A study on mitosis : role of kinesins and microtubule instability

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A dissertation to be submitted for the partial fulfilment of BS-MS dual degree in Science

Indian Institute of Science Education and Research, Mohali
April, 2020

Certificate of Examination

This is to certify that the dissertation titled "Study on mitotic kinesins" submitted by **Ms. Nimisha krishnan** (Reg. No. MS15206) for the partial fulfilment of BS-MS dual degree program of the Institute, has been examined by the thesis committee duly appointed by the Institute. The committee finds the work done by the candidate satisfactory and recommends that the report be accepted.

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(Supervisor)

Dated: June 15, 2020

Declaration

The work presented in this dissertation has been carried out by me under the guidance of Dr Abhishek Chaudhuri and Prof. Takashi Toda at the Indian Institute of Science Education and Research, Mohali and Graduate School of Integrated Sciences for Life, Hiroshima University.

This work has not been submitted in part or in full for a degree, a diploma, or a fellowship to any other university or institute. Whenever contributions of others are involved, every effort is made to indicate this clearly, with due acknowledgement of collaborative research and discussions. This thesis is a bonafide record of original work done by me and all sources listed within have been detailed in the bibliography.

Nimisha Krishnan
(Candidate)

Dated: June 15, 2020

In my capacity as the internal supervisor of the candidate's project work, I certify that the above statements by the candidate are true to the best of my knowledge.

Dr. Abhishek Chaudhuri
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In my capacity as the external supervisor of the candidate's project work, I certify that the above statements by the candidate are true to the best of my knowledge.

Prof. Takashi Toda
Graduate School of Integrated Sciences for Life
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I am very grateful and thankful to professor Takashi Toda for giving me a great opportunity of working in his lab and I would also like to thank Masashi Yukawa for guiding me, other lab members for helping me and Takashi sir ,Sachiko ma'am and other friends for making me feel like I am home during the time in Hiroshima .

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Nimisha Krishnan

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Abstract

Motor proteins play a very pivotal role in many cellular processes including cell cycle. Kinesin motor proteins move on microtubule tracks and are known to be extremely important during the process of mitosis. A defect in these proteins can adversely affect the cell cycle even causing cell death. In this thesis, we experimentally explored various situations. In fission yeast over expression of kinesin-5/cut7 and the presence of cut7-rigor are extremely toxic for cell growth and showed mono polar cell. But cut7-rigor when expressed with cut7-22 rescues temperature sensitivity of cut7-22. A few Kinsen-14 kifc1, kifc3, kif25 and Eg5 from the human and macaque when expressed in fission yeast cell resulted in varying level of toxicity for cell growth. We also theoretically explored dynamical instability of microtubules which is known to increase during mitosis. The model shows length versus time traces of microtubule instability which are similar to experimental results.

Chapter 1

Introduction

1.1 Cell division and life

Cell division is one of the most important processes. It not only ensures perpetuation of life but also plays a major role in maintenance and growth of an organism. All forms of life emerge from one cell and form an entire organism through the process of cell division. In unicellular organisms, cell division results in a new organism but in case of multicellular organisms a complex mechanism of maintaining continuous cell division, cell growth and cell death is needed for development of a functional new organism.

1.1.1 The centrosome

The center of the cell regulation is controlled by centrosome. It is a non-membranous, small dense-phase material which is surrounded by significantly less dense material over a large surface. In interphase cells the focus of the thin cytoplasmic fiber was the organelles and in mitotic cells it is the mitotic spindles. This region is surrounded by microtubules and pericentriolar material (PCM).

Centrosomes are found near the nucleus and occur as a pair of centrioles which is surrounded by the PCM. The two centriole lies at right angle to each other and is closer to the proximal end. PCM is a very important site for microtubule nucleation since it occurs as an interconnected matrix of protein complexes and

fibers [RR Gould 77]. Centrosome maturation is the development of the dynamic structure of centrosome in preparation for mitosis [Kimble 92, I A Vorobjev 87, Astrid Kal 93].

1.1.2 Mictrotubule organizing centres

Organizing microtubules is one of the key functions of the centrosome. Microtubule organizing centres (MTOC) are all heterogeneous organelles that nucleate, grow and attach to microtubules [Pickett-Heaps 69]. So along with centrosomal sites, there are some other sites like cytoplasm and nuclear envelope which can be termed as non-centrosomal sites which are a part of MTOC [Borisy 99]

Centrosome is that point from which tubulins from the depolymerised microtubules can polymerize again. For both cytoplasmic and spindle microtubule-nucleation, centrosome is a very important site. The minus end of microtubule is attached to the centrosome while the plus end keeps growing away from the centrosome. Other than these classical role the centrosome is also involved in regulating cytokinesis, DNA damage response, asymmetric cell division and other cellular processes. [Blanka Rebacz 07, Ody C. M. Sibon 00, cell 03, Sluder 02, Alexey Khodjako 00] In fission yeast the functional equivalent for centrosome is spindle pole body (SPB).

Fission yeast nucleates from three different types of MTOC. Though SPB Is always the main site of nucleation along with that there are other MTOCs as well. During interphase, microtubules additionally nucleate from the nuclear surface of pre-existing microtubules [Marcel E. Janson 05, Kenneth ESawin 04, Douglas R.Drummond 00, P.T. Tran 01] and during mitosis all microtubules only nucleate from SPB but at mitotic exit the post anaphase array nucleates from the middle of the cell. These non-SPB sites are called interface MTOC (IMTOC) and equatorial MTOC (EMTOC) [Molly J. Heitz 01] . (see Fig. 1.1).

1.1.3 The spindle pole body

Though there are structural difference between SPB and centrosome many components and protein localization are conserved in both cases. SPB in budding yeast

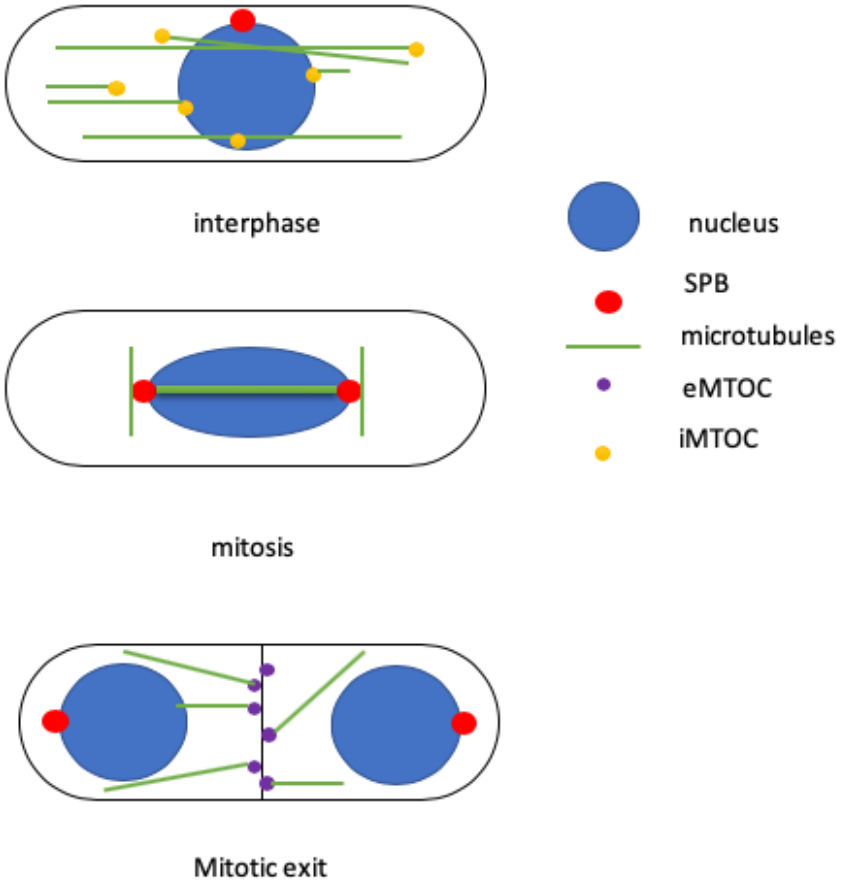


Figure 1.1: Stages of fission yeast cell cycle with equitorial and interphase MTOCs .

is observed to be a disc shaped organelle whose change in diameter is proportional to the ploidy. The radius of SPB in a diploid cell is 100nm , in tetraploid cell is 200nm while that in haploid cell is 50nm [Byers 74, Byers 75]. As the SPB size increases with ploidy, this means increase in DNA content is necessary for the increase in MT nucleation during chromosome separation

Although in other yeasts the SPB stays embedded in the nuclear envelope, it is not the same in case of fission yeast. SPB of fission yeast is present in the

cytoplasm for most of the interphase part of the cell cycle. The SPB duplicates in the cytoplasm during late G2 phase and the two daughter SPBs are connected by a bridge till the time spindles are formed. As the cell enters mitosis the nuclear envelope breaks at the SPB site resulting in formation of pockets in the cytoplasm for each SPB to fit in. Now each of the daughter SPB initiates intra nuclear microtubules and both of them start separating to form a bipolar spindle structure. Along with the spindle MTs, a small number of cytoplasmic MTs also form along with each SPB [R Ding 97].

1.2 Cell cycle

Cell cycle is the process of cell division or cell reproduction. This cycle involves replication and segregation of the genetic information to form a daughter cell. The daughter cells are exactly same as the mother cell in all aspects. To facilitate this exact duplication it is important to have equal duplication of all organelles and macromolecules in the cell which can then help in maintaining equal cell mass after each cell cycle.

A eukaryotic cell cycle has four phases which are Gap-1, Synthesis, Gap-2, and Mitosis phase (see Fig. 1.2). The increase in cell size and preparation for DNA replication is done in the G1 phase. The next phase is S-phase where the DNA replication takes place. In the G-2 phase the cell continues to grow and along with that acts as a checkpoint to ensure all requirements to enter M-phase is ready. Finally, it enters M-phase where the growth of cell stops and all the energy is channeled into dividing the mother cell into two daughter cell.

There are five stages of mitosis.

- Prophase : In this phase chromosome gets condensed and mitotic spindle formation is initiated
- Prometaphase : Early prometaphase shows the disintegration of the nuclear envelope and microtubules starts entering nuclear space and by late prometaphase microtubules start getting attached to the kinetochore.

- Metaphase : In this stage, the centrosomes starts pulling the chromosome toward itself and become aligned at the centre of the cell.

- Anaphase : In this stage the sister chromatids and other organelles are separated.

- Telophase : In this stage, the nuclear envelope starts forming again around the daughter cells and when the spindle microtubules start disassembling.

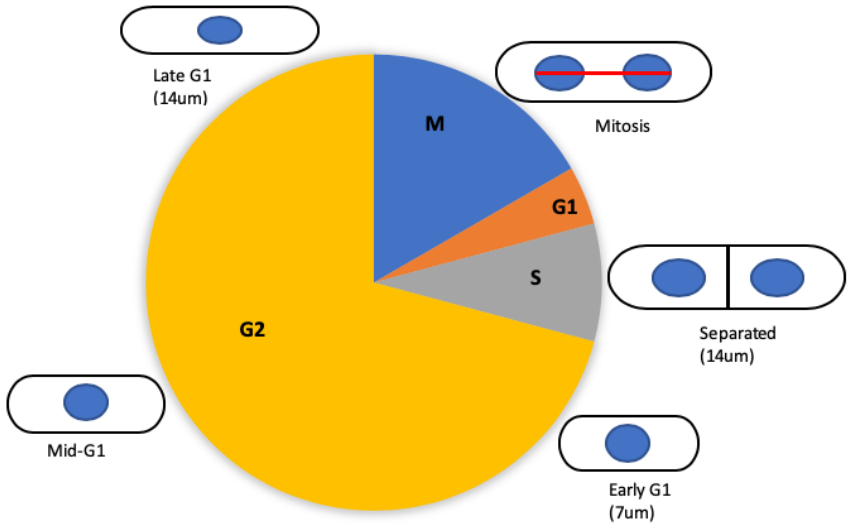


Figure 1.2: Schematic diagram of a cell cycle in fission yeast.

Cytokinesis is another main process in cell division where the cell cleaves into two parts and results in fully developed two daughter cells. The two new daughter cells finally enters interphase again where the cell starts preparing itself to divide and repeating the process by going into G1,s, and G2 phase. Alternatively, the cell can also enter a G0 phase where the cell stops dividing and exits the cell cycle. A cell can re-enter into interphase when the respective signal of the presence of growth factors is received by cell.

