

Experimental and Computational Investigation of Kinetochores Protein Dynamics



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This dissertation is submitted for the degree of

Doctor of Philosophy (D.Phil.)

Dedication

I dedicate my DPhil thesis to my mother Mrs. Nasrin Noorahmed Ladak and my father Mr. Noorahmed Noorali Ladak.

Declaration

I hereby declare that except where specific reference is made to the work of others, the contents of this dissertation are original and have not been submitted in whole or in part for consideration for any other degree or qualification in this, or any other university. This dissertation is the result of my own work and includes nothing that is the outcome of work done in collaboration, except where specifically indicated in the text.



Yaseen Noorahmed Ladak

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First and foremost, I would like to thank God for the opportunity I got to study at University of Oxford. I would like to thank my mother and father for their support, sacrifices and understanding. I would like to thank Prof. Bela Novak and Prof. David Glover for accepting me in their laboratories, supervising me and making sure that I got all the facilities to do my research.

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Statement of Changes Made

1. Abstract, Aim and Scope

- 1.1. The abstract now sets the overall aim of the thesis that is to use a systems approach to experimentally and computational investigate the kinetochore protein dynamics. The section also mentions the aim and scope
- 1.2. Subsections are now added to describe how each chapter achieves the above-mentioned aim, its scope and goals. The thesis starts with developing computational methods to analyse experimental live imaging data and set hypothesis. The biological predictions are experimentally verified and characterised. Further, a mathematical model for quantitatively simulating the verified experimental findings is developed. Furthermore, a software is developed that can be used for analysing and verifying experimentally data. Finally, a section on discussion and future work.

2. Introduction

- 2.1. Introduction chapter is a) Completely rewritten keeping in mind computational and experimental findings of the thesis that characterise kinetochore-microtubule formation and sister kinetochore tension as a part of error correction and spindle assembly checkpoint b) Starts with overview of mitosis and introduces key proteins gradually c) Appropriate illustrations are now included d) shorter sentences are used
- 2.2. In overall thesis the references now a) Refer to primary work or its application b) Relate to similar or opposing studies or in context relevant to what is being described c) A consistent method for siting references is adopted d) refer to more recent publications.

3. Materials and Methods

3.1. The methods section now has a) Summary in text form b) Detailed protocols, PCR primer list, gel images and fly genotypes are added to the appendix A c) The reproducibility is addressed in section 2.6

4. Results

4.1. General Comments: a) The errors bars represent the standard deviation and has been mentioned in section 3.1 b) Frame numbers are used on specific occasions where the experiment was defining a characteristic rather than the time point when the event happened c) Supporting findings have been reported specially in case of Polo and AurB in sections 4.3 and 4.4

4.2. Chapter 3: a) This chapter was added after the first viva to show comparison of image analysis approaches, its robustness and identify photobleaching effect b) This chapter is now appropriately placed after material and methods section c) It now states the assumption and the limitations of the method d) Polo dynamics are now characterised by more traditional method after discussion with an image analysis expert Dr. Ian Dobbie and its rational is explained

4.3. Chapter 4: a) R^2 values are now added in fig. 4.2 and 4.3 b) Non-linear relationship now is shown by doing a linear fit on Mis12 and Ndc80 in fig. 4.3 with R^2 low values c) The rate kinetic analysis in fig 4.5 is done on average data set of Polo in fig 4. with SD shown based on individual data set average d) Rational for early localisation of AurB dynamics compared to other CPC components discussed in chapter 4.4 and in section 1.7.4.5. e) Limitation of Mad2 was mainly due to background however and its role that was critically to find out time points when attachments are completed did not require the trends.

4.4. Chapter 5: a) The term wild type and control are the same in case of micro injection control experiments and clarified in section 5.1 b) For better evidence of Binucleine2 an additional fig. 5.2 with histone GFP is added c) Section 5.3 and 5.4 now explains the slight differences between them question being addressed in the experiments d) Cdc20 overexpression explanation added in section 4.5 e) Two rationales presented for first drop of Polo in section 5.9.

4.5. Chapter 6: a) Frame number relation to time points now explained in the text of section 6.4.

4.6. Chapter 7: This is a new chapter showing quantitative mathematical model.

4.7. Chapter 8: a) PPI term and my contribution to this chapter is explained in section 8.1 b) The embryo quantities for mass spectrometer analysis is mentioned in section 2.4 c) Examples of analysing binding partners using DAPPER and its uniqueness are not added in section 8.7 and 8.8

5. Discussion and Future work

5.1. This now gives a more coherent account of thesis and future work that can add value.

Abstract, Aim and Scope

The ultimate goal of mitosis is to achieve faithful chromosome segregation (CS) to form two genetically identical daughter nuclei. Kinetochore (KT) is one of the most vital machinery that governs and regulates CS. How the different KT proteins regulate such a complex process with robustness and precision has been an active area of research. Here, I present a system-approach using experimental and computational techniques to investigate the KT proteins in *Drosophila melanogaster* syncytial embryos.

The first aim of chapter 4 is to build a temporal dynamic map of some of the key KT proteins (associated with KMN network, Chromosomal Passenger Complex, Spindle Checkpoint and Polo) to understand loading and unloading kinetics. Although the scope of this thesis is to investigate the dynamics between Nuclear Envelope Breakdown (NEBD) and Anaphase (AO), the dynamics are plotted over an entire mitotic cycle for sake of completeness. Central to getting the dynamics is a robust image analysis method that is discussed in Chapter 3. The aim of chapter 3 was to compare various image analysis approaches to select the most appropriate one. The second aim of chapter 4 is to develop a computational/mathematical approach using differential equation to identify protein inter-dependencies using their dynamics. The predicted protein interdependencies were experimentally validated using KMN network protein kinetics. An additional goal of chapter 4 is to develop a computational method using rate kinetic analysis to predict the number and timing of biological events that shape the loading and unloading dynamics of Polo that were hypothesised to be monophasic and biphasic respectively. The aim of chapter 5 and 6 was to experimentally verify and characterise the biological events governing Polo loading and unloading dynamics as hypothesised in chapter

4. The scope and aim of chapter 7, is to build an initial quantitative model using Polo dynamics to simulate the experimental findings with the lens of Error Correction (EC) and Spindle Assembly Checkpoint (SAC). The aim of chapter 8 is to build software called DAPPER and describe its functionality using Polo and BubR1 protein-protein interaction (PPI) data that is experimentally obtained. It is a part of future work to experimentally and computational investigate Polo PPI in relation to other KT and more broadly other mitotic proteins using DAPPER. Chapter 9 covers the discussion and future work.

