct ttataaattc ttaattgatt ttaccatccc agtttaacta tgcttcgtcg
3781 gcatctctgc acatgtcgtg ttttcttacc gtattgtcct accaagaacc tctttttgc
3841 ttggatcgaa attaaaggtt taaaagcaaa gttatggatg ctagagtatt tcaaagctat
3901 tcagctagag ctgaggggat gaaaaatccc attgccaagg aattgttggc tttgatggaa
3961 gaaaagcaaa gcaacttgtc agtcgcggtc gatttgacga agaaatccga aatcttagaa
4021 ttggtagata aaattggacc ctatgtctgt gttatcaaga cacatattga cgttgtcgag
4081 gatttcgacc aggatatggt agaaaaactg gtggccttag gtaaaaagca tcgttttctt
4141 atctttgagg atcgcaaatt cgcagacatt ggaaataccg tcaagctaca atatgcatct
4201 ggtgtgtaca aaattgcttc ttgggctcat atcacaaatt gccatacagt gccaggcgag
4261 ggtattatac aaggcctcaa agaagttggt ttacctttgg gacgtggtct cttgcttttg
4321 gctgaaatgt cttccaaagg ctctttggct actggttcct acacagagaa aaccttagaa

4381 tggtttgaga agcataccga tttttgcttt ggctttatag ctggtcgtcg atttcctaac
4441 cttcaaagcg actacataac tatgtcccct ggtatcggct tggatgttaa aggagacggg
4501 ctgggacagc aatatcgtac tcctgaagaa gtgattgtaa actgcggtag cgatatcatc
4561 attgttggtc gtggagtcta tggagctggt cgtaatcctg ttgtcgaagc caagagatat
4621 agagaagctg gttggaaggc atatcagcaa agactttctc agcattaaaa aaagactaat
4681 gtaaaatttt tttggttggt tattgaaaaa gtcgatgcct tgtttgcgtt tgttttccta
4741 ggcgttttat gtcagaaggc atttagaatt agtatacaag tactctttgg taaaatttta
4801 tgtagcgact aaaatattaa ctattataga taaacacctt gggaataaaa agtaatttgc
4861 tatagtaatt tattaaacat gctcctacaa cattaccaca atcttttctc ttggattgac
4921 attgaataag aaaagagtga atttttttag acttgtaatg ataactatgt acaaagccaa
4981 tgaaagatgt atgtagatga atgtaaaata ccatgtagac aaacaagata aaacttggtt
5041 ataaacattg gtgttggaac agaataaatt agatgtcaaa aagtttcgtc aatatcacaa
5101 gccggctttc cccgtcaagc tctaaatcgg gggctccctt tagggttccg atttagtgct
5161 ttacggcacc tcgaccccaa aaaacttgat tagggtgatg gttcacgtag tgggccatcg
5221 ccctgataga cggtttttcg ccctttgacg ttggagtcca cgttctttaa tagtggactc
5281 ttgttccaaa ctggaacaac actcaaccct atctcggtct attcttttga tttataaggg
5341 attttgccga tttcggccta ttggttaaaa aatgagctga tttaacaaaa atttaacgcg
5401 aattttaaca aaatattaac gcttacaatt taggtg

Full sequence of plasmid pSMUY2-*cts1*:

1 cttttcgggg aaatgtgcgc ggaacccta tttgtttatt tttctaaata cattcaaata
61 tgtatccgct catgagacaa taaccctgat aaatgcttca ataatattga aaaaggaaga
121 gtatgagtat tcaacatttc cgtgtcgccc ttattccctt ttttgcggca ttttgccttc
181 ctgtttttgc tcacccagaa acgctggtga aagtaaaaga tgctgaagat cagttgggtg
241 cacgagtggg ttacatcgaa ctggatctca acagcggtaa gatccttgag agttttcgcc
301 ccgaagaacg ttttccaatg atgagcactt ttaaagttct gctatgtggc gcggtattat

361 cccgtattga cgccgggcaa gagcaactcg gtcgccgcat acactattct cagaatgact
421 tggttgagta ctcaccagtc acagaaaagc atcttacgga tggcatgaca gtaagagaat
481 tatgcagtgc tgccataacc atgagtgata acactgcggc caacttactt ctgacaacga
541 tcggaggacc gaaggagcta accgcttttt tgcacaacat gggggatcat gtaactcgcc
601 ttgatcgttg ggaaccggag ctgaatgaag ccataccaaa cgacgagcgt gacaccacga
661 tgcctgtagc aatggcaaca acgttgcgca aactattaac tggcgaacta cttactctag
721 cttcccggca acaattaata gactggatgg aggcggataa agttgcagga ccacttctgc
781 gctcggccct tccggctggc tggtttattg ctgataaatc tggagccggt gagcgtgggt
841 ctcgcggtat cattgcagca ctggggccag atggtaagcc ctcccgtatc gtagttatct
901 acacgacggg gagtcaggca actatggatg aacgaaatag acagatcgct gagataggtg
961 cctcactgat taagcattgg taactgtcag accaagttta ctcatatata ctttagattg
1021 atttaaaact tcatttttaa tttaaaagga tctaggtgaa gatccttttt gataatctca
1081 tgaccaaaat cccttaacgt gagttttcgt tccactgagc gtcagacccc gtagaaaaga
1141 tcaaaggatc ttcttgagat cctttttttc tgcgcgtaat ctgctgcttg caaacaaaaa
1201 aaccaccgct accagcggtg gtttgtttgc cggatcaaga gctaccaact ctttttccga
1261 aggtaactgg cttcagcaga gcgcagatac caaatactgt ccttctagtg tagccgtagt
1321 taggccacca cttcaagaac tctgtagcac cgcctacata cctcgctctg ctaatcctgt
1381 taccagtggc tgctgccagt ggcgataagt cgtgtcttac cgggttggac tcaagacgat
1441 agttaccgga taaggcgcag cggtcgggct gaacgggggg ttcgtgcaca cagcccagct
1501 tggagcgaac gacctacacc gaactgagat acctacagcg tgagctatga gaaagcgcca
1561 cgcttcccga agggagaaag gcggacaggt atccggtaag cggcagggtc ggaacaggag
1621 agcgcacgag ggagcttcca gggggaaacg cctggtatct ttatagtcct gtcgggtttc
1681 gccacctctg acttgagcgt cgatttttgt gatgctcgtc agggggggcgg agcctatgga
1741 aaaacgccag caacgcggcc tttttacggt tcctggcctt ttgctggcct tttgctcaca
1801 tgttctttcc tgcgttatcc cctgattctg tggataaccg tattaccgcc tttgagtgag
1861 ctgataccgc tcgccgcagc cgaacgaccg agcgcagcga gtcagtgagc gaggaagcgg
1921 aagagcgccc aatacgcaaa ccgcctctcc ccgcgcgttg gccgattcat taatgcagct