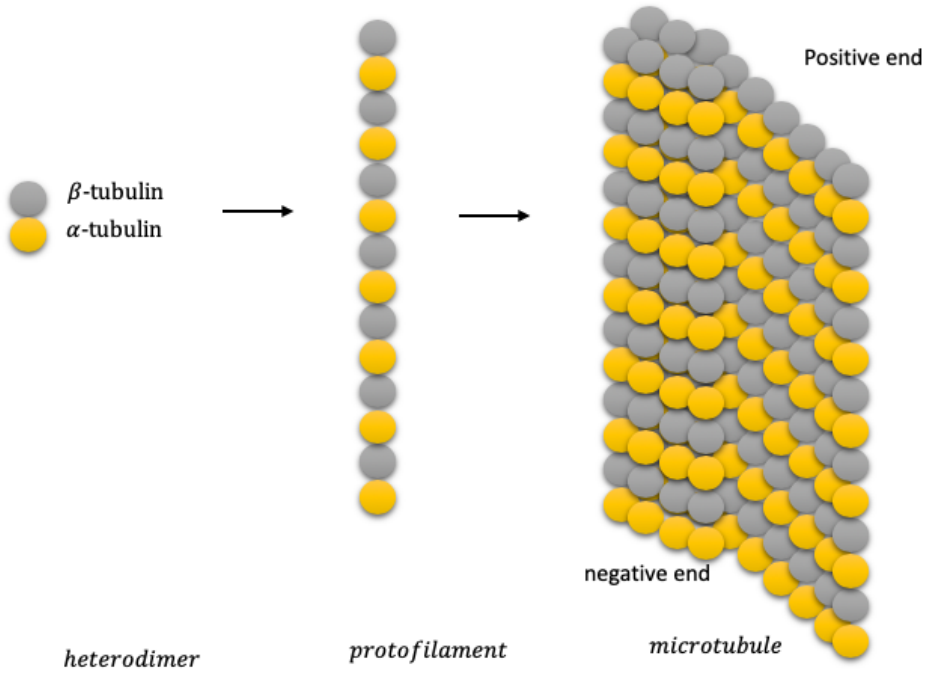


Figure 1.3: Different steps depicting assembly of Microtubule structure.

1.3 Microtubules

Microtubules are polymers made up of a heterodimer. α and β tubulin are the part of this heterodimer that form a microtubule. These heterodimer polymerises in one direction and forms one long chain called protofilament. Due to this uniderctionality in assemply there exists a polarity to the protofilament. When thirteen such protofilaments lies parallel to each other , a microtubule is formed. In the microtubule α tubulin end is the slow-growing end while β end is the fast growing end.

Microtubule is a cylindrical, hollow, rigid polymer with diameter of about 25nm and can polymerise upto a length of $20\mu-$m in cells. The ability of microtubule to constantly switch between depolymerization and polymerization is called dynamic instability. One of the most important as well as interesting events during cell division is segregation of chromosomes and to achieve this the dynamic nature of microtubules is very important [Kirschner 84].

In a cell free tubulin heterodimer exists as GTP bound form and the polymerization and depolymerization of microtubule depend on the status of the bound guanine-nucleotide. When a free GTP bound tubulin gets attached to microtubule the tubulin gets hydrolysed and forms a GDP bound tubulin [Eva Nogales 98]. When the rate of polymerization exceeds the rate of GTP hydrolysis to GDP, the end of microtubule has a GTP cap [David N.Drechsel 94]. But when the hydrolysis rate is more than the incorporation rate then the microtubule starts depolymerizing. This switch of rapid growth and shrinkage is called dyamic instability where “catastrophe" is when there is a rapid shrinkage and “rescue" is when depolymerised microtubule starts growing again (see Fig. 1.4).

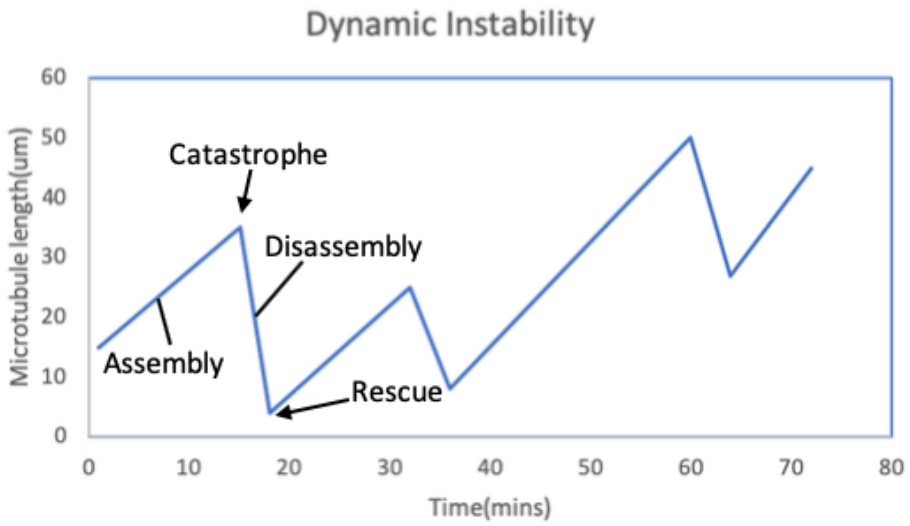


Figure 1.4: Dynamic instability in microtubules explaining switch of catastrophe and rescue.

1.4 Motor proteins

A class of molecular machines that can walk along the cytoplasm of a cell are called motor proteins. Motor proteins drive their energy by converting chemical energy to mechanical energy by the ATP hydrolysis. We have three groups of motor protein known to us dyneins, kinesins and myosins. Myosin is found to be

walking along actin filaments while dynein and kinesin move along microtubules (see Fig. 1.5). For the purpose of this thesis we will be concentrating on kinesin motor protein.

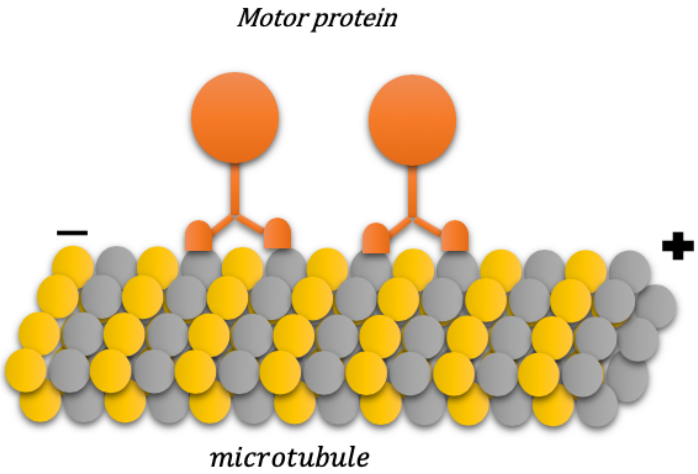


Figure 1.5: Motor proteins walking on microtubules.

1.4.1 Kinesin motor protein

Kinesins are the family of motor proteins which are involved in functions like transporting cargo within the cell, mitosis and meiosis. In the structure of kinesin there are 3 domains a N-terminal, a motor domain and a C-terminal. There is a presence of two binding sites on the motor domain, one for ATP and another to helps the protein to walk on the MT. Since MT has a polarity it is possible to assign directionality to motion. Previously kinesins were considered as positive directed proteins but recently in studies it has been observed that few kinesins can move in negative direction like klp2 (kinesin-14) in fission yeast and is important for sliding apart anti parallel microtubules during mitosis.

Kinesin family is divided into 3 sub groups on the basis of the the positioning of ATP binding site being in N-terminal, M-terminal or C-terminal and are

called N-kinesin , M-kinesin or C-kinesin respectively. Their detailed functionality are given in the table below (see Table 1.1). Few kinesins are involved in bipolar spindle assemble during mitosis [Shirasugi 19] and help in ensuring the coordinated chromosome segregation. These collectively are called mitotic kinesins [Marvin E.Tanenbaum 10, Amber L.Younta 15] for example kinesin-5 and kinesin-14.

N-kinesin	M-kinesin	C-kinesin
Position: N-terminal	Position : middle	Position: C domain
These motors show a plus directed motion	Instead of walking these motors act as MT-depolymerase	These motor show a minus end directed walk
Kinesin-1 to kinesin-12	Kinesin-13	Kinesin-14

Table 1.1: Types of kinesin motors

One way of understanding the importance of the roles played by a protein is by perturbing it. This can be done by changing the level of protein translated or by changing its gene sequence. I will be using these two methods to understand the role of kinesin-5 and kinesin-14 in fission yeast. A few questions that I will be addressing are:

1. How does the inability of motor skills of kinesin-5 that need Atp-binding activity affect the cell growth ?

2. How does over-production of kinsein-5 affect cell growth ?

3. What happens when different kinesin-14 is expressed in a fission yeast cell in terms of growth rate,terminal phenotype and localization ?

1.5 Plan of the thesis

The plan of the thesis is as follows. In Chapter 2, I describe all the methods used for the experimental part of the thesis. In Chapter 3, I will be discussing how the mutants of kinesin-5/cut-7 affect the growth rate and terminal phenotype of

a fission yeast cell. In Chapter 4, I will discuss the results obtained for human kinesin : kifc1, kifc2, kifc3, kif25, Eg5 and macaque kif25 in fission yeast host cell and how it affects the growth rate, terminal phenotype and localization. In Chapter 5, I will be discussing about a theoretical study of microtubule instability which is experimentally known to be important during mitosis. Finally, in Chapter 6, I will present a consolidated understanding of the entire work done in this thesis.

Chapter 2

Experimental Methodologies

In this chapter, we discuss the experimental procedures that we have used in order to explore the role of the motor protein kinesin during mitosis. The different methodologies that we have explored are transformation, spot test, growth curve analysis, terminal phenotype and localization experiments.

Transformation analysis

Transformation, as the word suggests, is the process of tweezing the cell to bring a change in it. One of the way is by inserting a plasmid DNA of interest into the cell. In this case my gene of intrest is kinesin i.e cut7, cut7-rigor, cut7-22, Hkifc1, Hkifc2, Hkifc3, Hkif25 and mkif25 whose plasmid DNA were introduced to a host cell which was then examined.

We outline the procedure involved in detail.

- About 50ml host strain was kept overnight to grow up to exponential level (7×10^2 cells/μl) and then centrifuged and collected.
- It was then washed with 1ml LiAc/TE buffer and re-suspended in the 100μl of the same.
- After this we add 7.5μll of pre-boiled salmon sperm DNA and 2μl of respective plasmids. Then we keep this on slow shaker for 10 minutes.

- Add 260μl of 40% PEG solution and keep on shaker for an hour at 30 degrees.
- After that we add 43μl of DMSO was and give a heat shock by keeping the mixture at 42 degree Celcius water bath for 10 minutes.
- Next we spin down and wash the cells with YES(yeast extract with 5 supplements), kept on shaker after re-suspeding in 500μl YES for one and half hours at 27 degree Celsius.
- Finally they are plated on PMG-Leu plates at different dilutions.

Spot test

The spot test or spot assay experiment is done on a media plate to check the growth of the organism in a particular media. The media used for this experiment was PMG−leu−thi and PMG−leu+thi since the marker on my transformants is leucine and the presence and absence of thiamine switches off and on NMT promoter.

We outline the procedure involved in detail.

- All of the strains has NMT promotor so the gene expresses itself only in the absence of thiamine. Firstly a 10ml of strain was grown in PMG-Leu+thia media until it reached log phase.
- Once it reaches log phase the cells were centrifuged washed with Sterile water.
- Now resuspend the cells in 1ml sterile water.
- After that sterilize the well plate with ethanol and flame.
- check the concentration of each culture and calculate how much volume is needed to make it 2×10^2 cells/μl.
- Now add the calculated volume into the well and the diluted it further with $1:10$ ratio for 5 times.

- Finally plated all the above cells from the well on PMG−Leu+thi and PMG−Leu−thi plates and observed the growths until $3-4$ days at 27 degree Celsius, 30 degree Celsius, 33 degree Celsius and 36 degree Celsius temperatures.

Growth curve analysis

Growth curve analysis is a technique to study and understand the rate of growth of the organism. For all the experiments the observation started at zero hour with concentration approximately 2×10^6 cells/ μl and then data was taken from $12-24$ hours.

We outline the procedure involved in detail.

- Day 1 : The cells were kept to grow in PMG−Leu culture till log phase and then it was filtered and shifted to a new culture where thiamine is absent i.e PMG−Leu−thiamine with a final concentration of 2×10^6 cells/ μl.

- Day 2 : After about 15 hours of shifting the concentration was checked. Data up to $24-25$ hours at an interval of 2 hours was taken using cell counter machine.

- The data recorded was plotted to analyse.

Terminal phenotype

Terminal phenotype as the name suggests is the characteristics shown by an organism at the end of its development or shown towards the last stage of development.For *Schizosaccharomyces pombe* that stage reaches in about $20-22$ hours.

We outline the procedure involved in detail.

- Day 1 : Let the cells grow in PMG−Leu culture till log phase 7×10^6 cells/ μl, then filter the culture and shift it to a new culture where thiamine is

absent i.e PMG−Leu−thiamine with a final concentration of 2×10^6 cells/ μl.

- Day 2 : After about 20 hours of shifting of cells we observed the culture under microscope at the end of 24 hours.

Localization experiments

Protein localization experiments gives us information about where that particular protein is present during the time of observation. This is achieved by adding a fluorescent colour tag to the protein during transformation and then observing it under a epi-fluorescence microscope.

We outline the procedure involved in detail.

- Day 1 : let the cells grow in PMG−Leu culture till log phase 7×10^6 cells/ μl. Then filter the culture and shift it to a new culture where thiamine is absent i.e PMG−Leu−thiamine with a final concentration of 2×10^6 cells/ μl.

- Day 2 : After about $14 - 16$ hours of shifting cells, start taking observations under microscope up to 20 hours at a gap of every two hours.