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Abbreviations

AO	Anaphase onset
APC/C	Anaphase promoting complex/Cyclosome
Cdc	Cell division cycle
Cdh	Cadherin
Cdk	Cyclin dependent kinase
CPC	Chromosomal passenger complex
CSF	Cytostatic factor
DM	<i>Drosophila melanogaster</i>
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
Emi	Early mitotic inhibitor
GFP	Green fluorescent protein
HeLa	Cervical cancer (Henrietta Lacks)
KMN	Kn1 complex, Mis12 complex and Ndc80 complex
INCENP	Inner centromere protein
kDa	Kilo Daltons
Mad	Mitotic arrest deficient
MAT	Metaphase to anaphase transition
M phase	Mitotic Phase/Mitosis
MPF	Maturation Promoting Factor/M phase Promoting Factor
NEBD	Nuclear envelope breakdown
PBD	Polo box domain
PCR	Polymerase chain reaction

Plk	Polo like kinase
PPI	Protein-protein interaction/s
RPF	Red fluorescent protein
RT	Room temperature
SAC	Spindle Assembly Checkpoint
SCF	Skp1, Cullin, F-box
SMC	Structural maintenance of chromosomes

Chapter 1 Introduction

1.1 Cell Cycle

The cell cycle is the most fundamental biological process to support life. Cells divide for several reasons e.g. reproduction, growth, maintenance, repair, etc. The cell cycle consists of DNA synthesis (S) phase and mitosis separated by gap (G) phases, in the order: G1-S-G2-M¹⁻⁴ (fig. 1.1 and 1.2). The S phase governs DNA replication initiated from its replication origins and enabled by DNA polymerase. During replication the DNA duplex is tied to its sister by Cohesin, that are ring like protein complexes, while the linked DNA duplexes are called as chromatids. G1, S, and G2 are collectively referred as interphase. Additionally, there is a quiescence (G0) phase in which cells that are not committed to cell cycle, due to absence of growth factors, are parked and may not enter G1. In order to achieve successful cell division eukaryotic cells, reorganise their microtubules (MTs) to form the mitotic spindle. MTs are metastable tubulin polymers of α and β -tubulin that switch between growth and shrinkage phase that is referred as dynamic instability^{5,6}. During mitosis, the spindle pulls apart the sister chromatids after the Cohesin complexes are cut and uses the actin cytoskeleton to divide the cells into two. In yeast cells as the spindle is built in the nucleus the division process is referred as closed mitosis. In other cell types, as the MTs capture the condensed chromatin in the cytoplasm after nuclear envelope break down (NEBD) the division process is called as open mitosis. Mitosis is followed by an interconnected process called as the cytokinesis that leads to physical cell division.

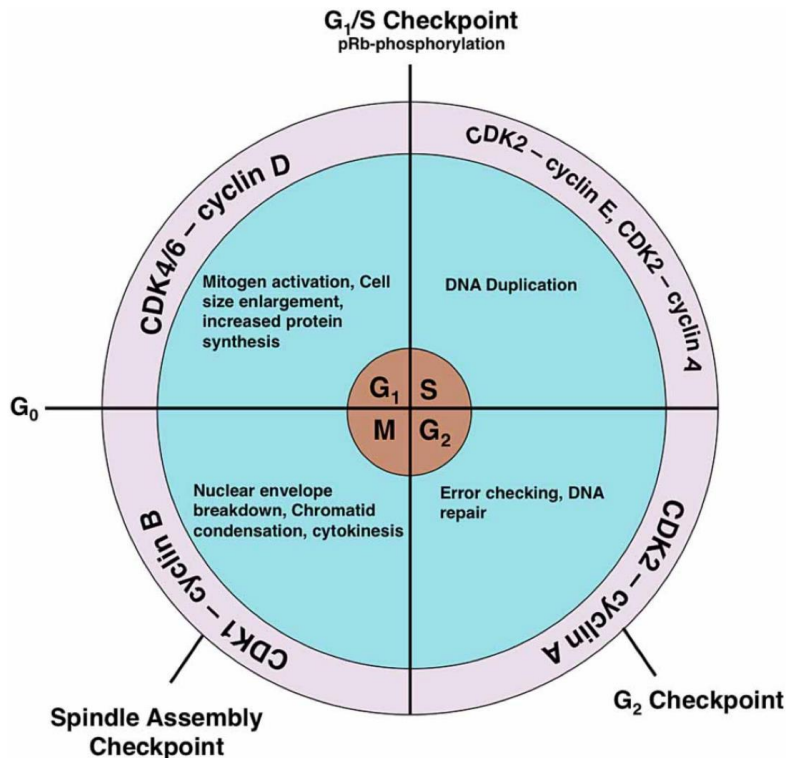


Figure 1.1. Cell division cycle

Cell enters cell cycle from G₀ phase based on extracellular signalling. G₁ phase involves activation of Cdk4/6 by CyclinD, leading to increase in size, transcriptional induction and organelle duplication. G₁/S checkpoint is overcome by phosphorylation of pRb protein. Cdk2 activation by CyclinE induces DNA synthesis while its activation by CyclinA enables completion of DNA synthesis and G₂ phase entry. After completing checks for error free DNA replication, M phase entry is promoted by increase in Cdk1-CyclinB activity. During M phase, nuclear envelope breakdown followed by chromatin condensation, spindle formation and separation of chromatids. Ultimately the cell separates into two daughter cells at cytokinesis. Picture and text adapted from adapted from: The sweet side of the cell cycle⁷

The signalling pathways that operate during G₂ focus on mitotic entry while those during mitosis regulate the chromosome segregation (CS). The two key roles of the signalling pathways are to coordinate independent events like mitosis and cytokinesis; and provide quality control checkpoints that arrest the process in case of any abnormalities. Checkpoints ensure the dependency of execution of later cell cycle events on successful completion of previous ones. The major checkpoints are: (a) G₁-S checkpoint which blocks entry into S phase until appropriate cellular signals appear (b) G₂/M check point governs entry into mitosis as a regulated response to DNA damage (c) Spindle assembly checkpoint (SAC) checks for unaligned and/or unstably attached chromatids, damaged spindles and governs sister chromatid separation that happens at anaphase onset (AO) for faithful CS.

Embryonic cell cycle undergoes rapid divisions without G phases, due to the maternally provided mRNA e.g. *Drosophila melanogaster* syncytial embryos, undergo 13 divisions before the zygotic contribution starts from cycle 14⁸⁻¹². The embryonic cell cycle works like an autonomous oscillator independent of the downstream chromosomal effects¹³⁻¹⁷. Conversely, a somatic eukaryotic cell cycle is more coordinated and controlled as the transcriptional input is modulated by system level feedbacks¹⁸⁻²¹.