1981 ggcacgacag gtttcccgac tggaaagcgg gcagtgagcg caacgcaatt aatgtgagtt
2041 agctcactca ttaggcaccc caggctttac actttatgct tccggctcgt atgttgtgtg
2101 gaattgtgag cggataacaa tttcacacag gaaacagcta tgaccatgat tacgccaagc
2161 tcgaaattaa ccctcactaa agggaacaaa agctggtacc gggcccctgt catgttggtc
2221 ccgaacaggt gctagctgtg catgatgtct ctagtacata tcacgttcct caactgcttg
2281 aagacaagct attggaatat ttgaaaattc gtttcgcact ggataagata agtgtatctc
2341 gcgaactcgc tctcgcaggc gagaatatgt ggagctcgtg gaaacattta acacagggat
2401 acgatcatct tttcaagaag gtcaccatcg tcctcgtagg aaaatacact catcttcaag
2461 attcctatat atcagtcatc aaagcattgg agcactctgc tatgcgctgt ggtcgtaaac
2521 ttgacctcca atgggtagaa gcttctcatt tggaagctag tacaaatact agtgacccac
2581 ttagttacca taaggcatgg catttagtct gttctgccaa tggtatccta gtgcctggcg
2641 gattcggctc tcgcggtgtc gaagggatga ttgccgcagc caagtgggct cgtgaaaata
2701 atacacccta cttgggcatt tgccttggta tgcaagttgc cgttattgaa ttcgcgagaa
2761 gtgtttgtgg aattgaaggt atgtttttg atgcttttat tggactccat tttaaatttt
2821 taactgcctt ctattatttt tgggatctat agattccatg cacttactag aggtttttgt
2881 cattttccgt tgactctaac tgtagacaag tgctacatga atagttaaac agttattatt
2941 aattggagca tatttttat tttgcttctt tcaaaagatt tttaactaac atttctaggt
3001 gcattttctg aagaattcga taaggaatgc gaaaacaatg ttgttgttta tatgcctgag
3061 attgataagg ataaactggg tggaactatg cgtttgggat tgagacctac ctttttccaa
3121 cccaattccg aatggagtaa acttcgtaaa cttcataaga tggttgatga agttttggaa
3181 agacatcgtc atagatatga aataaatcct gcgtttgtaa gccgtttgga acaaggtgga
3241 atttctttca tcggaaaaga tgaacgtggt gagcgtatgg aaattattga gaaacgtgac
3301 catccttact ttgttggcgt acaataccat cctgaatatt tatctaagcc acttaagccc
3361 tccccaccga tttttggatt ggttgcggcc agtgctggct tgttggatga atttattcag
3421 tctggtgagg aagtcgaatg gagcaacttt tctcatttta acgctgaatc tgccttagct
3481 gacatgaatg actctgttga agttactgaa gaagccactg tcgtcaccat cagtctcgag
3541 ggtagatctg gtgcccgggg tggtgctggt gccggagccg gtgctggtgc tgaagcttta

3601 atggtgagca agggcgagga gctgttcacc ggggtggtgc ccatcctggt cgagctggac
3661 ggcgacgtaa acggccacag gttcagcgtg tccggcgagg gcgagggcga tgccacctac
3721 ggcaagctga ccctgaagtt catctgcacc accggcaagc tgcccgtgcc ctggcccacc
3781 ctcgtgacca ccctgacctg gggcgtgcag tgcttcagcc gctaccccga ccacatgaag
3841 cagcacgact tcttcaagtc cgccatgccc gaaggctacg tccaggagcg taccatcttc
3901 ttcaaggacg acggcaacta caagacccgc gccgaggtga agttcgaggg cgacaccctg
3961 gtgaaccgca tcgagctgaa gggcatcgac ttcaaggagg acggcaacat cctggggcac
4021 aagctggagt acaactacat cagccacaac gtctatatca ccgccgacaa gcagaagaac
4081 ggcatcaagg cccacttcaa gatccgccac aacatcgagg acggcagcgt gcagctcgcc
4141 gaccactacc agcagaacac ccccatcggc gacggccccg tgctgctgcc cgacaaccac
4201 tacctgagca cccagtccgc cctgagcaaa gaccccaacg agaagcgcga tcacatggtc
4261 ctgctggagt tcgtgaccgc cgccgggatc actctcggca tggacgagct gtacaaggtc
4321 gacggatcct aaagagctcc aattcgccct atagtgagtc gtattacaat tcactggccg
4381 tcgttttaca acgtcgtgac tgggaaaacc ctggcgttac ccaacttaat cgccttgcag
4441 cacatccccc tttcgccagc tggcgtaata gcgaagaggc ccgcaccgat cgcccttccc
4501 aacagttgcg cagcctgaat ggcgaatggg acgcgccctg tagcggcgca ttaagcgcgg
4561 cgggtgtggt ggttacgcgc agcgtgaccg ctacacttgc cagcgcccta gcgcccgctc
4621 ctttcgcttt cttcccttcc tttctcgcca cgttcgccgg cttagctaca aatcccactg
4681 gctatatgta tgcatttgtg ttaaaaaagt ttgtatagat tatttaatct actcagcatt
4741 ctttctctaa ataggaattt gttacttaat ggagaaaaaa atgtttcgat ttacctagtg
4801 tatttgtttg tatactcacg tttaatttca aacatccatt ctatcttgtg taatttttgg
4861 catggtgaaa aagataatca gccttataat ctttacaaaa gtaagaaatt ctgtaaataa
4921 gccttaatgc ccttgcttta aattaaaatg gttctttttc atgataatgt ttgcactttg
4981 tgaatatatt ttagatagtt ctgtgaggta taattaagat gttttagaga cttatacaat
5041 tttgtcttta taaattctta attgatttta ccatcccagt ttaactatgc ttcgtcggca
5101 tctctgcaca tgtcgtgttt tcttaccgta ttgtcctacc aagaacctct tttttgcttg
5161 gatcgaaatt aaaggtttaa aagcaaagtt atggatgcta gagtatttca aagctattca