Recipe to make PMG media

- Add 3g/l of potassium hydrogen phthallate
- Add 2.2g/l of Disodium phosphate
- Add 3.75g/l of L-glutamic acid, monosodium salt
- Add 20gm/l of glucose
- Add 20ml/l of salt stock solution
- Add 1ml/l of vitamin stock solution

- Add 0.1ml/l of mineral stock solution
- Add 100ml/l Ade Ura mixture
- Add 20ml/l of Leu His Lis mixture
- Add 0.5ml/l if thiamine stock solution (Do not add in case of making PMG-thiamine solution)

Chapter 3

Kinesin-5 : Fission yeast

Kineisn motor protein play a very important role in cell cycle. In many eukaryotes, kinesin-5 along with kinsin-14 are responsible for anti parallel movement of spindle mictrotubules and for spindle pole body separation. In fission yeast kinsesin-5 protein is cut7 and kinesin-14 has two proteins klp2 and pkl1. Two very important step in mitosis is separation of SPB towards opposite ends and separation of chromosomes. Here I show that both the roles of cut7 are essential and repression or over-expressing of any would be toxic for the cell.

Kinesin-5 and its mutants

Kinesin-5 is a member of N-kinesin family. Hence it follows a plus ended motion as was originally seen in *Aspergillus nidulans*. In most of the eukaryotes, Kinesin-5 (budding yeast : Cin8 and Kip1, fission yeast : Cut7 and human : Eg5) is essential for mitosis [Blangy 95, Enos 90, Hagan 90, Heck 93, Le Guellec 91].

Talking about fission yeast, outward force generated by kinesin-5/cut7 is necessary to establish and maintain the bipolar spindle assembly (see Fig 3.2). Cut7 forms a homotetramer from four identical sub units which helps it to crosslink antiparallel MTs starting from the opposite poles of the mitotic spindle and induces sliding apart of microtubules [Kashina 96, Kapitein 05] (see Fig 3.1).

Cut7 plays an essential role for SPB separation [Hagan 90, Shirasugi 19]. Dele-

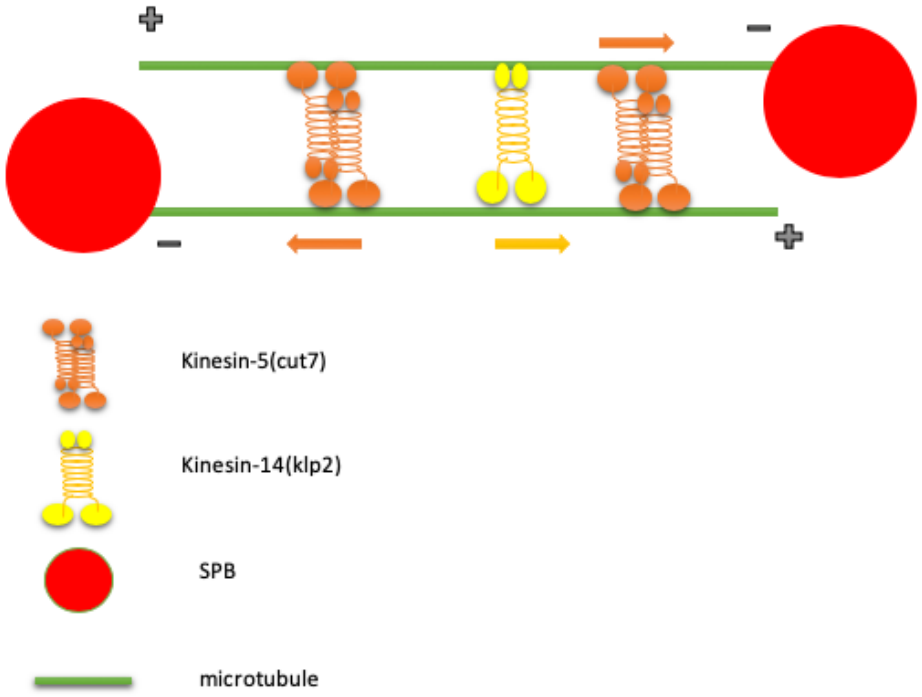


Figure 3.1: Schematic of kinesin-5 forming a cross linker and exerting a outward force on the anti parallel spindle microtubules.

tion of cut7 is lethal [Masashi Yukawa 18] and results in monopolar spindle and an unseparated spb and hence resulting in a mitotic arrest [Enos 90, Hagan 90, Heck 93, Hoyt 92, Mayer 99, Roof 92].

cut7-rigor

Cut7-rigor is a point mutation of cut7 within the N-terminal motor domain. This mutation results in a dis functional ATP binding site. This results in a inability of the kinesin motor protein to do most of the motor activities since it involves conversion of chemical energy into mechanical energy by hydrolysis of ATP.

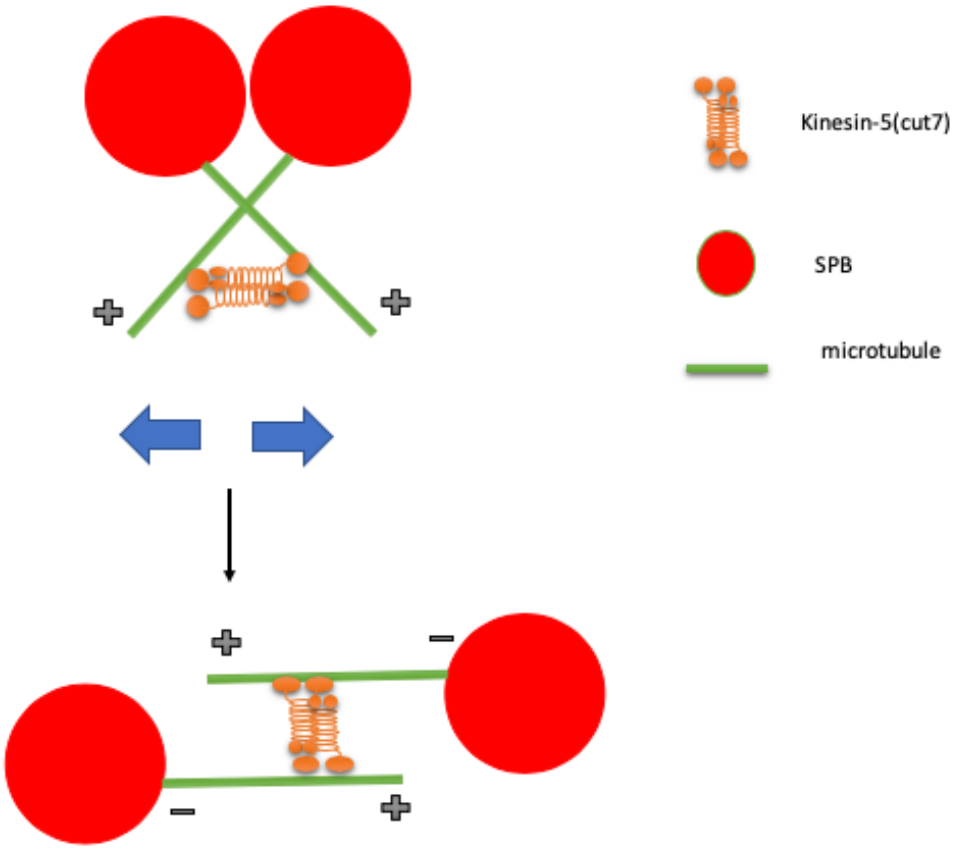


Figure 3.2: Schematic of force exerted by kinsein-5 during SPB separation.

cut7-22

Cut7-22 is a point mutation of cut7 at within the C-terminal tail. this mutant is a temperature sensitive mutant. cut7-22 is sensitive to temperature higher than 30 degree Celsius [Masashi Yukawa 18].

All the plasmids had nmt-promoter (no message in thiamine) on them. The presence of this promoter affects the transcription activity in a small concentration of thiamine. There are three variants of this promoter nmt1 which results in a over production of the protein. nmt41 results in a mild expression of protein while nmt81 is used for minimal expression of protein. So in the presence of thiamine

the promotor will be off and there will no expression of protein from plasmid (gene of intrest) except that from the host cell. In the absence of thiamine, the protein will be expressed along with that of the host cell. These promoters were used to control the protein expression levels throughout the experiments.

3.1 Results

Cut7-over expression (oe)

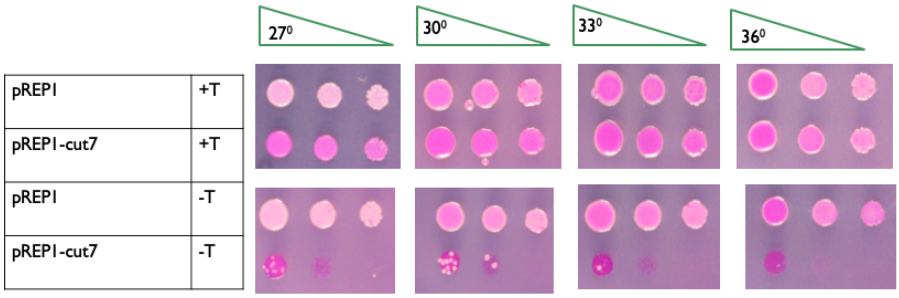


Figure 3.3: Spot assay results for four different temperatures at the end of day-3 in PMG-Thiamine and PMG+Thiamine media for cut7-over expression

- In Fig. 3.3 pREP1-cut7 is the strain showing over expression cut7. The presence of phloxine B dye on agar plates helps in identifying dead cell (pink coloured) and hence indicates toxicity. pREP1 acts as a control and +T and −T shows presence and absence of thiamine. So pREP1-cut7 in the absence of thiamine shows the result for over expressed cut7.

- In Fig. 3.4 the growth curve shows a decrease in growth rate in the absence of thiamine hence indicating toxicity and are similar to spot assay results .

- As indicated in the Fig. 3.5, the green colour (GFP tagged) represents nuclear envelope and SPB (globular structure) and red colour (mcherry tagged) indicates MTs.

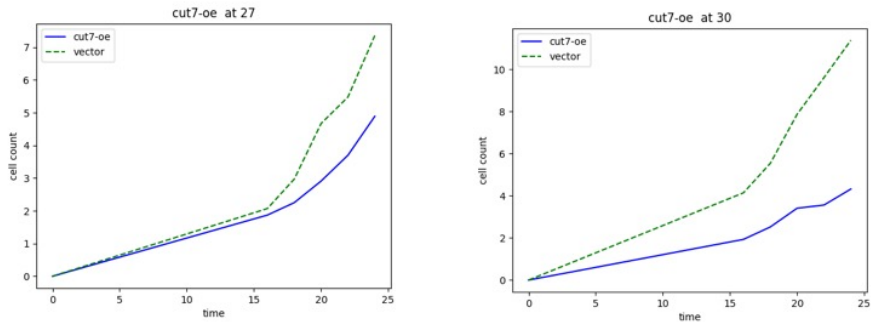


Figure 3.4: Growth curve results of cut7-oe at two different temperatures in PMG-Thiamine and PMG+Thiamine media .

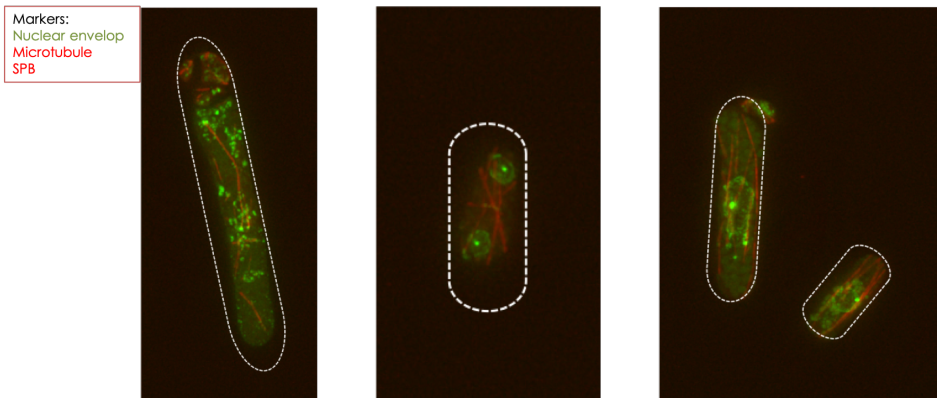


Figure 3.5: Terminal phenotype of cut7-oe.

- In Fig. 3.5, after about 24 hours almost 80% of cells were observed to be longer than usual and an unusual nuclear shape was also observed in more than 50% of cells.

- As indicated in the Fig. 3.6, the green colour(GFP tagged) represents SPB and red colour(mcherry) indicates MTs.

Cut7-rigor

- During transformation we insert a plasma DNA into a host cell. Hence here we have taken two different host cells, one with wild type background and another is a mutant background with cut7-22 in it instead of wild type cut7.