Two major mitotic events for successful mitosis are G2/M transition (mitotic entry) and the metaphase to anaphase transition (MAT). These transitions are regulated by cyclin-dependant kinase 1 (Cdk1) and anaphase-promoting complex/Cyclosome (APC/C) respectively. Cdk1 activation is the main driver of G2/M transition, as it is a necessary and sufficient trigger for mitotic entry. At mitotic entry, activation of Cdk1 enables APC/C activity. Activated APC/C leads to sister chromatid separation and inactivation of Cdk1, thus leading mitotic exit. As APC/C is sufficient and necessary for AO, the mitotic exit is guarded by SAC, that arrests any aberrant cell division.

1.2 Cyclin Dependant Kinase 1 and Mitotic Entry

Cdk1 is a 34-KDa protein kinase that works as a trigger for mitotic entry²². It phosphorylates many substrates and is tightly regulated. Cdk is maintained at a constant level throughout the cell cycle, however its periodic activity is modulated by a regulatory subunit called cyclin (Cyc) that brings about a conformational change in Cdk thus activating it. Different Cdk-Cyc heterodimers regulate different phases of cell cycle^{1,4} (fig. 1.2). In mammalian cells, the G1 cyclins in conjunction with Cdk4, Cdk6 and Cdk2 modulate entry into cell cycle. While the S phase and mitosis is modulated by Cdk1 (Cdk2 also functions in S

phase) along with B-type family of cyclins (CycB). Cdk1-CycB complex is also called as M-phase promoting factor (MPF).

In somatic cells, CycB is generally expressed during late S and G2 phases leading to its accumulation. However, at MAT, APC ubiquitinates CycB marking it for degradation by 26S proteasome, thus inactivating it. APC is a part of the broader ubiquitin-proteasome system (UPS) that ubiquitinates target proteins thus marking them for proteasome mediated destruction^{23,24}.

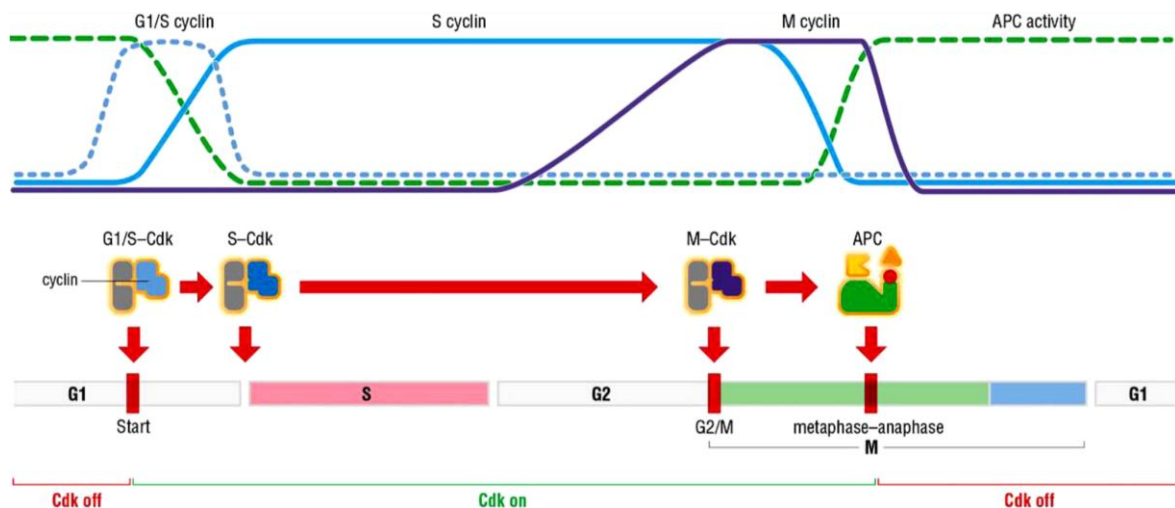


Figure 1.2. Cell cycle control via stage specific activity of Cyclin-dependant kinases
Picture and text adapted from: The Cell cycle: Principles of Control²⁵

In yeast, a relatively high Cdk1 activity is required for mitotic entry compared to a lower levels of Cdk1 for modulating events in S phase^{26,27}. Different cyclins are expressed at different times and enabling Cdk1 to confer substrate specificities, thus adding a layer of fine tuning to the basic model²⁸. Although Cyc binding is necessary but not sufficient for full Cdk activation. Cdk1 requires phosphoactivation by Cdk-activating kinase (Cak) which requires Cdk in complex with Cyc^{29,30}. Cak activity does not change during cell cycle and in some

organisms, there are multiple Caks. The Caks thus enable full activation of Cdk1 but do not regulate the timing.

Entry into M phase is restricted by Wee1 and Myt1 (fig. 1.3) that keep the activity of S and G2 phase accumulated Cdk cyclins low^{4,31-33}. Wee1 and Myt1 phosphorylates Cdk1 on tyrosine (Y15) residue and threonine (T14) residue once Cdk1 is bound to cyclin. The phosphorylation inhibits the substrate binding capacity of Cdk³⁴. Cdc25 which is a dual specificity protein phosphatase dephosphorylates Cdk1 thus enabling its mitotic entry³⁵⁻³⁷. Thus, the opposing activities of Wee1 and Cdc25 are the key ingredients of the switch like activation of Cdk1 which is itself governed by a feedback mechanism¹⁴. Cdk1 can phosphoinhibit Wee1 (in vertebrates, Cdk1 activated Plk1 marks Wee1 for degradation), inactivate Myt1 and phosphoactivate Cdc25 (by binding it via Polo box domain in a Cdk1 phosphorylation dependant manner)^{1,38-46}. These feedback loops create a bi-stable system in which after a point the balance tips from low to high Cdk1 activity, thus allowing entry into mitosis.

Analogous to the Cdk1 activation loop there exists a feedback mechanism in animal cells that inactivates protein phosphatase 2A (PP2A) that is involved in dephosphorylating Cdk1 substrates⁴⁷. Cdk1 activates Greatwall (Gwl) kinase which in turn activates Arpp19 and α -endosulfine, which are the two inhibitors of PP2A-B55^{48,49}. Antagonising the PP2A activity increases the effectiveness of Cdk1 activity by reducing the rate at which Cdk1 substrates are dephosphorylated. These feedback loops are vital for committed G2/M transition⁵⁰. In addition to protein activity, cellular localisation is also important during G2/M transition. During G2 phase Cdk1-CycB complexes have critical nucleoplasmic targets. However, the complexes are maintained in the cytoplasm inhibited by Wee1 and sequestered away from nuclear Cdc25

activation. One of the early events that happen during G2/M transition is the nuclear localization of Cdk1-CycB enabling the complex to reach its targets⁵¹.