5221 gctagagctg aggggatgaa aaatcccatt gccaaggaat tgttggcttt gatggaagaa
5281 aagcaaagca acttgtcagt cgcggtcgat ttgacgaaga aatccgaaat cttagaattg
5341 gtagataaaa ttggacccta tgtctgtgtt atcaagacac atattgacgt tgtcgaggat
5401 ttcgaccagg atatggtaga aaaactggtg gccttaggta aaaagcatcg ttttcttatc
5461 tttgaggatc gcaaattcgc agacattgga aataccgtca agctacaata tgcatctggt
5521 gtgtacaaaa ttgcttcttg ggctcatatc acaaattgcc atacagtgcc aggcgagggt
5581 attatacaag gcctcaaaga agttggttta cctttgggac gtggtctctt gcttttggct
5641 gaaatgtctt ccaaaggctc tttggctact ggttcctaca cagagaaaac cttagaatgg
5701 tttgagaagc ataccgattt ttgctttggc tttatagctg gtcgtcgatt tcctaacctt
5761 caaagcgact acataactat gtcccctggt atcggcttgg atgttaaagg agacgggctg
5821 ggacagcaat atcgtactcc tgaagaagtg attgtaaact gcggtagcga tatcatcatt
5881 gttggtcgtg gagtctatgg agctggtcgt aatcctgttg tcgaagccaa gagatataga
5941 gaagctggtt ggaaggcata tcagcaaaga ctttctcagc attaaaaaaa gactaatgta
6001 aaatttttt ggttggttat tgaaaaagtc gatgccttgt ttgcgtttgt tttcctaggc
6061 gttttatgtc agaaggcatt tagaattagt atacaagtac tctttggtaa aattttatgt
6121 agcgactaaa atattaacta ttatagataa acaccttggg aataaaaagt aatttgctat
6181 agtaatttat taaacatgct cctacaacat taccacaatc ttttctcttg gattgacatt
6241 gaataagaaa agagtgaatt tttttagact tgtaatgata actatgtaca aagccaatga
6301 aagatgtatg tagatgaatg taaaatacca tgtagacaaa caagataaaa cttggttata
6361 aacattggtg ttggaacaga ataaattaga tgtcaaaaag tttcgtcaat atcacaagcc
6421 ggctttcccc gtcaagctct aaatcggggg ctccctttag ggttccgatt tagtgcttta
6481 cggcacctcg accccaaaaa acttgattag ggtgatggtt cacgtagtgg gccatcgccc
6541 tgatagacgg tttttcgccc tttgacgttg gagtccacgt tctttaatag tggactcttg
6601 ttccaaactg gaacaacact caaccctatc tcggtctatt cttttgattt ataagggatt
6661 ttgccgattt cggcctattg gttaaaaaat gagctgattt aacaaaaatt taacgcgaat
6721 tttaacaaaa tattaacgct tacaatttag gtggca

Appendix 3: *S. pombe* library

number	name	genotype
001**	*wild type h+*	*h+;ade6-M216, leu1-32, ura4-D18,*
002**	*wild type h-*	*h-;ade6-M216, leu1-32, ura4-D18,*
003	*cts1-YFP*	*h+; ura+*
004	*cts1-CFP*	*h-; ura+*
005	*gua1-CFP*	*h-; kanMX6*
006	*RNRBiFC; cts1-CFP*	*h-; suc22promoter-VC155-suc22-kanMX6; ade6-M210; ura4-D18; leu1-32;cdc22promoter-VN173-cdc22-kanMX6; cts1(C-Term)-YFP;*
007	*pFA6a-kanMX6-P3nmt1-cts1-YFP*	*h-; kanMX*
008**	*ade6-M210, leu1-32, ura4-D18*	*h+, ade6-M210, leu1-32, ura4-D18*
009**	*ade6-M210, leu1-32, ura4-D18*	*h-, ade6-M210, leu1-32, ura4-D18*
010**	*ade6-M216, leu1-32, ura4-D18*	*h+, ade6-M216, leu1-32, ura4-D18*
011**	*ade6-M216, leu1-32, ura4-D18*	*h-, ade6-M216, leu1-32, ura4-D18*
012**	*diploid strain*	*h+/h-, ade6-M210/ade6-M216, leu1-32/leu1-32, ura4-D18/ura4-D18*
013	*cts1 GATase del (YFP)*	*h-; ura+*
014	*cts1 CTPsynth del*	*h-; ura+*
015	*cts1 (2-18 del)*	*h-; ura+*
016	*cts1 (19-37 del)*	*h-; ura+*
017	*cts1 (38-52 del)*	*h-; ura+*
018	*cts1 (53-79 del)*	*h-; ura+*
019	*cts1 (80-129 del)*	*h-; ura+*