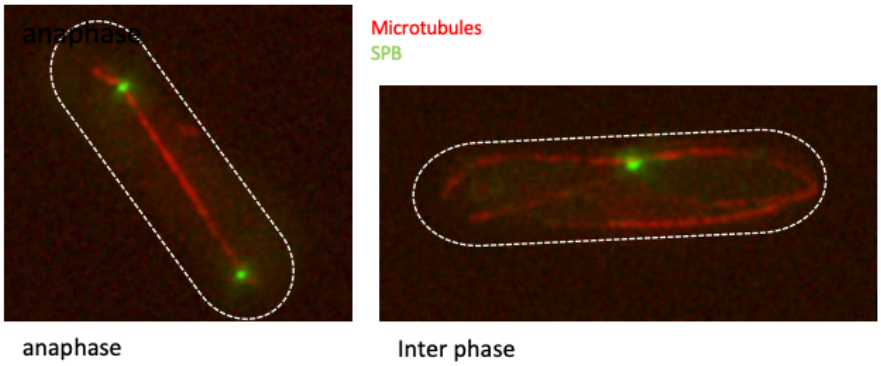
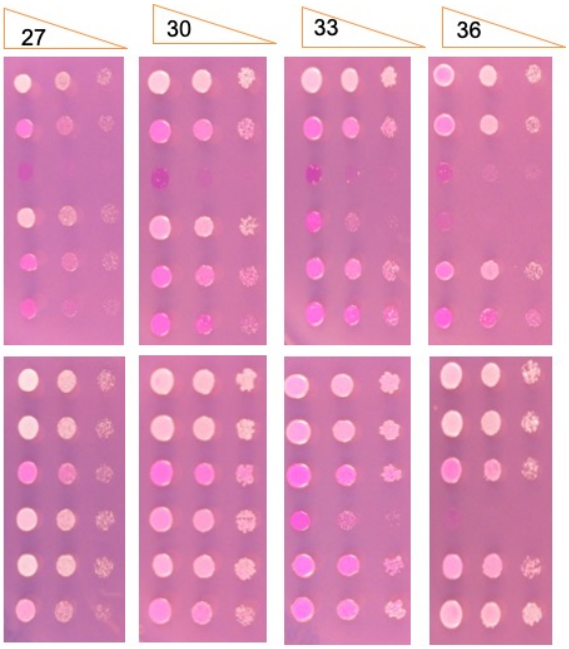


Figure 3.6: Fission yeast vector cells showing position of bipolar SPB and monopolar SPB in anaphase and interphase of mitosis respectively .



pREP81(bg:WT)	-T
pREP81-cut7(bg:WT)	-T
pREP81-cut7-rigor(bg:WT)	-T
pREP81(bg:cut7-22)	-T
pREP81-cut7(bg:cut7-22)	-T
pREP81-cut7rigor(bg:cut7-22)	-T

pREP81(bg:WT)	+T
pREP81-cut7(bg:WT)	+T
pREP81-cut7rigor(bg:WT)	+T
pREP81(bg:cut7-22)	+T
pREP81-cut7(bg:cut7-22)	+T
pREP81-cut7rigor(bg:cut22)	+T

Figure 3.7: Spot assay for four different temperatures at the end of day-3 in PMG-Thiamine media for cut7-rigor in the presence of wild type and cut7-22.

- pREP81-Cut7rigor (bg:WT) in the absence of thiamine will produce very small amount of cut7-rigor protein along with cut7 and even that small amount is found to be highly toxic for the cell.
- While pREP81-cut7rigor (bg:cut7-22) produces small amount of cut7-rigor

protein along with cut7-22.as cut7-22 is a temperature sensitive mutant hence it is not visible at high temperature. As the previous results suggest, small amount of cut7-rigor is toxic for wild type cell but in case of cut7-rigor along with cut7-22 mutant as host rescues the temperature sensitivity of the cell.

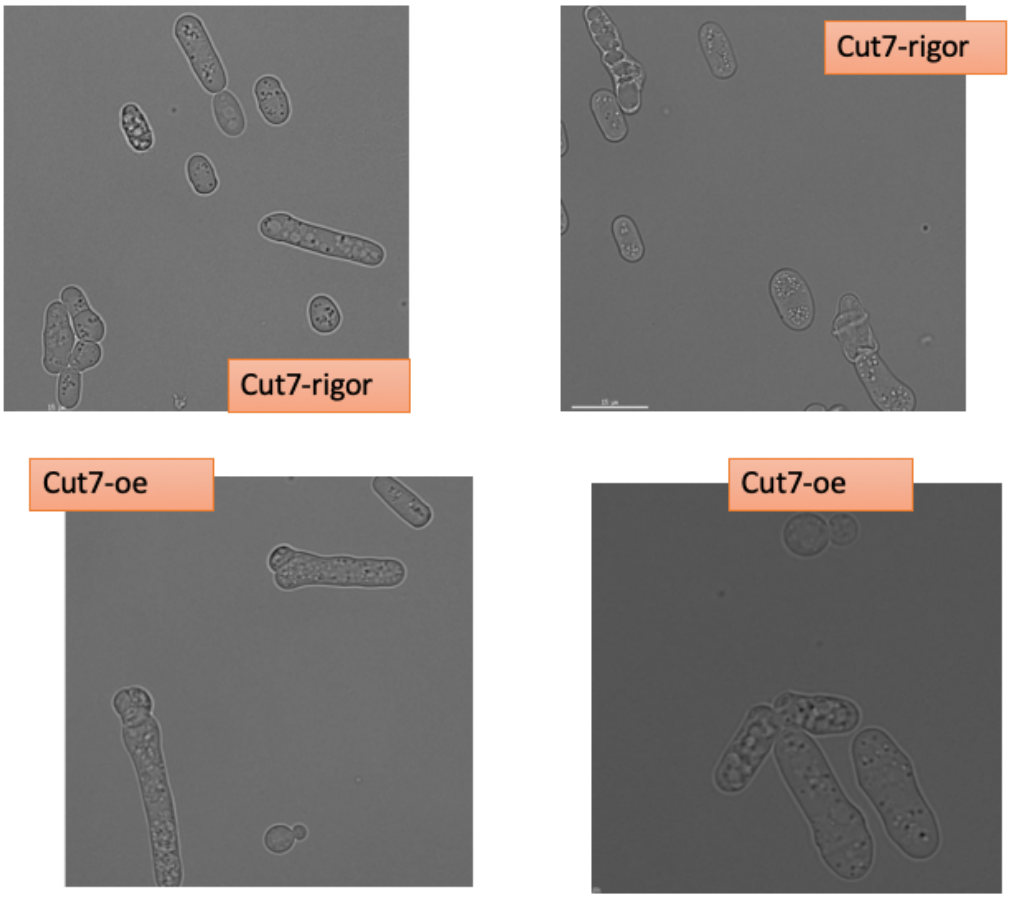


Figure 3.8: Abnormal cell size of cut7-oe and cut7-rigor.

- In Fig. 3.9, cut7-rigor in wild type background shows a lower growth rate curve in the absence of thiamine when compared to that in the presence of it. This result co-relates to that observed in the spot assay.

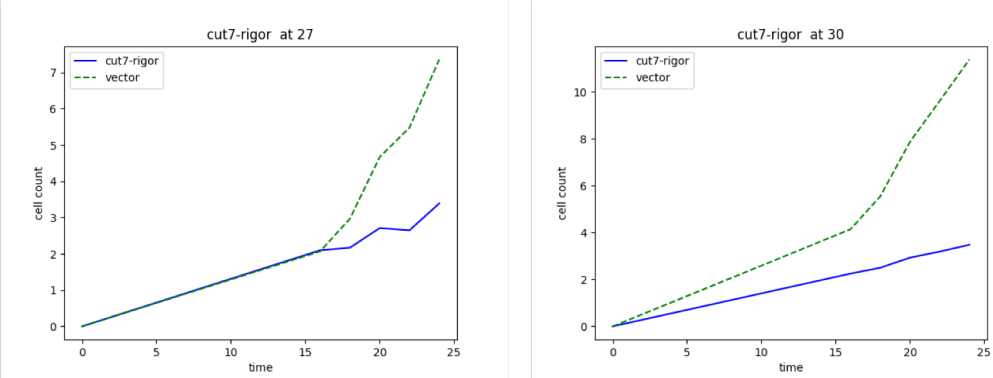


Figure 3.9: Growth curve of cut7-rigor at two different temperatures in PMG-Thiamine and PMG+Thiamine media.

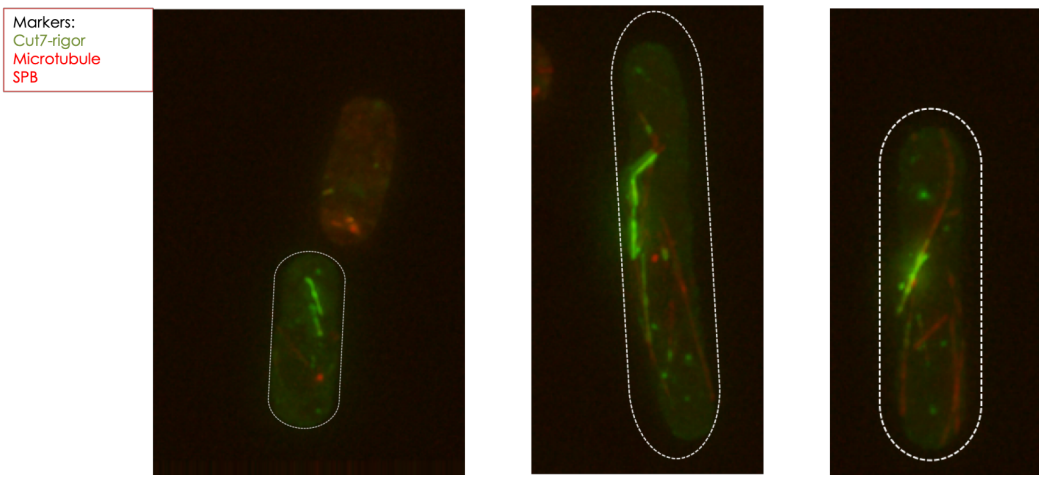


Figure 3.10: Localization of cut7-rigor.

- As indicated in the Fig. 3.10, the green colour (GFP tagged) represents cut7-rigor protein while red colour (mRFP and mcherry tag) indicates SPB (globular) and MTs (tubular structure) respectively.
- In total by the end of 24 hours 80% of cells were longer than usual and had distorted nuclear shape. Very few mitotic cells were observed and the localization was majorly observed on the microtubules.
- As indicated in the Fig. 3.11, the green colour(GFP tagged) represnts nuclear envelope and SPB(globular) while red colour(mcherry tag) indicates MTs(tubular structure)

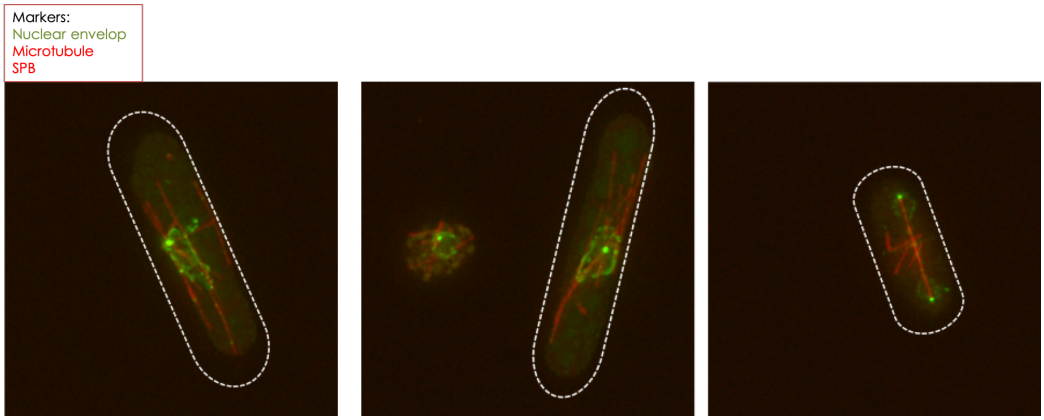


Figure 3.11: Terminal phenotype of cut7-rigor

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- Terminal phenotype and localization of cut7-rigor in temperature sensitive background was not observed.

Chapter 4

Kinesin-14 : The human kinesin

In this chapter, I am going to report observations and analysis of human kinesins : kifc1, kifc2, kifc3, kif25 and macaque kinsein kif25 expresses itself in a fission yeast cell.

4.1 Different kinesin-14 members in mammals

Kinesin-14 is a part of *c* family kinesin which means motor domain is in the *c* terminal and walks in the direction of the negative end of the microtubule. These three domains are : an N-domain, a central coiled-domian and a C-terminal motor domain that possess the ATP binding activity [Zhen-Yu She 17].

The kinesin-14 motor proteins (,Kar3 in *Saccharomyces cerevisiae* ,Pkl1 and klp1 in *Saccharomyces pombe* and XCTK2 in *Xenopus laevis:*) has a motor domainin C-terminal and is important for the organization of spindle microtubule during mitosis [G. Fink 09, M. Braun 09]. Kifc1, Kifc2 and kifc3 are the three members of kinesin-14 family in mammals :

4.1.1 kifc1

One of the kinesin-14 in human is HSET/KIFC1 which works fine in cell containing two chromosomes and is responsible for centrosome clusturing in brest cancer cells with supernuemary chromosomes [Masashi Yukawaa 18, Pannu 15,

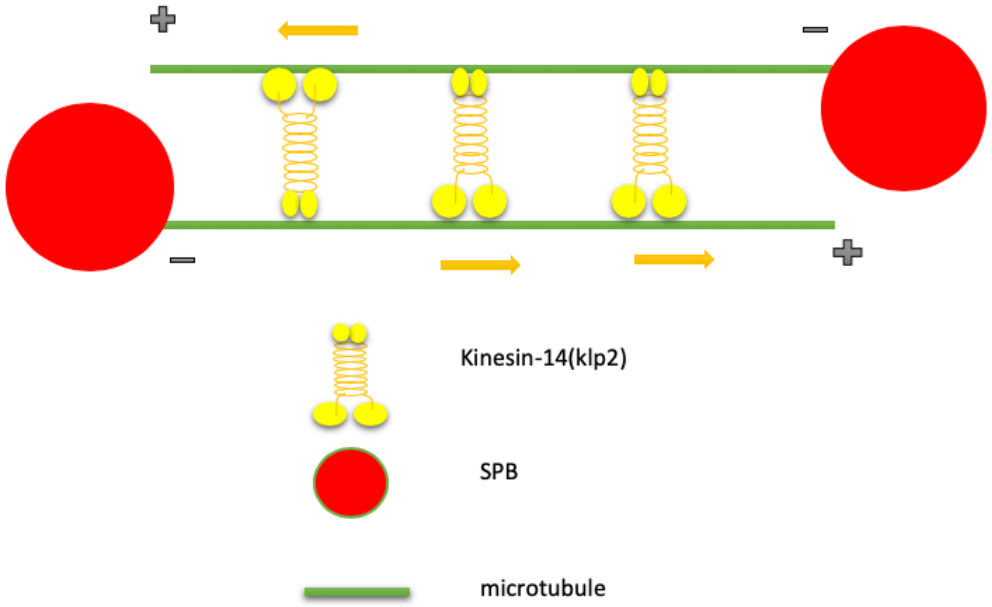

Figure 4.1: Schematic of kinesin-14 motor exerting inward force on anti-parallel spindle microtubule during anaphase.