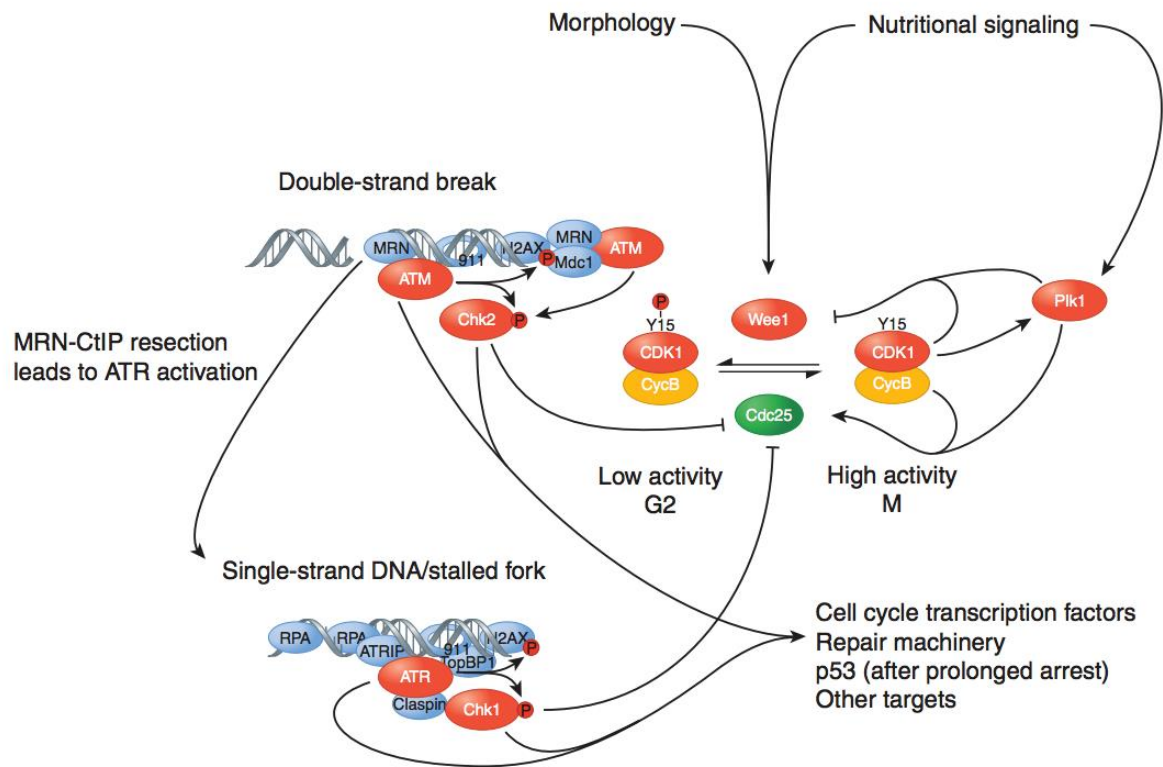


Figure 1.3. Mitotic Entry
Picture and text adapted from: Signalling pathways that regulate cell division¹

1.3 Metaphase to Anaphase Transition

Metaphase is a mitotic stage where the chromosomes are centrally aligned between the two poles of a cell. Once the chromosomes are aligned anaphase is triggered via APC⁴. APC is required to ubiquitylate mitotic substrates by marking them for degradation via 26S proteasome. APC activity is modulated by binding two subunits namely Cdc20 and Cdh1⁵². Cdc20 is active during MAT transition while Cdh1 is active during telophase and G1 phase. APC-Cdc20 has two key substrates, Securin and CycB. Securin is a Separase inhibitor while Separase is a protease that cleaves Cohesin complexes that hold the sister chromatids together⁵³. In yeast Cdc14 phosphatase is also indirectly activated when Cdk1 is inactivated,

thus allowing rapid dephosphorylation of Cdk1 substrates⁵⁴. In animal cells, PP2A and PP1 are involved in dephosphorylation⁴⁷. There is a lag between activation of Cdk1 and APC, thus giving the cell enough time to make correct load bearing KMT attachments via a process called as error correction (EC) allowing chromosomes to form a compact plate at the cell equator. However, in case of a delay to achieve metaphase (which is mostly the case in metazoan cells) SAC is activated. SAC prevents APC activation ultimately preventing CS. SAC has evolved from yeast to be a quality control to a prime signalling pathway in metazoans that initiates AO.

1.4 Ubiquitin-Proteasome System

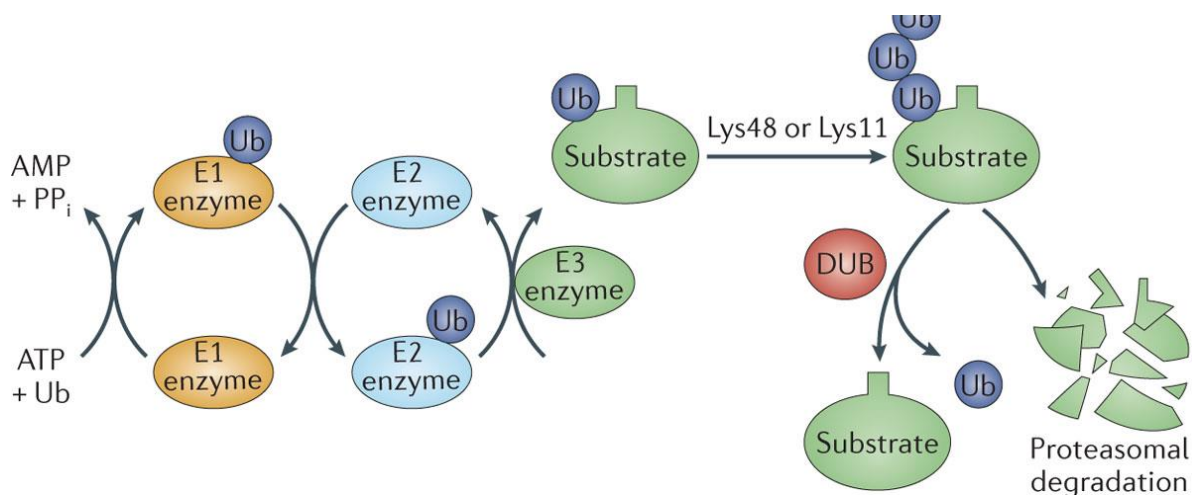


Figure 1.4. Ubiquitin-Proteasome System

Picture and text adapted from adapted from: SCF ubiquitin ligase-targeted therapies⁵⁵.

UPS modulates pathways by regulating abundance, activity and localisation of proteins via ubiquitin code^{24,55–60}. In poly/multi-ubiquitination, multiple small Ub (~8.6 kDa) attach to each other via the Lys/K residue within Ub or the amino-terminal (NT) methionine (Met1) of Ub to extend the chain on substrate proteins. Different Ub configurations are recognised by effector proteins with linkage specific Ub-binding domains (UBD) forming ubiquitin code influencing the outcome of the substrate protein, e.g. K48, K11 or K48-K11 mixed linked chains commit to proteasome degradation, except in yeast^{61–63}. Mono or multi-mono-

ubiquitination does not form chains but plays a role by altering intra or inter molecular interactions which affects protein localisation, complex formation or activity and plays a role in DNA repair and chromatin remodelling^{58,64,73,65–72}.