020	*cts1 (2-56 del)*	*h-; ura+*
021**	*cdc25-22; ura4-D; h+*	*h+;cdc25-22; ura4-D;*
022**	*cdc25-22; ura4-D; h-*	*h-;cdc25-22; ura4-D;*
023	*pSMUY2+ cts1; cdc25-22; ura4-D; h+*	*h+;cts1-YFP; cdc25-22; ura4-D;*
024	*pFA6a-kanMX6-cts1-YFP*	*h-;cts1-YFP; kanMX6;*
025	*pFA6a-kanMX6-cts1(19-37aa del)-YFP*	*h-;cts1(19-37aa del)-YFP; kanMX6;*
026	*pFA6a-kanMX6-cts1(52-79aa del)-YFP*	*h-;cts1(52-79aa del)-YFP; kanMX6;*
027	*pFA6a-kanMX6-cts1(80-129aa del)-YFP*	*h-;cts1(80-129aa del)-YFP; kanMX6;*
028	*cdc25-22; cts1-YFP*	*h+; cdc25-22; cts1-YFP, ura4+*
029	*RNRBiFC; cts1-YFP*	
030	*pFA6a-kanMX6-cts1(G31A)-YFP*	
031	*pFA6a-kanMX6-cts1(V34A)-YFP*	
032	*pFA6a-kanMX6-cts1(C403G)-YFP*	
033	*pFA6a-kanMX6-cts1(C403S)-YFP*	*h-;cts1(C403S)-YFP; kanMX6;*
034	*pFA6a-kanMX6-cts1(C403A)-YFP*	*h-;cts1(C403A)-YFP; kanMX6;*
035	*pFA6a-kanMX6-cts1(C403 del)-YFP*	
036	*pFA6a-kanMX6-cts1(T459A)-YFP*	*h-;cts1(T459A)-YFP; kanMX6;*
037	*pFA6a-kanMX6-cts1(S468A)-YFP*	*h-;cts1(S468A)-YFP; kanMX6;*
038	*pFA6a-kanMX6-cts1(S573A)-YFP*	*h-;cts1(S573A)-YFP; kanMX6;*
039	*cts1-YFP*	*cts1-YFP; +L, -H, -A, -R, -U*
040	*gua2-YFP*	*gua2-YFP; +L, -H, -A, -R, -U*
041	*gua2-YFP*	*gua2-YFP; +L, -H, -A, -R, -U*
042	*cts1-YFP*	*cts1-YFP; +L, -H, -A, -R, -U*
043	*gua1*	
044	*gua1*	
046***	*Ch 16 Stain*	*h+; leu1-32 ura4-D18 ade6-M210/M216*

047***	Ch 16 Stain	*h-; leu1-32 ura4-D18 ade6-M210/M216*
048***	*scf1Δ*	V2-30-B09; *scf1Δ*
049***	*hsp9Δ*	V2-10-F06; *hsp9Δ*
050***	*hsp16Δ*	V2-26-A12; *hsp16Δ*
051***	*hsp20Δ*	V2-09-C10; *hsp20Δ*
052***	*pdr13Δ*	V2-06-E07; *pdr13Δ*
053***	*sks2Δ*	V2-11-D04; *sks2Δ*
054*	*cts1-YFP crf1(V2-14-D06)::KanMX6*	*cts1-YFP-ura3 crf1::KanMX6 leu1-32*
055*	*cts1-YFP nrm1(V2-02-B07)::KanMX6*	*cts1-YFP-ura3, nrm1::KanMX6, leu1-32*
056*	*cts1-YFP toc1(V2-31-B12)::KanMX6*	*cts1-YFP-ura3, toc1::KanMX6, leu1-32*
057*	*cts1-YFP, ste20(V2-07-G02)::KanMX6*	*cts1-YFP-ura3, ste20::KanMX6, leu1-32*
058***	WT h- sequenced by Sanger	*h-; wt*
059*	*cts1-YFP vgl1(V2-10-F05)::KanMX6*	*cts1-YFP-ura3 vgl1::KanMX6 leu1-32*
060*	*cts1-YFP ssa1(V2-11-G11)::KanMX6*	*cts1-YFP-ura3 ssa1::KanMX6 leu1-32*
061*	*cts1-YFP slx4(V2-17-G04)::KanMX6*	*cts1-YFP-ura3 slx4::KanMX6 leu1-32*
062*	*cts1-YFP SPAC18G6.12c(V2-26-G04)::KanMX6*	*cts1-YFP-ura3 SPAC18G6.12c::KanMX6 leu1-32*
063*	*cts1-YFP mph1(V2-27-A02)::KanMX6*	*cts1-YFP-ura3 mph1::KanMX6 leu1-32*
064*	*cts1-YFP rad3(V2-03-G10)::KanMX6*	*cts1-YFP-ura3 rad3::KanMX6 leu1-32*
065*	*cts1-YFP ppk29(V2-08-A05)::KanMX6*	*cts1-YFP-ura3 ppk29::KanMX6 leu1-32*
066*	*cts1-YFP SPCC132.04c(V2-08-D12)::KanMX6*	*cts1-YFP-ura3 SPCC132.04c::KanMX6 leu1-32*
067*	*cts1-YFP tor1(V2-12-G08)::KanMX6*	*cts1-YFP-ura3 tor1::KanMX6 leu1-32*
068*	*cts1-YFP SPAC22H10.11c(V2-14-D06)::KanMX6*	*cts1-YFP-ura3 SPAC22H10.11c::KanMX6 leu1-32*
069*	*cts1-YFP SPAC26A3.17c(V2-27-A06)::KanMX6*	*cts1-YFP-ura3 SPAC26A3.17c::KanMX6 leu1-32*
070*	*cts1-YFP mep33(V2-21-A06)::KanMX6*	*cts1-YFP-ura3 mep33::KanMX6 leu1-32*
071*	*cts1-YFP raf1(V2-01-A07)::KanMX6*	*cts1-YFP-ura3 raf1::KanMX6 leu1-32*