Shrikant Pawar 14, Helena Grinberg-Rashi 09]. Targeted inhibition of Hset might give way to kill cancer cells.

4.1.2 kifc3

One of the main events in mitosis is separation of chromosomes. Tto avoid premature separation a strong cohesion force is required. Premature centrosome separation can lead to abnormalities like microtubule-depending nuclear translocation, which rreaches high eccentric nuclear positioning leading to disruption of the cortical spindle positioning machinery. Kifc3 exist as a hometetramer and holds the two chromosomes together via a special microtubule network. This acts as a main driving force to avoid premature spindle formation which later gets deactivated by NEK2 [Yujie Cao 20, Shoji Hata 19].

4.1.3 EG5

EG5 is an evolutionary conserved kinesin. Bipolar-spindle formation is one of the very essential function carried out by EG5. Being a kinesin-5 it produces push force in the outwards direction between the two centrosomes and the presence of Eg5 as a homotetramer helps this motor to cross-link and slide apart antiparallel microtubules leading to bipolar-spindle formation. It is also observed that premature centrosome separation can lead to wrong spindle orientation at the begining of mitosis and aberrant nuclear positioning, which may account for the error in chromosome segregation [Shoji Hata 19, Justin Decarreau 17].

4.1.4 Kif25

Kif25 is minus-end microtubule dependent motor protein and behaves like a negative regulator of separation of centrosome and is important to avoid premature separation of centrosome during interphase see Fig.4.2. This is required to maintain a centered nucleus which further ensure the stability of spindle at the onset of mitosis. During interphase maintenance of the nucleus at the centre is ensured by Kif25 and it is also to ensure a stable orientation of spindle at the start of mitosis [Justin Decarreau 17]. Apart from mitotic functions that we know, kinesin-14

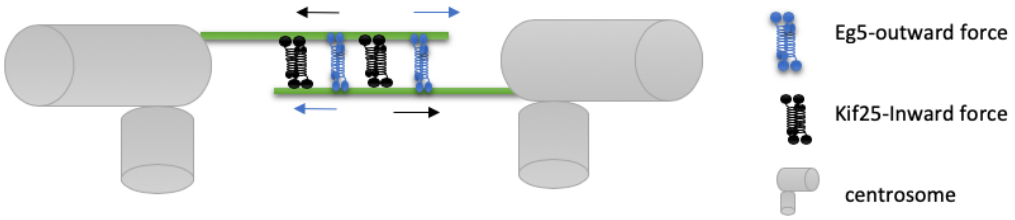

Figure 4.2: Schematic showing microtubule-dependent centrosome tethering pathway.

motor KIFC3 plays an important role in controlling dendrite development and organizing dendritic microtubules [Justin Decarreau 17].

4.2 Results

Hkif-c1

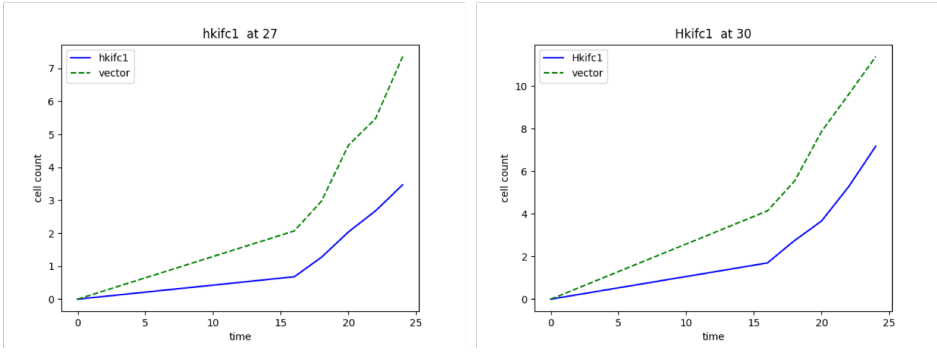

Figure 4.3: Growth curve of Hkif-c1 at two different temperatures in PMG-Thiamine and PMG+Thiamine media.

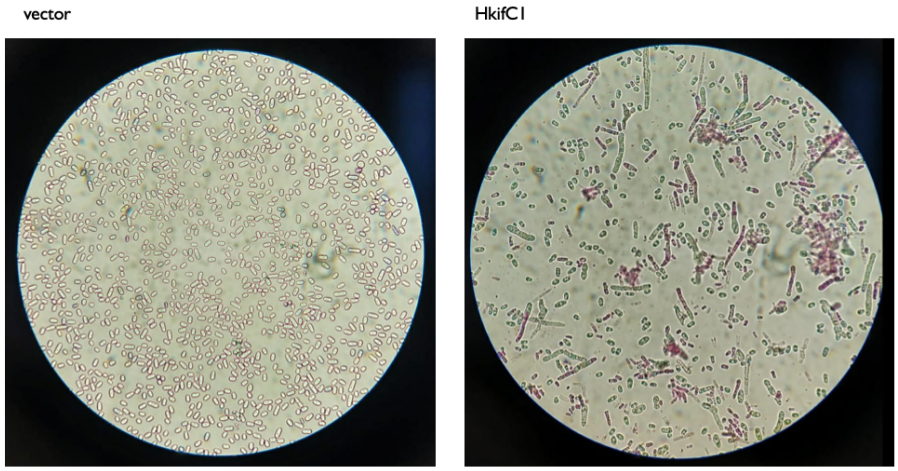

Figure 4.4: Comparison of cell size of vector and hkifc1 on a Simple Microscope.

- pREP41-Hkif-c1 plasmid was transformed into fission yeast host cell hence we have mild production of human kinesin protein along with host cell proteins. In Fig. 4.3, we see that the absence of thiamine shows a decline in growth curve of hkif-c1 in suggests that it is toxic for the cell.

- Fig. 4.4 is an image from a table microscope with the same setting which can help in comparing the cell size and structure. As we can observe, the cell shape was evidently abnormal suggesting that there might a hindrance during mitosis (cells were incubated at 27 degrees).

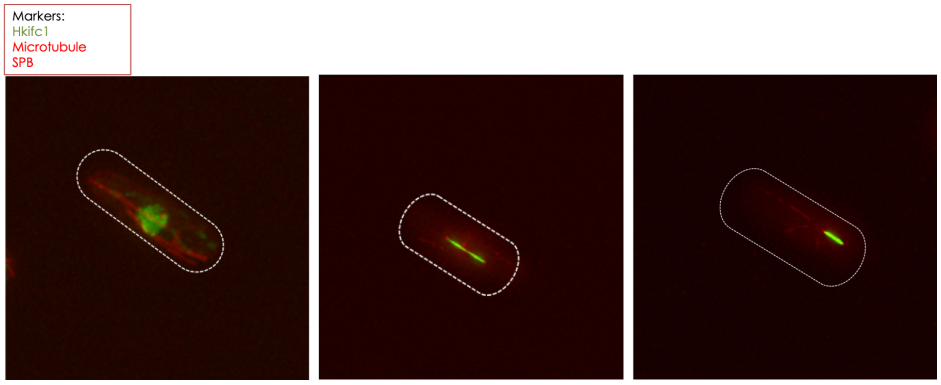

Figure 4.5: Localization of Hkif-c1.

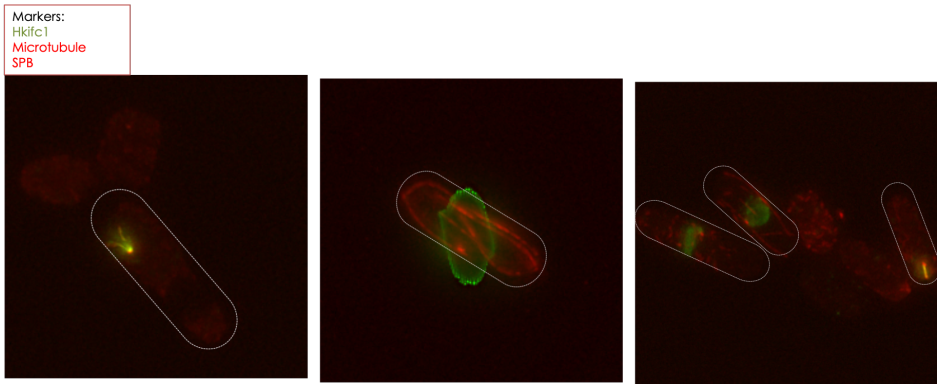

Figure 4.6: Terminal phenotype of hkif-c1.

- As indicated in the Fig. 4.5, the green colour (GFP tagged) represents hkif-c1 protein while red colour (mRFP and mcherry tag) indicates SPB (globular) and MTs (tubular structure) respectively.
- At the end of 26 hours 70% of the cell size were a little longer than usual and in $5-10\%$ of them were monopolar.
- Cut7-rigor was getting localized to spindle in mitotic cells while to nucleus in interphase cells.

Hkif-c2

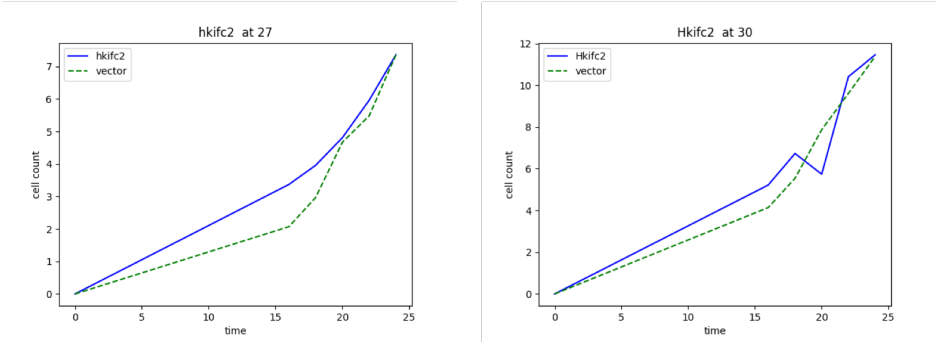

Figure 4.7: Growth curve of Hkif-c2 at two different temperature at two different temperatures in PMG-Thiamine and PMG+Thiamine media

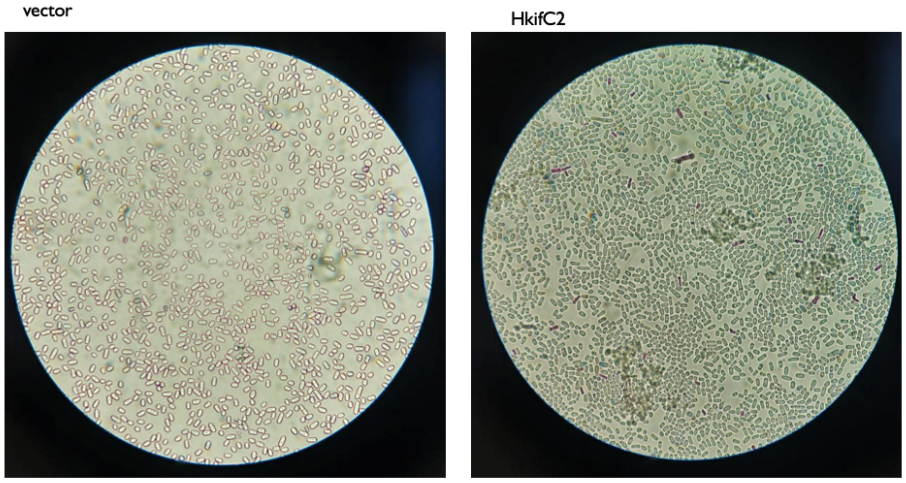

Figure 4.8: Comparison of cell size of vector and hkifc2 n a Simple Microscope

- pREP41-Hkif-c2 plasmid was transformed into fission yeast host cell. Hence we have mild production of human kinesin protein along with host cell proteins. In Fig. 4.7 absence of thiamine does not shows a significant decline in growth curve of hkif-c2.
- Fig. 4.8 is an image from a table microscope with the same setting which can help in comparing the cell size and structure. There was not much

difference observed in cell size suggesting that hkif-c2 might not be toxic at this moderate level of expression (cells were incubated at 27 degrees).