Ub attachment to target proteins is a sequential multi-enzymatic process requiring, ubiquitin activating (E1) enzyme, ubiquitin-conjugating (E2) enzyme and a ubiquitin ligase (E3) (fig. 1.4). Deubiquitinating enzymes (DUBS) can reversely modify ubiquitylated proteins. E3 ligases are generally classified based on the structure of the E2 harbouring domain and mechanism of Ub transfer⁵⁶. Members of the RING family (APC being one of them) play a vital role in many aspects of cell cycle. K11 linked chains are preferentially produced during mitosis and early G1 phase enabled by E3 APC^{62,74,83,84,75–82}. In humans, E3 APC activity is enabled by two distinct E2 namely, Ube2C (UbcH10) and Ube2S for Ub chain initiation and elongation respectively^{75,85,86}. UbcH10 can initiate mixed chains of K11, K48 and K63 (non-proteolytic roles e.g. DNA repair, proteins trafficking, kinase activation and translation) while the Ube2S can only produce K11 chains which when bound to those produced by UbcH10 is recognised by 26S proteasome^{62,79,87}. Although, a recent report has suggested role for K63 in degradation⁸⁸. Despite the conventional view that chain elongation and polyubiquitination by Ube2S is essential for degradation, E2 Ube2S is not required for degradation of CycB1, a bonafied APC substrate^{77,89}. Hence, multi-mono Ub by UbcH10 suffices for degradation signal in case of CycB1^{81,89}.

1.5 Mitosis

Faithful chromosome segregation (CS) is the overarching goal of mitosis (M) and its dysregulation leads to aneuploidy (via chromosome instability, CIN) and more broadly genomic instability (GIS)⁹⁰. M phase governs the equatorial segregation of replicated

chromatids from one mother nucleus into two genetically identical daughter nuclei and consists of prophase, pro-metaphase, metaphase, anaphase and telophase⁹¹ (fig. 1.5). Prophase, begins with condensation of chromatin followed by dispersion of nucleolus and Golgi complex^{92–99}. MT nucleation usually begins before the nuclear envelope break down (NEBD) and MTs are radiated from the centrosomes that are either close or well separated^{95,100}.

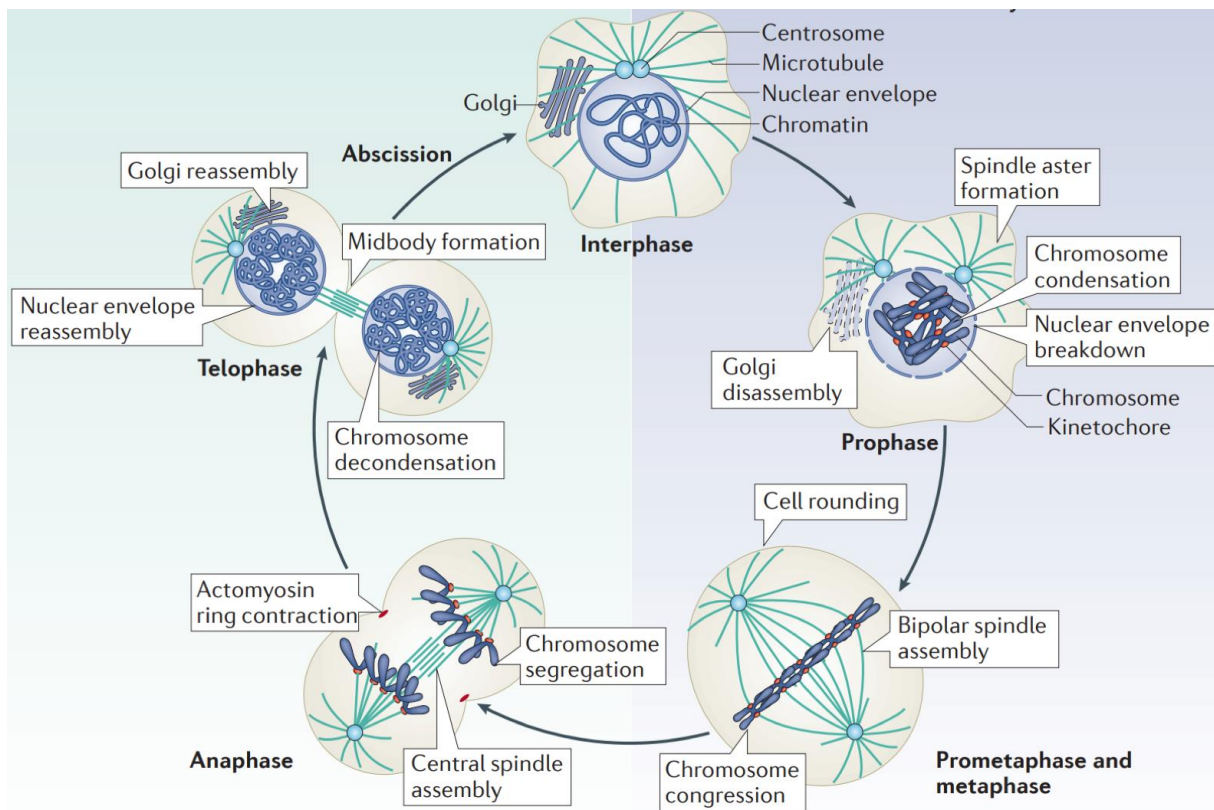


Figure 1.5. Mitosis schematic overview

Picture and text adapted from adapted from: Phosphatases: providing safe passage through mitotic exit⁴⁷

Metaphase, beings when the sister KT's have been captured by MTs emanating from opposite poles and ends with amphitelic attachments that are equatorially aligned and has congressed chromosomes. The mitotic spindle assembly (organisation) plays a vital role in chromosome congression by generating mechanical forces^{101–103}. The equatorial alignment of chromosomes gives an unbiased starting point for the chromosome to split equally into two daughter nuclei, though some cells further show tightening of the metaphase aligned chromosomes^{104–107}. The end of metaphase is marked by silenced SAC as a result of which