072*	*cts1-YFP SPAC13G7.11(V2-01-A08)::KanMX6*	cts1-YFP-ura3 SPAC13G7.11::KanMX6 leu1-32
073*	*cts1-YFP SPCP1E11.11(V2-01-C07)::KanMX6*	cts1-YFP-ura3 SPCP1E11.11::KanMX6 leu1-32
074*	*cts1-YFP dad3(V2-01-C08)::KanMX6*	cts1-YFP-ura3 dad3::KanMX6 leu1-32
075*	*cts1-YFP dss1(V2-01-C12)::KanMX6*	cts1-YFP-ura3 dss1::KanMX6 leu1-32
076*	*cts1-YFP SPBC1604.09c(V2-01-D05)::KanMX6*	cts1-YFP-ura3 SPBC1604.09c::KanMX6 leu1-32
078*	Same as 003 but Leu+	ade6-M216, ura4-D18,
079*	Same as 003 but Leu+	ade6-M216, ura4-D18,
080*	*sad1*-GFP	
081*	*cts1-YFP pop3(V2-07-C09)::KanMX6*	cts1-YFP-ura3 pop3::KanMX6 leu1-32
082*	*cts1-YFP tsc2(V2-12-G04)::KanMX6*	cts1-YFP-ura3 tsc2::KanMX6 leu1-32
083*	*cts1-YFP tsc1(V2-22-H06)::KanMX6*	cts1-YFP-ura3 tsc1::KanMX6 leu1-32
084*	*rhp55Δrqh1Δ Ch16*	rhp55::ura3 rqh1::ura3 urg1::HPH
085*	*exo1Δrqh1Δ Ch16*	exo1::ura3 rqh1::ura3 Ch16 urg1::HPH
086*	*WT ch16*	WT Ch16 urg::HPH
087*	*rqh1Δ Ch16*	rqh1::ura3 Ch16 urg1::HPH
088*	*exoΔ Ch16*	exo1::ura3 Ch16 urg1::HPH
089*	*rhp55Δ Ch16*	rhp55::ura3 Ch16 urg1::HPH
090*	*Wee1-50*	Wee1-50
091*	*cts1-YFP tco89(V2-21-H09)::KanMX6*	cts1-YFP-ura3 tco89::KanMX6 leu1-32
092*	*cts1-YFP sin1(V2-14-H10)::KanMX6*	cts1-YFP-ura3 sin1::KanMX6 leu1-32
093*	*cts1-YFP bit61(V2-21-E11)::KanMX6*	cts1-YFP-ura3 bit64::KanMX6 leu1-32
094*	*cdc13*-GFP	cdc13-GFP::kanR
095*	*res2 cts1-YFP*	cts1-YFP-ura3 res2::KanMX6 leu1-32
096***	*atf1::ura4*	h-; leu1-32; ura4+; his+; ade6-d1;
097***	*spc1-m13*	h-; spc1-m13; ura4-d18; lue1-32; ade6-d1;
098***	*hsp9Δ* (V2-30-B09) (scf1)	h+ ; ade6-M210(ade6-M216); ura4-D18; leu1-32; kanMAX6

099***	*hsp104Δ (V2-10-F06)*	*h+ ; ade6-M210(ade6-M216); ura4-D18; leu1-32; kanMAX6*
100***	*hsp16Δ (V2-26-A12)*	*h+ ; ade6-M210(ade6-M216); ura4-D18; leu1-32; kanMAX6*
101***	*hsp20Δ (V2-09-C10)*	*h+ ; ade6-M210(ade6-M216); ura4-D18; leu1-32; kanMAX6*
102***	*pdr13Δ (V2-06-E07)*	*h+ ; ade6-M210(ade6-M216); ura4-D18; leu1-32; kanMAX6*
103***	*sks2Δ (V2-11-D04) (hsc1)*	*h+ ; ade6-M210(ade6-M216); ura4-D18; leu1-32; kanMAX6*
104***	*cdc10 C4-MYChyg*	*h+ ura4-D18; leu1-32; cdc10-C4–13xmyc::KAN nrm1-3xHA::KAN*
105***	*cdc10 C4-MYChyg ct1-YFP*	*h+ cts1-YFP-ura3 ura4-D18; leu1-32; cdc10-C4–13xmyc::KAN nrm1-3xHA::KAN*
106***	Strain for Edu	
107	*hsp9::kanMX6; cts1-YFP-ura4*	*h+; ura+; hsp9Δ; cts1-YFP; ade6-M210(ade6-M216); leu1-32; kanMAX6*
108	*hsp16::kanMX6; cts1-YFP-ura4*	*h+; ura+; hsp16Δ; cts1-YFP; ade6-M210(ade6-M216); leu1-32; kanMAX6*
109	*hsp20::kanMX6; cts1-YFP-ura4*	*h+; ura+; hsp20Δ; cts1-YFP; ade6-M210(ade6-M216); leu1-32; kanMAX6*
110	*hsp104::kanMX6; cts1-YFP-ura4*	*ura+; hsp104Δ; cts1-YFP; ade6-M210(ade6-M216); leu1-32; kanMAX6*
111	*pdr13::kanMX6; cts1-YFP-ura4*	*h+; ura+; pdr13Δ; cts1-YFP; ade6-M210(ade6-M216); leu1-32; kanMAX6*
112	*sks2::kanMX6; cts1-YFP-ura4*	*h+; ura+; sks2Δ; cts1-YFP; ade6-M210(ade6-M216); leu1-32; kanMAX6*
113***	Bioneer diploid	*h+/h- ; ade6-M210/ade6-M216; ura4-D18/ura4-D18; leu1-32/leu1-32*
114***	wis1Δ (V2-16-F02)	*h+ ; ade6-M210(ade6-M216); ura4-D18; leu1-32; kanMAX6*
115***	*pyp1*Δ (V2-05-E12)	*h+ ; ade6-M210(ade6-M216); ura4-D18; leu1-32; kanMAX6*
116***	*pyp2*Δ (V2-03-C05)	*h+ ; ade6-M210(ade6-M216); ura4-D18; leu1-32; kanMAX6*
117***	Bioneer haploid	*h+/h- ; ade6-M210/ade6-M216; ura4-D18; leu1-32; kanMAX6*
118***	*ppk15Δ (V2-06-B10)*	*h+ ; ade6-M210(ade6-M216); ura4-D18; leu1-32; kanMAX6*
119	*ppk15::kanMX6; cts1-YFP-ura4*	*h+; ura+; ppk15::kanMX6; cts1-YFP; ade6-M210(ade6-M216); leu1-32;*
120	*h+; pSMUY2+cts1*	*h+; cts1-YFP-ura4; ade6-M216, leu1-32;*
121	*h-; pSMUY2+cts1*	*h-; cts1-YFP-ura4; ade6-M216, leu1-32;*
122	*h+; wis1::kanMX6; cts1-YFP-ura4*	*h+; wis1Δ::ura4; cts1-YFP; ade6-M210(ade6-M216); leu1-32; kanMX6*
123	*h-; wis1::kanMX6; cts1-YFP-ura4*	*h-; wis1Δ::ura4; cts1-YFP; ade6-M210(ade6-M216); leu1-32; kanMX6*