Hkif-c3

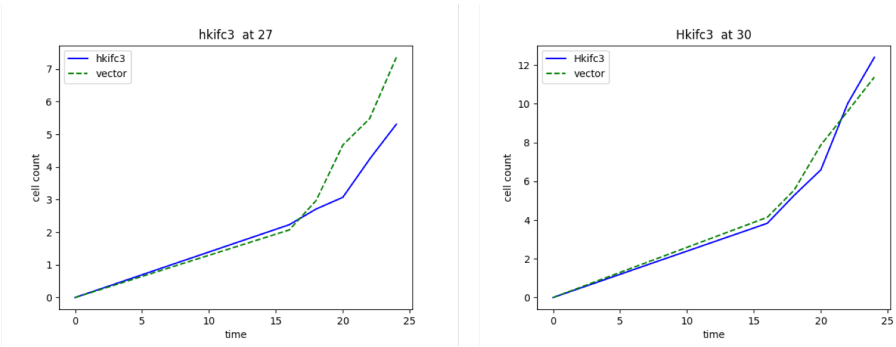

Figure 4.9: growth curve of Hkif-c3 at two different temperatures in PMG-Thiamine and PMG+Thiamine media

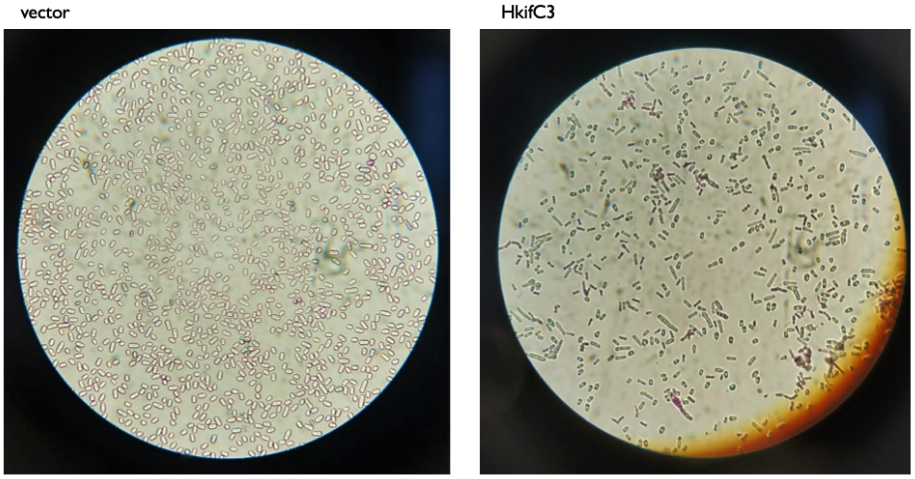

Figure 4.10: Comparison of cell size of vector and hkifc3 n a Simple Microscope

- pREP41-Hkif-c3 plasmid was transformed into fission yeast host cell. Hence we have mild production of human kinesin protein along with host cell proteins. In Fig. 4.9, absence of thiamine shows a slight decline in growth curve of hkif-c3 at 27 degrees but no difference was observed at 30 degrees.

- Fig. 4.10 is an image from a table microscope with the same setting which can help in comparing the cell size and structure. We see difference in cell size suggesting some abnormality in growth (cells were incubated at 27 degrees).

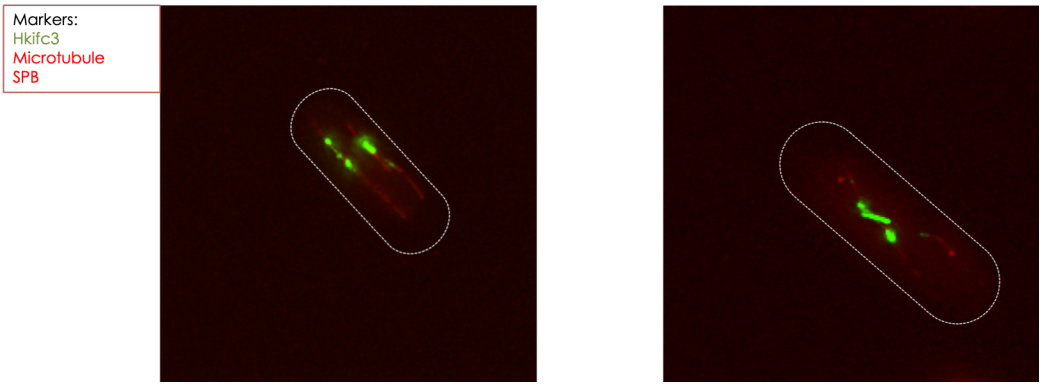

Figure 4.11: Localization of Hkif-c3.

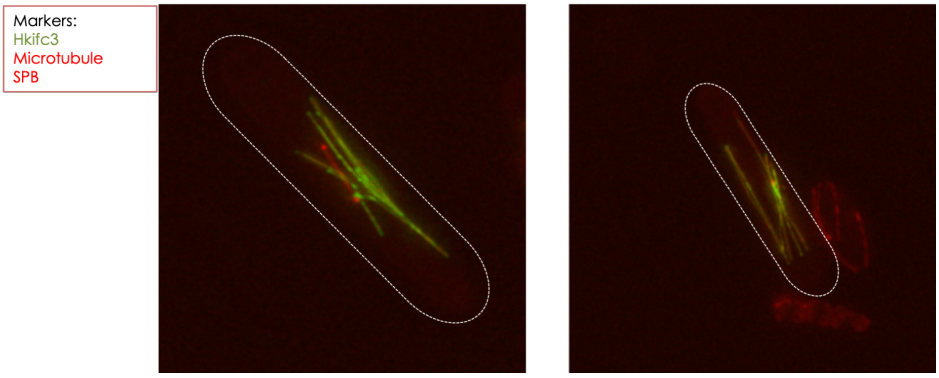

Figure 4.12: Terminal phenotype of Hkif-c3.

- As indicated in the Figs. 4.11 and 4.12, the green colour(GFP tagged) represents hkif-c3 protein while red colour (mRFP and mcherry tag) indicates SPB (globular) and MTs (tubular structure) respectively.
- By the end of 24 hours of incubation at 27 degree Celsius 20% cells were longer observed and very few mitotic cells were observed.
- Hkifc3 was localized to interface spindles 80%.

Hkif25

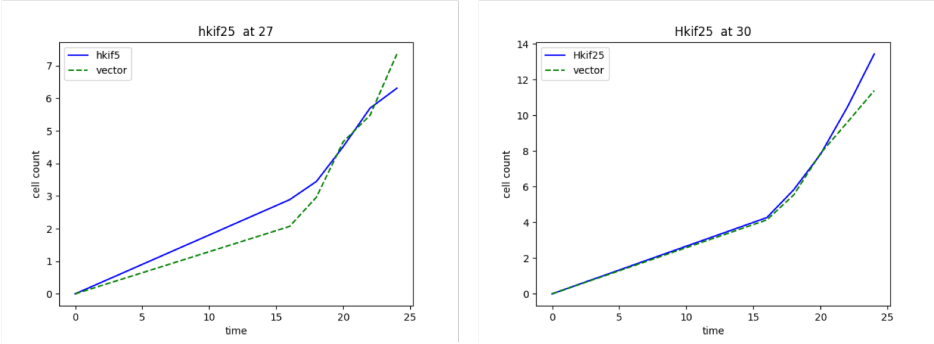

Figure 4.13: Growth curve of Hkif25 at two different temperature in PMG-Thiamine and PMG+Thiamine media.

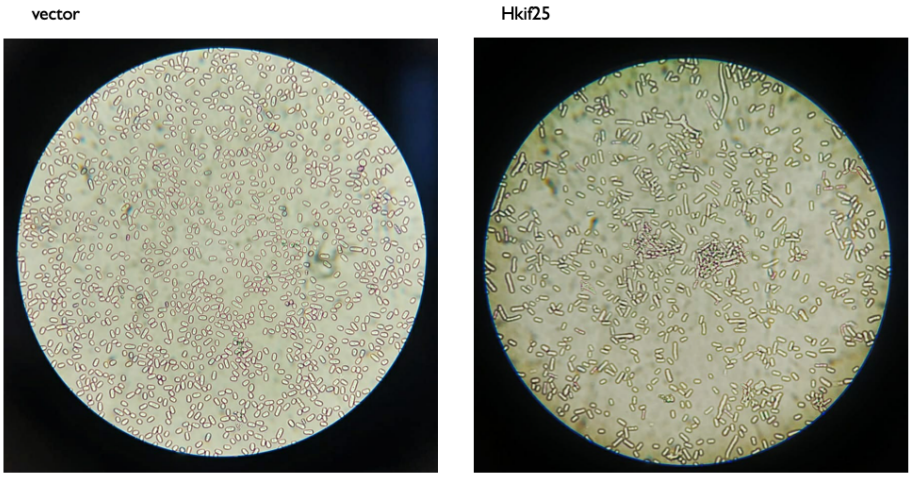

Figure 4.14: comparison of cell size of vector and hkif25 n a Simple Microscope

- pREP41-Hkif25 plasmid was transformed into fission yeast host cell hence we have mild production of human kinesin protein along with host cell proteins .In Fig.4.13 absence of thiamine doesn not shows a significant decline in growth curve of hkif-c3 at 27 degrees or at 30 degrees.
- In Fig.4.14 is a image from a table microscope with the same setting which can help in comparing the cell size and structure shows a few abnormally long cells. (cells were incubated at 27 degrees)

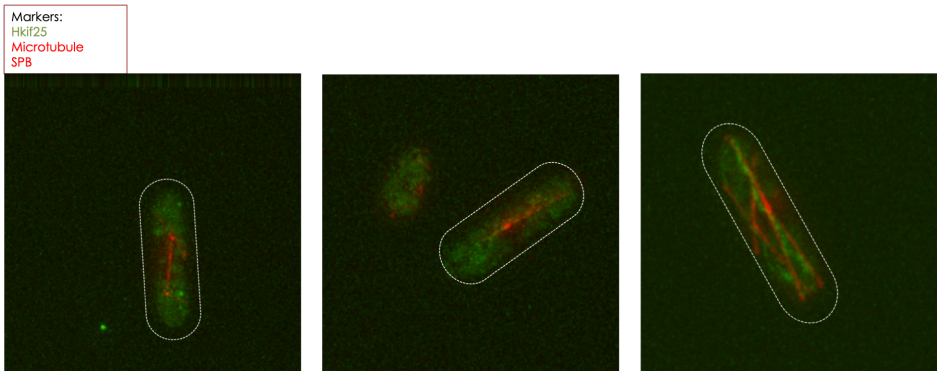

Figure 4.15: Localization of Hkif25

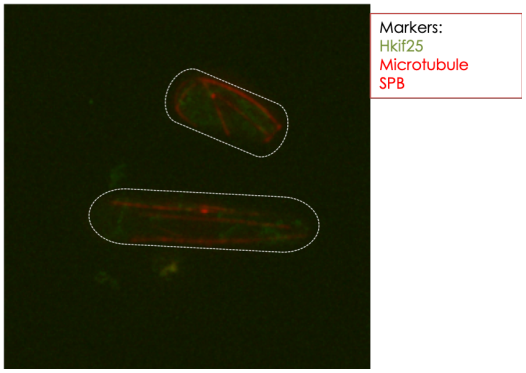

Figure 4.16: Terminal phenotype of Hkif25

- As indicated in the Figs.4.15 and 4.16 the green colour(GFP tagged) represents hkif25 protein while red colour(mRFP and mcherry tag) indicates SPB(globular) and MTs(tubular structure) respectively. Hkif-25 and Hkif-c2 was not very toxic according to curve test and localization was not clear to observe.

Eg5

- pREP41-Eg5 plasmid was transformed into fission yeast host cell hence we have mild production of human kinesin protein along with host cell proteins .In Fig.4.17 absence of thiamine shows a significant decline in growth curve of Eg5 at 27 degrees and a very little difference was at 30 degrees.

- Fig. 4.18 is a image from a table microscope with the same setting which can help in comparing the cell size and structure shows a few abnormally

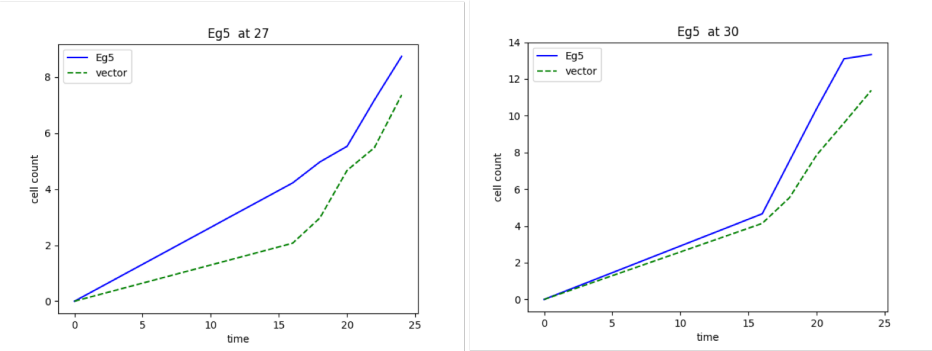

Figure 4.17: Growth curve of Eg5 at two different temperature in PMG-Thiamine and PMG+Thiamine media

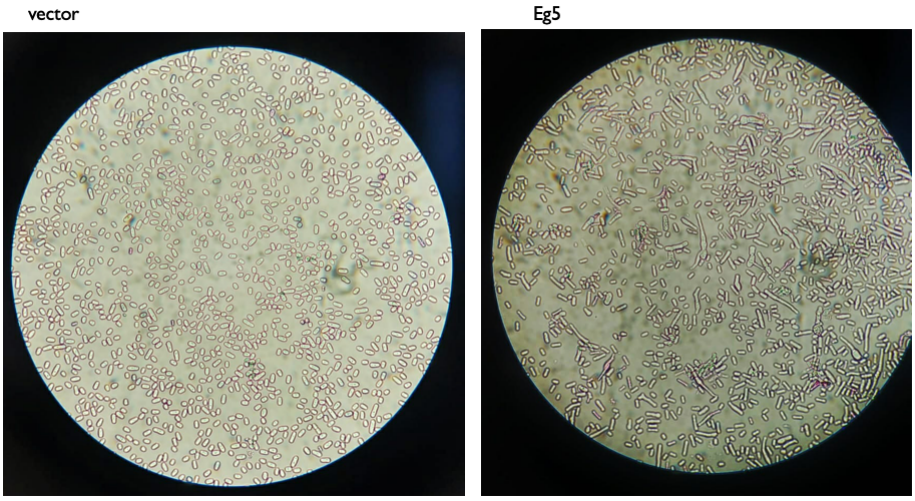

Figure 4.18: comparison of cell size of vector and Eg5 n a Simple Microscope

long cells rest looked of regular size. (cells were incubated at 27 degrees)