following events happen^{19,108–113}: a) the generation of MCC is stopped b) the already produced MCC disassembles during metaphase c) APC is activated d) CycB is degraded triggering the mitotic exit process e) Separase is activated that leads to cleavage of Cohesin rings that bind replicated chromatids. During Prometaphase, the MTs are captured by the kinetochores (KTs) enabled by search and capture (SC) mechanism that stems from dynamic instability of MTs^{5,114–116}. SC is guided by biochemical pathways and by geometry of the cell and its components^{102,115,117–119}. In addition to dynamic instability MTs pivot to maximise being captured by the KT's and once captured its plus ends are capped and dynamics are suppressed¹²⁰. SAC generates the wait signal (mitotic check point complex, MCC), giving enough time for the EC machinery to enable formation of amphitelic kinetochore-microtubule (KMT) attachments^{121–126}. AO denotes the time point at which cleavage of centromeric Cohesin holding the two sisters is complete^{127,128}. Anaphase-A (early anaphase) is marked by the movement of the chromosome towards the spindle poles and involves shortening of the MTs while Anaphase-B (late anaphase) involves separation of poles via spindle elongation, both are governed by microtubule dynamics^{129,130}. Anaphase-B (late anaphase) is marked by central spindle formation enabling cleavage furrow during telophase^{131–134}. Decrease in Cdk1-CycB activity leads to drop in activity of other kinases and gives phosphatases an upper hand^{135–138}. During Telophase, segregated chromatin near each of the spindle poles undergoes compaction^{139,140}. Nuclear envelope proteins mingle with the condensed chromosome followed by assembly of nuclear envelope^{94,141–143}. Finally, the chromatin decondenses and transcription is initiated. Cytokinesis, leads to cleavage furrow ingression under direction from actin-myosin ring, from late anaphase onwards, where it gathers bundles of MTs forming narrow bridge that ruptures forming the midbody that persists till the interphase of the next cycle^{134,144,145}. There is little separation between end of anaphase, start of telophase and cytokinesis and some of the events happen in parallel.

1.6 Kinetochore and Centromere

1.6.1 Kinetochore

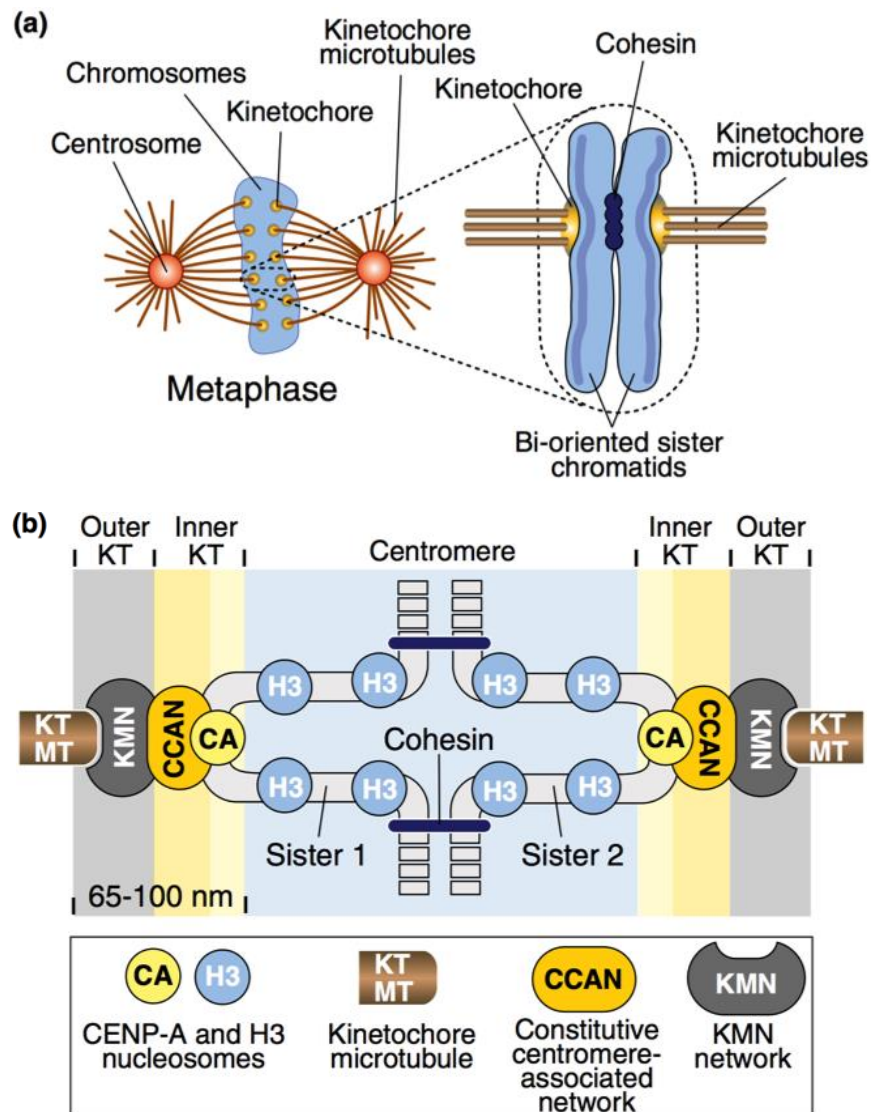


Figure 1.6. Schema representing the relationship between centromeres and kinetochores

Picture and text adapted from: Progress in the structural and functional characterization of kinetochores¹⁴⁶

CS requires formation of correct load bearing amphitelic KMT attachments^{147,148}.

Microtubules originate from centrosomes, and those that bind to the kinetochores are referred as KMTs. KTs are multi-subunit proteinaceous assembly on centromeres (CTs) – the evolutionary conserved constriction^{149–153} (fig. 1.6). The KT is described in terms of layers (based on its distance from centromere) built from specific proteins (fig. 1.6 and 1.7). It is vital

that correct configuration of KMT attachments are selectively stabilised by the KTs over incorrect ones. The EC mechanism is governed by the binding affinity of MTs via changes in KT structure and chemistry (e.g. post translational modifications, etc.), although the molecular basis of EC is under active research^{121,154,155}. It has been suggested that tension is the key regulator for EC and incepted from the pioneering work of Nicklas and recent functional studies although recently a tension independent EC pathway has been reported^{121,154,156–159}. KTs plays a vital role in EC and SAC machinery, thus governing the sister chromatid bi-orientation and initiation of mitotic exit^{124,160–162}. There is an overlap at the apex of molecular mechanisms between SAC and EC, as SAC works as a timekeeper to allow formation of correct KMT attachments via EC mechanism.