124*	*atb-GFP 3GFP-NLS HA1149*	*h90; ade6-M216 ura4-D18 leu1-32 lys1+::Pnmt-3GFP-NLS atb2::CFP-atb2+::kanMX6*
125*	*cut11+-GFP-HA-KanMX6 H14/C04*	*h90; ade6-M216 ura4-D18 leu1-32 lys1-131 Cut11+-GFP-HA::kanMX6*
126*	*ish1+::mCherry-kanMX6 HA742-8C*	*h+; his2 ade6-M210 leu1 ish1+::mCherry-kanMX6*
127*	*ish1+::mCherry cts1 - YFP*	*h+; his2- ade6- leu1 ish1+::mCherry-kanMX6 cts1-YFP-ura3*
128	*h+, hsp104::kanMX6, cts1-YFP*	*h+, hsp104::kanMX6, cts1-YFP-ura4, leu1-32, ade6-M210(ade6-M216)*
129	*h-, hsp104::kanMX6, cts1-YFP*	*h-, hsp104::kanMX6, cts1-YFP-ura4, leu1-32, ade6-M210(ade6-M216)*
130	*h+, pyp1::kanMX6, cts1-YFP*	*h+, pyp1::kanMX6, cts1-YFP-ura4, leu1-32, ade6-M210(ade6-M216)*
131	*h-, pyp1::kanMX6, cts1-YFP*	*h-, pyp1::kanMX6, cts1-YFP-ura4, leu1-32, ade6-M210(ade6-M216)*
132	*h+, pyp2::kanMX6, cts1-YFP*	*h+, pyp2::kanMX6, cts1-YFP-ura4, leu1-32, ade6-M210(ade6-M216)*
133	*h-, pyp2::kanMX6, cts1-YFP*	*h-, pyp2::kanMX6, cts1-YFP-ura4, leu1-32, ade6-M210(ade6-M216)*
134	*h+/h-, cts1(E161A)-YFP/cts1*	*h+/h-, ade6-M210/ade6-M216; ura4-D18/ura4-D18; leu1-32/leu1-32; cts1(E161A)-YFP-kanMX6/cts1,*
135	*h+/h-, cts1(S468A)-YFP/cts1*	*h+/h-, ade6-M210/ade6-M216; ura4-D18/ura4-D18; leu1-32/leu1-32; cts1(S468A)-YFP-kanMX6/cts1,*
136	*h+/h-, cts1(T459A)-YFP/cts1*	*h+/h-, ade6-M210/ade6-M216; ura4-D18/ura4-D18; leu1-32/leu1-32; cts1(T459A)-YFP-kanMX6/cts1,*
137	*h+/h-, cts1(S573A)-YFP/cts1*	*h+/h-, ade6-M210/ade6-M216; ura4-D18/ura4-D18; leu1-32/leu1-32; cts1(S573A)-YFP-kanMX6/cts1,*
138	URA7-GFP (budding yeast)	
139	*h+, atf1::ura4; cts1-YFP*	*h+; atf1::ura4; cts1-YFP-ura4; ade6-M216, leu1-32*
140	*h-, atf1::ura4; cts1-YFP*	*h-; atf1::ura4; cts1-YFP-ura4; ade6-M216, leu1-32*
141	*h+, spc1-m13; cts1-YFP*	*h+, spc1-m13-ade6, cts1-YFP-ura4, leu1-32*
142	*h-, spc1-m13; cts1-YFP*	*h-, spc1-m13-ade6, cts1-YFP-ura4, leu1-32*
143	*h+, pyp1::kanMX6, pyp2::kanMX6, cts1-YFP*	*h+, pyp1::kanMX6, pyp2::kanMX6, cts1-YFP-ura4, ade6-M216, leu1-32*
144	*h-, pyp1::kanMX6, pyp2::kanMX6, cts1-YFP*	*h-, pyp1::kanMX6, pyp2::kanMX6, cts1-YFP-ura4, ade6-M216, leu1-32*

145*	JP598 *gad8::ura4* (sterile)	*gad8::ura4+ ura4.d18*
146*	JP1511 *gad8::ura4* (sterile)	*gad8.K259R*
147*	URA7-GFP (budding yeast)	
148*	URA8-GFP (budding yeast)	
149*	*gad8::ura4 cts1-YFP*	*gad8::ura4+, ura4.d18 cts1-YFP, kanMX6*
150*	*gad8.K259R cts1-YFP*	*gad8.K259R, ura4.d18 cts1-YFP, kanMX6*
151*	All +ve 972	*h-, ura+, leu+, ade+, his+*
152*	All -ve	*h+, leu2-32, ura4-D18, ade6-M210, his-*
153*	All -ve	*h-, leu2-32, ura4-D18, ade6-M210, his-*
154***	WT mini chromosome 16	*h-, leu2-32, ura4-D18, ade6-M210, his- Ch16 ade6-M216, rad21::MataG418, Cig2::his4*
155***	WT mini chromosome 16	*h+, leu2-32, ura4-D18, ade6-M210, his- Ch16 ade6-M216, rad21::MataG418, Cig2::his4*
156***	ade point mutant	*h-, ade6-485*
157***	ade point mutant	*h+, ade6-(GTCC)2*
158***	ade point mutant	*h+, ade6-(GTCC)2*
159***	ade point mutant	*h-, ade6-(GTCC)2*
160*	*psk1::hphMX6*	*h-,psk1::hphMX*
161*	*tsc2::kanMX6*	*h-,tsc2::kanMX*
162*	*tor1::kanMX6*	*tor1::KanMX6 leu1-32 ura4-D18*
163*	*tor1::kanMX6*	*tor1::KanMX6 leu1-32 ura4-D18 leu1-32::TOR1-YFP-HA-FLAG*
165*	*tor1::KanMX6 ade6-485*	

* The strains numbered 59-95, 124-127, 145-153, 160-165 are constructed by Lydia Hulme.