- As indicated in the Figs. 4.19 and 4.20 the green colour(GFP tagged) represnts Eg5 protein while red colour(mRFP and mcherry tag) indicates SPB(globular) and MTs(tubular structure) respectively Eg5 was slightly toxic as reflected in the growth curve graph.and at the end 24 hour incubation at 27 degree celcius 40% cells were longer and though very few mitotic cells were observed still Eg5 showed localization at interface spindles

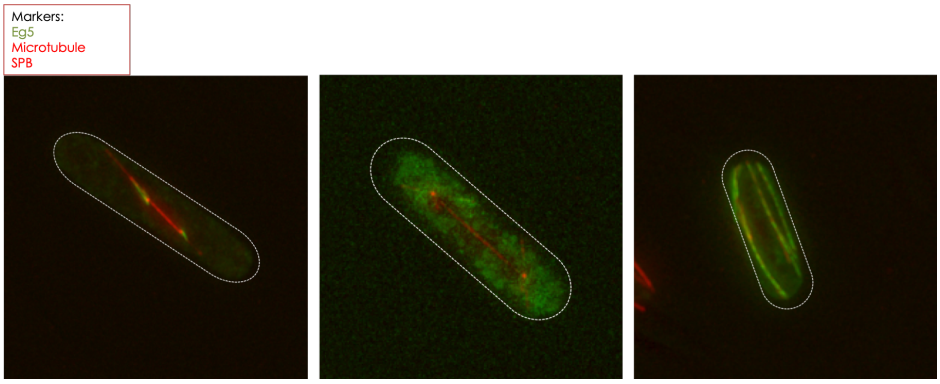

Figure 4.19: Localization of Eg5

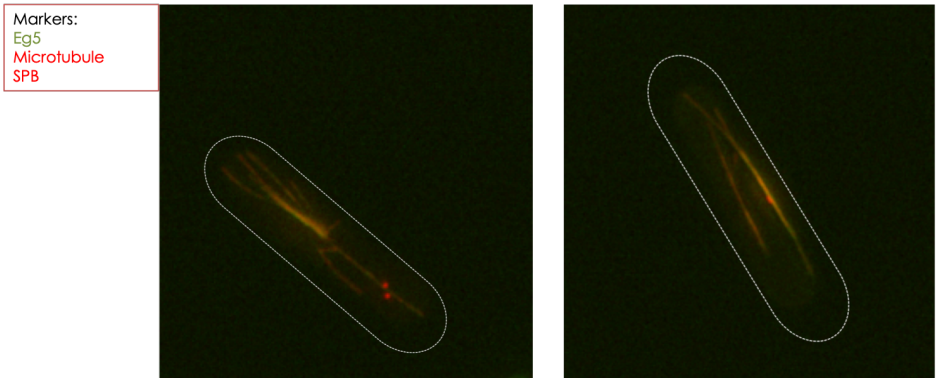

Figure 4.20: Terminal phenotype of Eg5

Mkif25

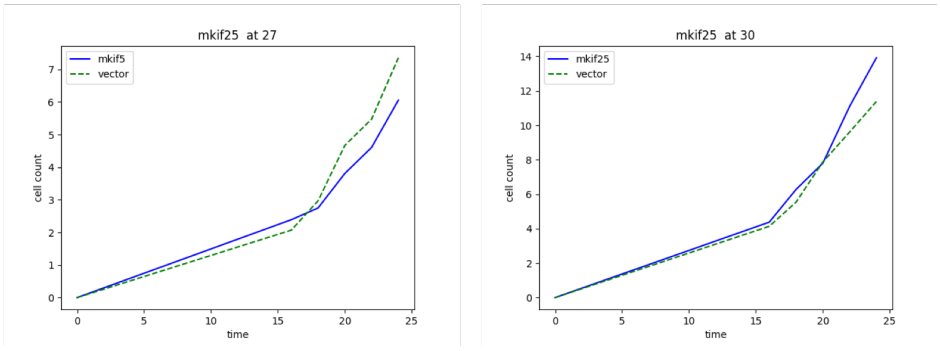

Figure 4.21: Growth curve of Mkif25 at two different temperature in PMG-Thiamine and PMG+Thiamine media

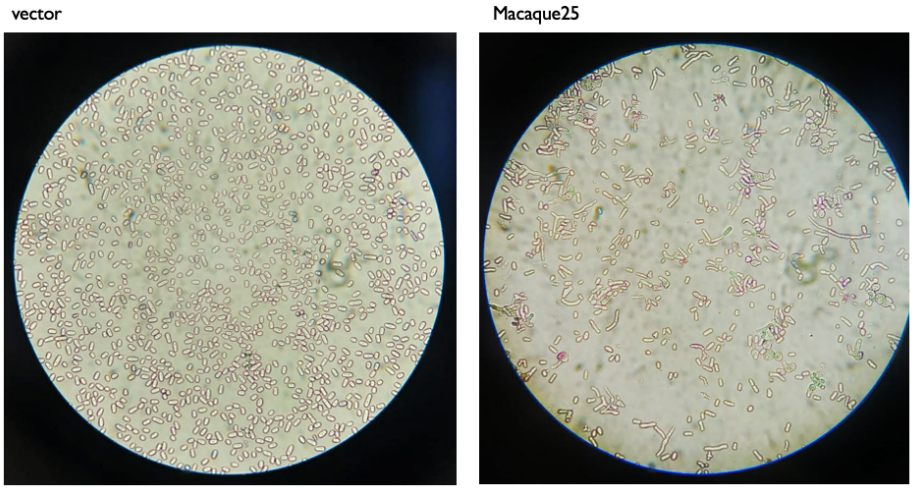

Figure 4.22: comparison of cell size of vector and Mkif15 n a Simple Microscope

- pREP41-Mkif25 plasmid was transformed into fission yeast host cell hence we have mild production of macaque kinesin protein along with host cell proteins .In Fig. 4.21 absence of thiamine shows a little decline in growth curve at 27 degrees and at 30 degrees so we coan say there was a little toxicity observed.

- Fig.4.18 is a image from a table microscope with the same setting which can help in comparing the cell size and structure shows a either longer cells than usual or shorter than a regular cell. (cells were incubated at 27 degrees)

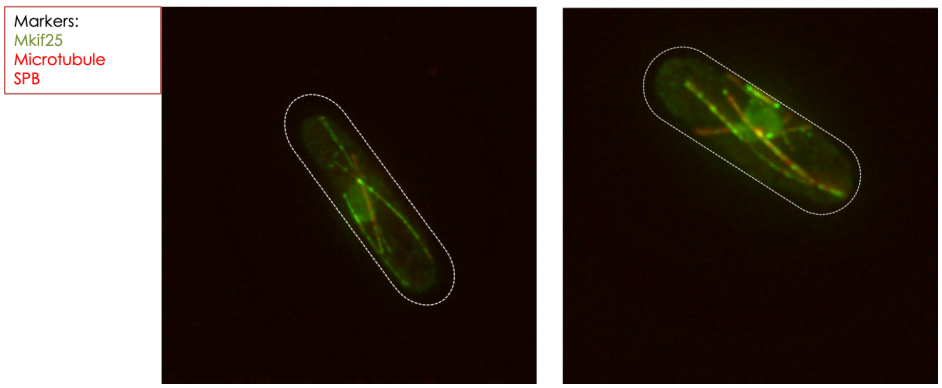

Figure 4.23: Localization of Mkif25

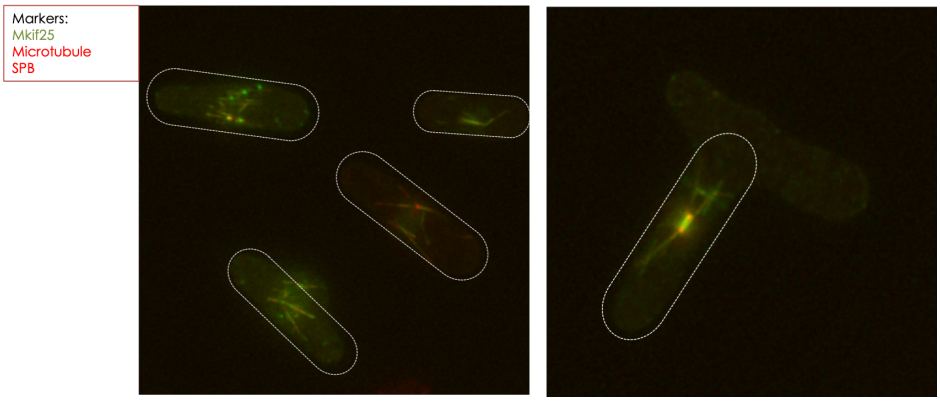

Figure 4.24: Terminal phenotype of Mkif25

- As indicated in the Figs. 4.19 and 4.20 the green colour(GFP tagged) represnts Mkif25 protein while red colour(mRFP and mcherry tag) indicates SPB(globular) and MTs(tubular structure) respectively
- At the end of 24 hour incubation about 60% of the Cell size were a little longer than usual and 10% of cells had T shape observed and in 5 – 10% of them were monopolar.
- Mkif25 localized to spindle in mitotic cells and to nucleus in interphase cells.

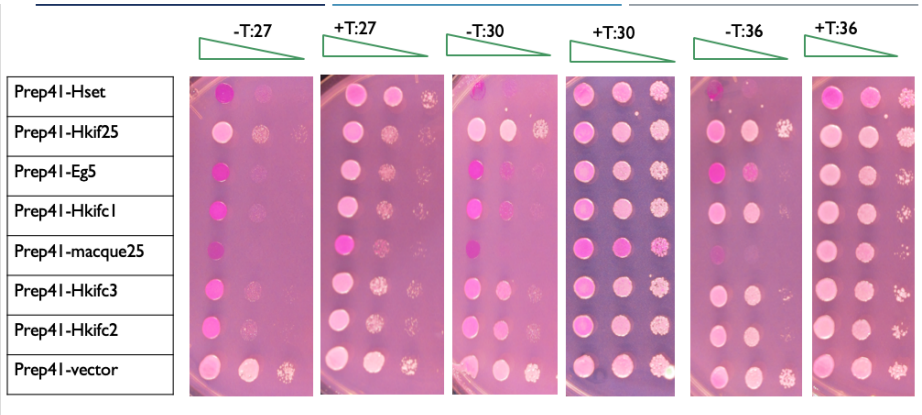

Figure 4.25: spot assay of all above mentioned kinesins for four different temperatures at the end of day-3 in PMG-Thiamine and PMG+Thiamine media

This consolidated data of spot assay shows toxicity level of each of the kinesin discussed above and match with the growth curve results as well.

Chapter 5

Dynamic instability of microtubules

Microtubules (MTs) form the cytoskeleton of a cell hence there are a wide variety of activities that MTs are associated with and which needs a varying level of stability. In this chapter, I will discuss microtubule instability theoretically using kinetic Monte Carlo simulations.

5.1 Dynamic instability in microtubules

Depending on the spatial position within cell and the necessity of the process MTs are involved in various cellular activities like during cell division they play a important role by forming mitotic spindles. One of the other such a major role is acting like a road for motor proteins to walk on and facilitate cargo transport [Alberts 02, Etienne-Manneville 10]. These variations require MTs be able to be in diverse state of stabilities. For example during mitosis, in case of kinetochore capture, MTs are required to be dynamic but at the same time for interphase, MTs have to be more stable in nature.

Experiments have shown that a micrtoubule filament which is growing can suddenly depolymerize leading to an event termed “catastrophe" and then again start polymerizing so that there is growth, an event called “rescue". This is a very good example of dynamic instability and is of great importance. In mitosis, it has been observed experimentally that dynamic instability in microtubules increases. In

interphase, MTs are long while in prophase there are a large number of short and dynamic MTs. In late prophase, MTs are again stabilized. It is in this context that we study theoretically the dynamic instability of MTs.

To achieve this stability MTs interacts with a variety of proteins. Experiments have confirmed that dynamic behavior is a result of the irreversible hydrolysis of GTP-bound tubulin to GDP-bound tubulin in the polymer, rendering the system to be out of equilibrium, and was suppressed in the presence of non-hydrolysable analogues of GTP [Desai 97]. It is also observed that GTp-tubulins on MT exist as a straight on the axis of filament unlike GDP-tubuline which shows a bend conformation. The GTP cap at the end of filament is assosciated with the growth phase, losing this GTP-cap due to depolymerization or hydrolysis results a GDP—tubulin end which further results in bend shape [Mandelkow 91, Brouhard 14].