1.6.2 Centromere

Centromeres in different organism occupy different locations on chromosome arms which is depicted in their names like metacentric (middle), acrocentric (separated at different lengths), telocentric (close to end), holocentric (along entire length) and monocentric (spatially restricted). The size of the DNA segment required to assemble a KT varies from ~125bp in *S. cerevisiae* (budding yeast) and up to several million in humans. It is the epigenetic (chromatin) signature (CENP-A/CenH3) and not the DNA sequence that defines the centromeres^{149,151,163–165}. The nucleosomes at the centromeric chromatin contain a variant of histone H3, CENP-A/CenH3/Cse4, embedded in H3 chromatin. In *Drosophila* unlike humans, Cenp-A is required and sufficient to recruit all KT proteins as seen by artificial ectopic expression of Cenp-A^{166–168}. Additionally, H3 chromatin directly interacts with a complex called as Chromosomal Passenger Complex (CPC) that forms a part of the inner centromeric region¹⁶⁹

S. cerevisiae (budding yeast), has point centromeres, which are built on a single nucleosome containing CENP-A^{170,171}. In *S. pombe* (fission yeast) and higher eukaryotes e.g. humans, the CENP-A/Cse4 nucleosomes are interspersed with canonical H3 nucleosomes to make up regional centromere (~0.2-4.0 Mbps with repetitive elements including retro-transposon or tandem repeats or both) upon which a more complex KT is built that binds the MTs^{149,151,172–174}. Except budding yeast, most organisms with more complex centromeres do not show an absolute correlation between centromere sequences and its ability to build KTs^{149,173,175}. The repetitive DNA sequence is dispensable for converting a non-functional centromere to a functional one¹⁷⁶, insufficient to form the KT^{177,178}, not necessary for centrosome functionality (as neo-centromeres in humans function independently of repeats)^{179–182} and completely absent in centromeres of several organisms^{183–187}. However, it does support the centrosome organisational stability.

1.6.3 Kinetochore Architecture

Despite spatial and compositional diversities of the KT, there is a common architectural framework in eukaryotes (except kinetoplastids)^{188–190} that forms the inner and outer KT (fig. 1.6 and 1.7). Most of the inner KT proteins (CENPs), interact with each other while showing constant centromeric association, thus forming the constitutive centromere-associated network (CCAN). In humans, CCAN consists of CENP-LN, CENP-TWSX, CENP-OPQRU and CENP-HIKM complexes, thus imparting structural stability^{191,192}. CENP-C and N interact directly with CENP-A and show higher specificity for CENP-A compared to H3 nucleosomes^{193–195}.

In contrast to humans, in *Drosophila melanogaster* and *C. elegans* majority of CCAN is absent except CENP-C which maintains its link with chromatin¹⁹⁶ (fig. 1.7). Majority of CCAN subunits associate with CENP-A foci although their interdependencies and protein

and Spc25) together called as the KMN network¹⁹⁶. KMN network proteins are chief conductors of KT orchestration by playing direct or indirect role in KMT attachments, modulating SAC and regulatory proteins, modulation of the MT stability via the plus end of the tip, etc. For e.g. a) KTs can hold depolymerising MT ends via Ndc80 clusters generating force capable of moving chromosomes^{129,147,205–208} b) Loading and unloading of SAC and regulatory proteins like Mad1, Mps1, BubR1, Aurora-B (AurB), Plk1 etc., to enable formation of KMT attachment, EC, activating and silencing SAC, etc. to allow faithful CS^{209–214}.

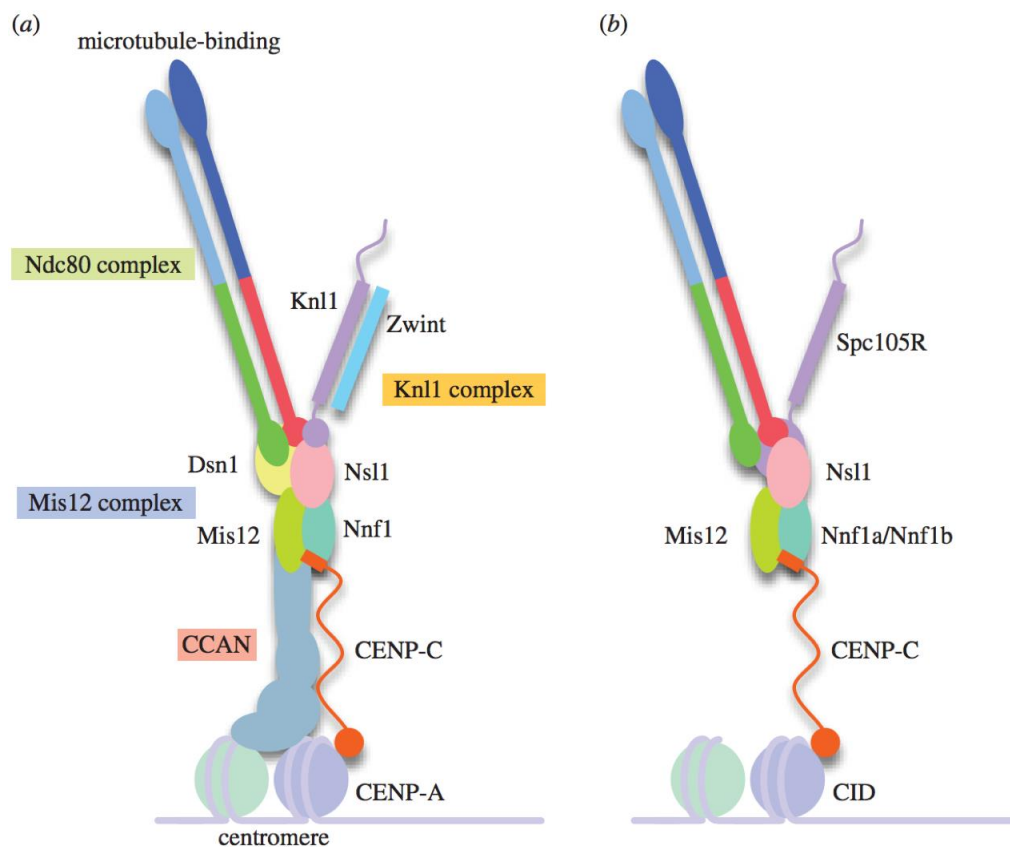


Figure 1.8. Differences between human and *D. melanogaster* kinetochore
Picture and text adapted from: Getting to the heart of an unusual kinetochore¹⁹⁶

Ndc80C acts as primary KT receptor (docking-site) for MTs^{215–217}. MT binding is enabled by amino-terminus(NT) of Ndc80 and Nuf2 subunits while KT targeting is enabled by carboxyl-terminus(CT) or Spc24 and Spc25^{218–223}. Additionally, Nuf1 exists as paralogues,

Nuf1a and Nuf2b^{224,225}. During M phase in higher eukaryotes, the outer KT is linked to inner KT by two mechanisms: a) CENP-C binds directly to Mis12-C, which in turn binds Ndc80C and Knl1C^{168,224,226–232} b) Ndc80C subunits Spc24 and Spc25 interact directly with Cenp-T^{227,233–238}. Thus, Mis12C acts as a hub connecting Ndc80C and Knl1C complexes which connects KMN to CCAN via CENP-C and CENP-T(humans). Knl1 forms a dimer with Zwint subunit to recruit checkpoint proteins^{124,239–242}. Knl1 CT via RWD domains mediates direct interaction with Mis12C (via its stalk and CT of Nsl1) while Zwint binds to a more extended domain of Knl1^{223,226,229}. *D. melanogaster*, Mis12C lacks Dsn1 however is stable as the C-terminal of Knl1 (*D. melanogaster* Spc105R homologue) has evolved to substitute for Dsn1, and is essential for Mis12C recruitment and overall KT assembly^{196,243}. The lack of Zwint subunit in *D. melanogaster* is characteristic as well²²⁴.