** The strains 001, 002, 008-012, 021, 022 are the kindly gift from Stephen's Lab.

*** The strains 046-053, 058, 096-106, 113-118, 154-159 are the kindly gift from Lydia's old Lab.

Appendix 4: media used in experiments

A. YES: Yeast Extract with supplements

Component	Amount	Final concentration
yeast extract	5 g/L	0.5% w/v
glucose	30 g/L	3.0% w/v
supplements: 225 mg/L adenine, histidine, leucine, uracil and lysine hydrochloride.		
Solid media is made by adding 2% Difco Bacto Agar		

B. EMM: Edinburgh minimal medium

Component	Amount	Final concentration
potassium hydrogen phthallate	3 g/L	14.7 mM
Na2HPO4	2.2 g/L	15.5 mM
NH4Cl	5 g/L	93.5 mM
glucose	20 g/L	2% w/v
50x salt stock	20 ml	
1,000x vitamin stock	1 ml	
10,000x mineral stock	0.1 ml	
Solid media is made by adding 2% Difco Bacto Agar		

C. PMG: Pombe Glutamate medium

Component	Amount	Final concentration
potassium hydrogen phthallate	3 g/L	14.7 mM
Na2HPO4	2.2 g/L	15.5 mM
L-glutamic acid, monosodium salt (Sigma G-5889)	3.75 g/L	
glucose	20 g/L	2% w/v
50x salt stock	20 ml	
1,000x vitamin stock	1 ml	
10,000x mineral stock	0.1 ml	
PMG can be used for G418 selection, which EMM is not compatible with.		
Solid media is made by adding 2% Difco Bacto Agar		

D. SPAS mating media

Component	Amount	Final concentration
glucose	10 g/L	1% (w/v)
KH2PO4	1 g/L	7.3 mM
1000x vitamin stock	1 ml	(w/v)
supplements: 45 mg/L adenine, histidine, leucine, uracil and lysine hydrochloride (1/5 of normal amount used in EMM and YES media).		
Solid media is made by adding 3% Difco Bacto Agar		

E. Stock Solutions

50x Salt stock

Component	Amount	Final concentration
MgCl2.6H20	52.5 g/L	0.26 M
CaCl2.2H20	0.735 g/L	4.99 mM
KCl	50 g/L	0.67 M
Na2SO4	2 g/L	14.l mM

1000x Vitamin stock

Component	Amount	Final concentration
pantothenic acid	1 g/L	4.20 mM
nicotinic acid	10 g/L	8l.2 mM
inositol	10 g/L	55.5 mM
biotin	10 mg/L	40.8 uM

10,000x Mineral stock

Component	Amount	Final concentration
boric acid	5 g/L	80.9 mM
MnSO4	4 g/L	23.7 mM
ZnSO4.7H2O	4 g/L	13.9 mM
FeCl2.6H2O	2 g/L	7.40 mM
molybdic acid	0.4 g/L	2.47 mM
KI	1 g/L	6.02 mM
CuSO4.5H2O	0.4 g/L	1.60 mM
citric acid	10 g/L	47.6 mM

F. YPD media

Component	Amount	Final concentration
Bacteriological peptone	20 g/L	2%
Yeast extract	10 g/L	1%
Glucose	20 g/L	2%
Solid media is made by adding 2% Difco Bacto Agar		

G. LB media

Component	Amount	Final concentration
tryptone	10 g/L	1%
Yeast extract	5 g/L	0.5%
NaCl	5 g/L	0.5%
Add antibiotic if needed (50 μg/mL of Amp or Carb). Keep plates in fridge at 4 ˚C.		
Solid media is made by adding 2% Difco Bacto Agar		

Appendix 5: CTPS related diseases

Disease	Publication
Breast Neoplasms	Cyclopentenyl cytosine induces senescence in breast cancer cells through the nucleolar stress response and activation of p53.
Carcinoma	Enhancement of the toxicity and DNA incorporation of arabinosyl-5-azacytosine and 1-beta-D-arabinofuranosylcytosine by cyclopentenyl cytosine.
	[Cytidine triphosphate synthetase gene expressed in the homogenous nasopharyngeal carcinoma tissue cells]
Carcinoma, Ehrlich Tumor	CTP synthetase from Ehrlich ascites tumor cells. Subunit stoichiometry and regulation of activity.
	Purification and properties of CTP synthetase from Ehrlich ascites tumor cells.
	Role of GTP in CTP synthetase from Ehrlich ascites tumor cells.
	Synthesis of N4-substituted CTP by mammalian CTP synthetase.
	The effect of some platinum compounds on the activity of the CTP synthetase of Ehrlich ascites tumor cells: in vitro and in vivo studies.
Carcinoma, Hepatocellular	Biochemical pharmacology of acivicin in rat hepatoma cells.
	Increased concentration of CTP synthetase in hepatoma 3924A: immunological evidence.
	Multi-enzyme-targeted chemotherapy by acivicin and actinomycin.
	Oncolytic activity and mechanism of action of a novel L-cysteine derivative, L-cysteine, ethyl ester, S-(N-methylcarbamate) monohydrochloride.
	Origin of increased deoxycytidine excretion into urine of rats bearing Yoshida ascites sarcoma.
	Potentiation of antimetabolite action by dibromodulcitol in cell culture.
	Rapid in vivo inactivation by acivicin of CTP synthetase, carbamoyl-phosphate synthetase II, and amidophosphoribosyltransferase in hepatoma.
	Uridine and cytidine nucleotide synthesis in renal hypertrophy: biochemical differences in response to the growth stimulus of diabetes and unilateral nephrectomy.