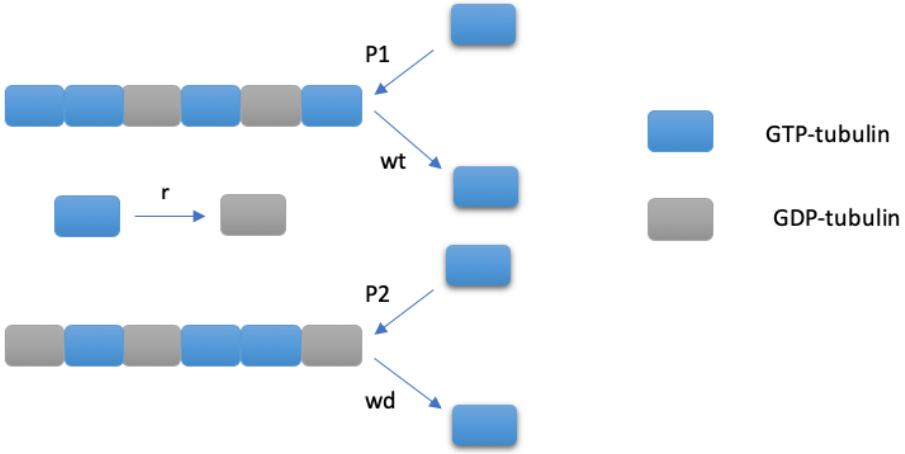

Figure 5.1: A statistical model for dynamic instability in MTs.

5.2 A statistical model

In this section, we describe the statistical model that we use to study dynamical instability in MTs. This model was proposed by Aparna et. al. [Aparna 17]. We

consider a single MT filament which is in a bath of GTP tubulin subunits. The bath of these subunits is kept at a fixed concentration ϕ. We can start with a filament made up of only GTP bound units. However, random GTP bound units in the filament can hydrolyse to GDP. The rate of hydrolyis is taken to be r. Therefore at any time the filament is made up of both GTP bound and GDP bound units. Thus the end of the filament can either be in the GTP bound or GDP bound state. If the filament is in the GTP bound state, then a GTP unit can get bound to the filament end with a rate $p_1 = k_1\phi$, where k_1 is the polymerization rate corresponding to the filament end state. Both GTP and GDP bound end units can depolymerize with rates ω_T and ω_D respectively. Microtubules with GDP bound end is known to depolymerize much faster than that with GTP bound end. Therefore, we assume $\omega_D >> \omega_T$. If the filament end is in the GDP bound state, then a GTP unit can get bound with a rate $p_2 = k_2\phi$. Experiments suggest that if the tip of the MT is in the GDP bound state, then it is less likely to polymerize. Therefore, $p_2 < p_1$.

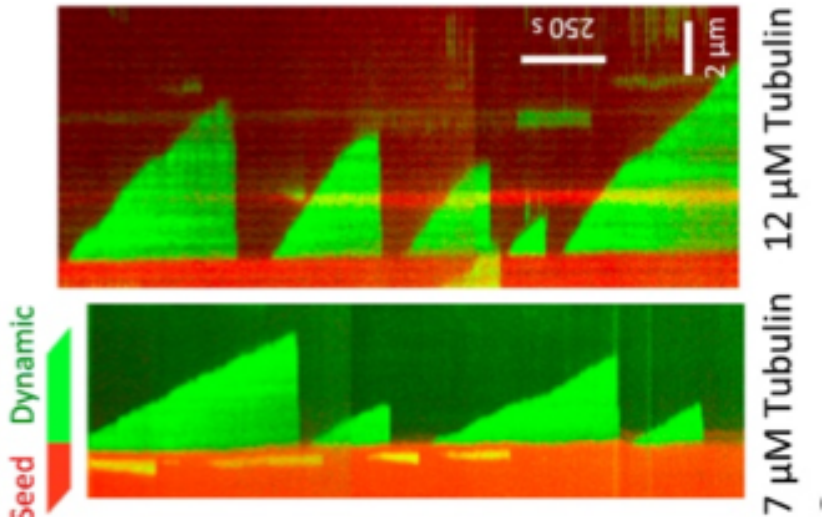

Figure 5.2: Experimental results for dynamic instability with $\phi = 11\mu$M and $\phi = 7\mu$M in [Gardner 11].

Kinetic Monte Carlo simulation

There are many aspects of biological systems for which complexities associated with their dynamics and structure which is difficult to study them analytically. Such problems can be unferstood as a chemical reaction these and hence be modelled as chemical reactions with specific kinetic rates. As with any stochastic process, the possibility of next event occurring depends on the rates of all possible events. The Gillespie Exact Stochastic Simulation [Gillespie 76] is a widely used algorithm which helps in simulating the behaviour of a system of chemical reactions. This algorithm tracks the time evolution of the system and its variables using the parameters (in our case, rates of polymerization/depolymerization and hydrolysis).

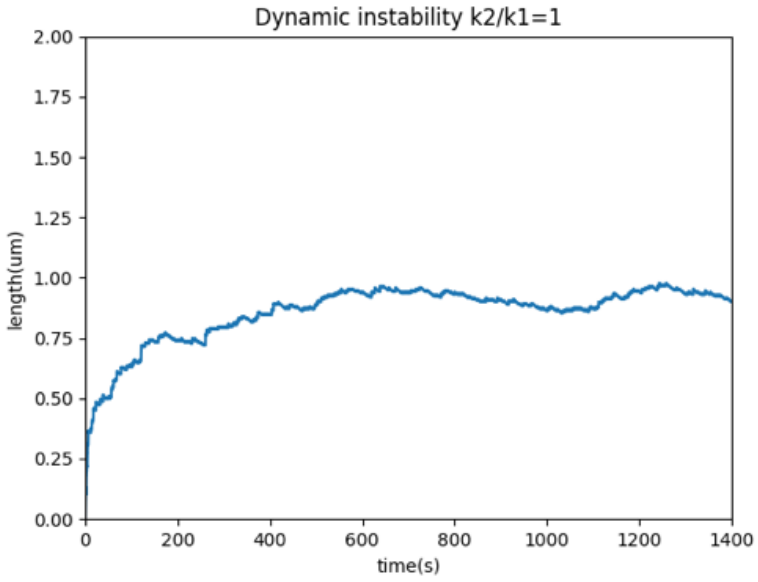

Figure 5.3: Dynamic instability curve for $k_2/k_1 = 1$ with $\phi = 10.45\mu$M.

The algorithm for the Gillespie simulations are as follows. Consider a system where there are N_1 reacting species with initial concentrations $\phi_i(0)(i = 1, ..., N_1)$ and N_2 reactions. Then the steps are :

1. At time $t = 0$, initialize rates $a_i(i = 1, ..., N_2$ and concentrations $\phi_i(0)(i = 1, ..., N_1)$. The sum of the reaction rates is given as $a_t = \sum a_i$.

2. A uniform random number r lying between 0 and 1 is drawn and the next reaction j is executed, if the following condition is satisfied :

$$\sum_{i=1}^{j-1} a_i \leq r \times a_t < \sum_{i=1}^{j} a_i \tag{5.1}$$

3. Concentrations of all other reacting species are updated according to the update of the specific reaction.

4. The time is updates as $t + (-\log(r)/a_t)$.

5. Continue till you reach $t = t_m$, where t_m is the maximum time set in the simulation.

This procedure is followed for the two reacting species in our system which are the GTP and GDP bound tubulin units. The reactions and their rates have been described in the model.

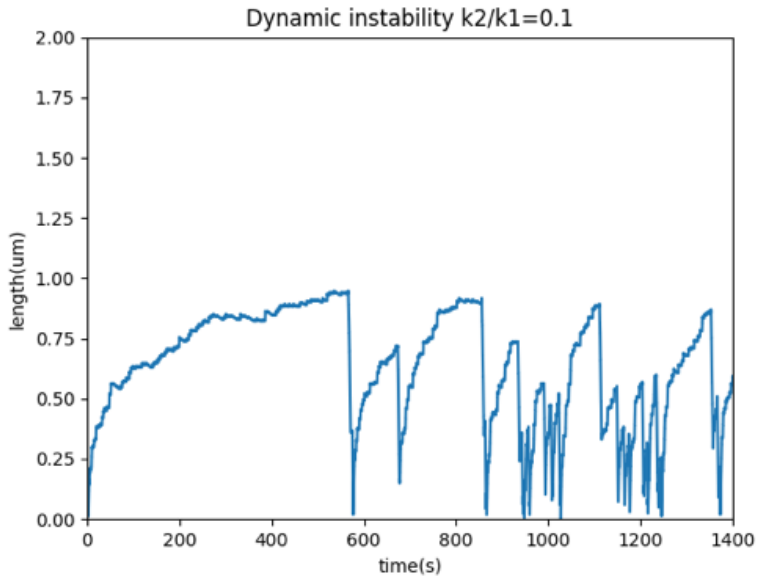

Figure 5.4: Dynamic instability curve for $k_2/k_1 = 0.1$ with $\phi = 10.45\mu$M.

5.3 Results

We check the results of this model by comparing them with experimental kymographs which are length versus time traces. We show experimental plots by [Gardner 11] (see Fig. 5.2) which are taken for a value of concentration $\phi = 12\mu$M. In our simulations, we start with a concentration of $\phi = 0.95\phi_c$ where ϕ_c is the critical concentration above which the filament shows a mean positive growth.

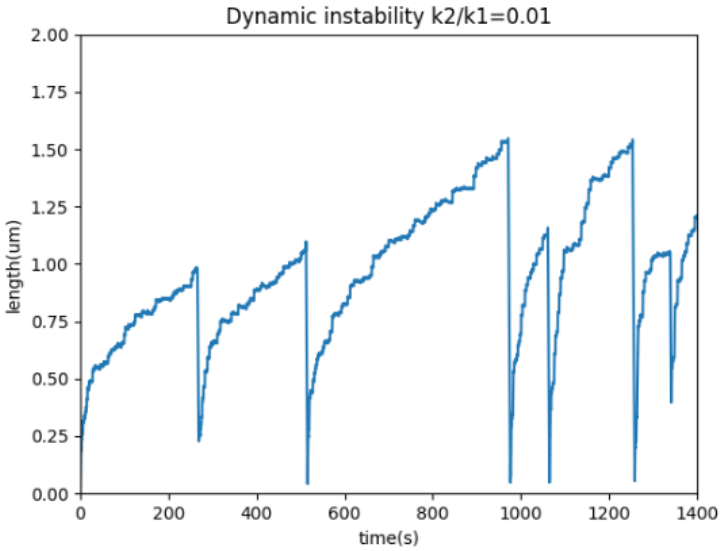

Figure 5.5: Dynamic instability curve for $k_2/k_1 = 0.01$ with $\phi = 10.45\mu$M.

In Fig. 5.3, we show the length versus time plot for the case $k_2 >> k_1$. In this situation, the polymerization of the GTP bound state increases. When this happens, the end of the filament is predominantly GTP bound. Hence the filament gets stabilized. As we see in the plot, the length of the filament largely saturates to a constant value.

In Fig. 5.4, we show the length versus time plot for the case $k_2 \sim k_1$. Here, the polymerization rates for both GTP and GDP to bind to the filament end are similar. We can see the onset of catastrophe and rescue events similar to experimental features. As we make $k_2 << k_1$, the end of the filament is predominantly GDP

bound and the dynamic instability observed is more robust as shown in Fig. 5.5.

Chapter 6

Discussion

6.1 Experimental results

Below we give the table for the consolidated Cut-7 experimental conclusion and summarize.

cell	Binding -activity	Force generation	viability	Experimental results
Cut7-WT	yes	yes	Alive cells	yes
Cut7-rigor	yes	no	Toxic	no
Cut7-22	No-at higher temp	yes	Rescues ts at higher temp	yes

Table 6.1: Cut-7 results

1. We can conclude that only in the presence of appropriate amount of protein can a cell efficiently perform its mitotic activities. Excess production of protein in cut-7-oe results in lower cell division rate.

2. In cut7-r with cut7-wt as host cell the presence of nmt81 promotor results in lower amount of cut7-r than the naturally occurring cut7-wt. It is still enough to make the cell toxic showing that the stationary cut7-rigors might be causing hindrance during chromosome separation even in such small concentration.

3. At higher temperature cut7-22 in WT background is lethal while cut7-rigor in WT background is lethal at all temperatures. In case of cut7-rigor in temperature sensitive mutant (CUT7-22), host cell gets rescued from temperature sensitivity. This shows that cut7-rigor compensates for the loss of activity in cut7-22 and vice versa. Since cut7-rigor cannot perform motor activities like applying an outward force by walking on MTs and cut7-22 fails to separate SPB, both are now compensated by cut7-22 and cut7-rigor respectively. In this case there are only one set of proteins doing each activity resulting in a rescue from temperature sensitivity (see 6.2).

	cut7-WT(bg)	cut7-22(bg)
Cut7-22	overproduction of walking protein since both have functioning motor activity	NA
Cut7-rigor	overproduction of binding protein since both can seperate SPB	only cut7-rigor can separate SPB and cut7-22 has motor activity
cut7-WT	both walking and sepeartion is over expressed (same as cut7-oe)	NA

Table 6.2: Summary of results.

4. The results of human kifc1 shows similarity with that of HSET which is previously known. This was done as a control experiment. In case of other human kinesins kifc3 ,kif25 ,Eg5 and macaque kinesin kif25, there was varying level of toxicity as well as abnormality in cell size observed but there was no significant toxicity seen in kifc2 .

6.2 Theoretical Results

We have done preliminary simulations of dynamical instability in MTs using an existing model. The results show similarity with that observed in experiments in kymographs. We would like to build a comprehensive model to understand the dynamic behavior of MTs during mitosis.

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