Carcinosarcoma	Origin of increased deoxycytidine excretion into urine of rats bearing Yoshida ascites sarcoma.
Colorectal Neoplasms	Low cytosine triphosphate synthase 2 expression renders resistance to 5-fluorouracil in colorectal cancer.
Hepatitis B	Another ten stories in antiviral drug discovery (part C): 'Old' and 'new' antivirals, strategies, and perspectives.
Herpes Simplex	Another ten stories in antiviral drug discovery (part C): 'Old' and 'new' antivirals, strategies, and perspectives.
HIV Infections	Another ten stories in antiviral drug discovery (part C): 'Old' and 'new' antivirals, strategies, and perspectives.
Hypersensitivity	DNA vaccination with the major outer-membrane protein gene induces acquired immunity to Chlamydia trachomatis (mouse pneumonitis) infection.
Infection	Another ten stories in antiviral drug discovery (part C): 'Old' and 'new' antivirals, strategies, and perspectives.
	DNA vaccination with the major outer-membrane protein gene induces acquired immunity to Chlamydia trachomatis (mouse pneumonitis) infection.
	Escherichia coli nucleoside diphosphate kinase interactions with T4 phage proteins of deoxyribonucleotide synthesis and possible regulatory functions.
Leukemia	Evidence for transformation-related increase in CTP synthetase activity in situ in human lymphoblastic leukemia.
	Resistance to cytosine arabinoside in acute leukemia: the significance of mutations in CTP synthetase.
	The IMP dehydrogenase inhibitor mycophenolic acid antagonizes the CTP synthetase inhibitor 3-deazauridine in MOLT-3 human leukemia cells: a central role for phosphoribosyl pyrophosphate.
Leukemia, Myeloid, Acute	Cyclopentenyl cytosine inhibits cytidine triphosphate synthetase in paediatric acute non-lymphocytic leukaemia: a promising target for chemotherapy.
Lupus Nephritis	A22: consensus treatment plans for induction therapy in childhood proliferative lupus nephritis-status of use in daily clinical care.

Lymphoproliferative Disorders	Cytidine triphosphate synthetase activity in lymphoproliferative disorders.
Melanoma	Circulating Tumor Cells, DNA, and mRNA: Potential for Clinical Utility in Patients With Melanoma.
Neoplasms	Activities of various enzymes of pyrimidine nucleotide and DNA syntheses in normal and neoplastic human tissues.
	Ara-C metabolism: implications for drug resistance and drug interactions.
	Comments on: cylopentenyl cytosine inhibits cytidine triphosphate synthetase in paediatric acute non-lymphocytic leukaemia: a promising target for chemotherapy. A.C. Verschuur et al. Eur J Cancer 2000, 36, 627-635.
	CTP synthetase from Ehrlich ascites tumor cells. Subunit stoichiometry and regulation of activity.
	Cyclopentenyl cytosine (CPEC): an overview of its in vitro and in vivo activity.
	Glutamine deprivation initiates reversible assembly of mammalian rods and rings.
	Increased CTP synthetase activity in cancer cells.
	Increased cytidine 5'-triphosphate synthetase activity in rat and human tumors.
	Low cytosine triphosphate synthase 2 expression renders resistance to 5-fluorouracil in colorectal cancer.
	Molecular cloning of the human CTP synthetase gene by functional complementation with purified human metaphase chromosomes.
	Oncolytic activity and mechanism of action of a novel L-cysteine derivative, L-cysteine, ethyl ester, S-(N-methylcarbamate) monohydrochloride.
	Penetration of 3-deazauridine into human brain, intracerebral tumor, and cerebrospinal fluid.
	Purification and properties of CTP synthetase from Ehrlich ascites tumor cells.
	Role of antimetabolites of purine and pyrimidine nucleotide metabolism in tumor cell differentiation.
	Role of GTP in CTP synthetase from Ehrlich ascites tumor cells.
	Structure of the synthetase domain of human CTP synthetase, a target for anticancer therapy.
	Synthesis of N4-substituted CTP by mammalian CTP synthetase.

	The effect of some platinum compounds on the activity of the CTP synthetase of Ehrlich ascites tumor cells: in vitro and in vivo studies.
	Uridine and cytidine nucleotide synthesis in renal hypertrophy: biochemical differences in response to the growth stimulus of diabetes and unilateral nephrectomy.
Neuroblastoma	Cyclopentenyl cytosine-induced activation of deoxycytidine kinase increases gemcitabine anabolism and cytotoxicity in neuroblastoma.
	Retinoic acid reduces the cytotoxicity of cyclopentenyl cytosine in neuroblastoma cells.
Pain	Myofascial trigger points are very prevalent in patients with chronic tension-type headache: a double-blinded controlled study.
Poxviridae Infections	Therapy and short-term prophylaxis of poxvirus infections: historical background and perspectives.
Precursor Cell Lymphoblastic Leukemia-Lymphoma	Determination of CTP synthetase activity in crude cell homogenates by a fast and sensitive non-radiochemical assay using anion-exchange high-performance liquid chromatography.
	Evidence for transformation-related increase in CTP synthetase activity in situ in human lymphoblastic leukemia.
	Increased activity of cytidine Triphosphate synthetase in pediatric acute lymphoblastic leukemia.
Smallpox	Therapy and short-term prophylaxis of poxvirus infections: historical background and perspectives.
Trypanosomiasis, African	Activation and inhibition of CTP synthase from Trypanosoma brucei, the causative agent of African sleeping sickness.
	Expression, purification, characterization, and in vivo targeting of trypanosome CTP synthetase for treatment of African sleeping sickness.
	Synthesis and Biological Evaluation of CTP Synthetase Inhibitors as Potential Agents for the Treatment of African Trypanosomiasis.
	Trypanosoma brucei CTP synthetase: a target for the treatment of African sleeping sickness.
Tuberculosis	Thiophenecarboxamide Derivatives Activated by EthA Kill Mycobacterium tuberculosis by Inhibiting the CTP Synthetase PyrG.
Vascular System Injuries	Smooth muscle-specific drug targets for next-generation drug-eluting stent.

http://www.brenda-enzymes.org/enzyme.php?ecno=6.3.4.2#printtable
http://www.brenda-enzymes.org/enzyme.php?ecno=6.3.4.2&organism%5B%5D=Homo+sapiens&showtm=0&UniProtAcc=Q9NRF8&onlyTable=Disease#printtable

Appendix 5: Publication

Chen, K., Zhang, J., Tastan, O. Y., Deussen, Z. A., Siswick, M. Y. and Liu, J. L. (2011). Glutamine analogs promote cytoophidium assembly in human and Drosophila cells. J Genet Genomics 38, 391-402.

Shen, Q. J., Kassim, H., Huang, Y., Li, H., Zhang, J., Li, G., Wang, P. Y., Yan, J., Ye, F. and Liu, J. L. (2016). Filamentation of Metabolic Enzymes in Saccharomyces cerevisiae. J Genet Genomics 43, 393-404.

Zhang, J., Hulme, L. and Liu, J. L. (2014). Asymmetric inheritance of cytoophidium in Schizosaccharomyces pombe. Biol Open 3, 1092-7.