There have been some estimates of stoichiometry from human KT composition^{146,244}. A single CENP-A binds two CCAN complexes¹⁹⁷. CENP-C and CENP-T bind Mis12C independently such that two CCAN complex's (one of each KT) have four Mis12C²³⁵. As each Mis12C binds Ndc80C and Knl1C, resulting in four Ndc80C and Knl1C per CENP-A nucleosome. Further, CENP-T can also directly recruit up to two Ndc80C independently which depends on phosphorylation and binding^{234,235,245}.

1.7 Error Correction

1.7.1 Kinetochore-Microtubule Attachments

KMT attachments show a transient intermediate configurations before converting to permanent amphitelic configuration²⁴⁶ (fig. 1.9). Mono-oriented configuration has two categories, monotelics in which only one of the sisters of KT pair is attached and syntelics in which both sisters of KT pair are attached to the MTs from same pole. Lateral attachments are

those in which KT binds to the side wall of MTs. Most end on KMT attachments begin with lateral configuration enabled MT pivoting that increases the chances of being capture by KTs¹²⁰. Monotelics are resolved as unattached sister is correctly captured by the MTs from opposing pole. Alternately, the unattached sister KT is captured by the MTs emerging from the same pole as the captured sister KT, converting monotelic to syntelic attachments. Syntelic attachments can be formed independently of monotelics and rarely found in cells, suggesting they are resolved or converted to other forms¹²³. Monotelics engage SAC due lack of attachment while syntelic attachments engage SAC due to lack of tension²⁴⁷. The SAC arrested cells can have several fates, including apoptosis or mitotic slippage potentially leading to aneuploidy^{248–250}.

Live imaging data suggest that syntelics instead of undergoing immediate detachment from the KTs are first pulled towards the poles and then detached by means of AurA mediated gradient and depolymerised as result of AurB activity^{251–253}, although invitro complete detachments have been observed^{215,254,255}. A more common erroneous KMT attachment is called merotelic in which a single sister KT is occupied by MT from opposite poles, potentially generating enough tension to evade SAC (unlike the on and off tension for syntelics) and initiate anaphase¹²³. Merotelics impede chromosome motion towards correct pole and generate lagging chromosomes constituting the most common pathway to aneuploidy^{122,256}. Merotelics, like syntelics, can face loss of inter-KT tension if the number of incorrect MTs is high in which there is a complete detachment. However, this is not a common process as observed in somatic cells and merotelics improve as a cell progress to metaphase and can be viewed as tension based Darwinian selection process²⁵⁷. Additionally, conserved visco-elastic KT response contributes to merotelic-amphitelic conversation²⁵⁸, while some studies point to spindle elongation mechanism for force balancing to correct merotelics²⁵⁹. Imbalanced merotelics can

be sorted by spindle mechanics of tug-of-war (referred as backup mechanism) while equi-merotely can lead to aberrant outcomes like formation of micronuclei etc.^{260–262}.

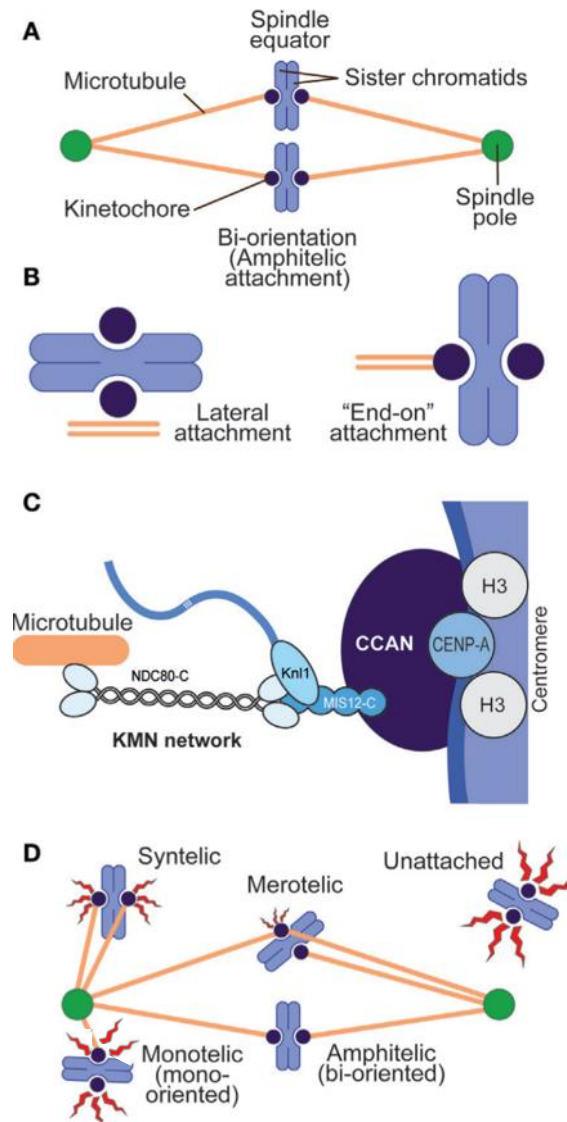


Figure 1.9. Types of microtubule-kinetochore attachment

(A) Metaphase bi-oriented chromosome with amphitelic attachments. (B) Early lateral attachments are converted to end-on. (C) Schema of MT attaching to KMN network that is built on CCAN being finally associated to inner centromere protein CENP-A. (D) Different kinds of attachment given different levels of wait signal (shown by red intensity) that is fed to SAC mechanism via feedback mechanism.

Picture and text adapted from: The Aurora B kinase in chromosome bi-orientation and spindle checkpoint signalling²⁶³

1.7.2 Correcting Syntelic Attachments

EC of syntelic attachments is enabled by Darwinian selection of amphitelic attachments over others the foundations of which were laid by B. Nickals²⁶⁴. The mechanism stems from

the understanding that KMT attachments are inherently unstable and that EC happens via trial and error. The MTs are released from the KT while the new ones are captured, a phenomenon called as KMT turnover. This trial and error constitute of KMT attachment and detachment cycles until the correct configuration is achieved. For any selection process there is a feedback mechanism which in this case is inter KT tension generated by KMT attachments. Amphitelic attachments generate maximum tension due to spindle geometry thus providing the ability to differentiate correct configuration over the others. It was shown that syntelic attachments are inherently unstable and were stabilised by application of intermediate tension using micromanipulation needle in grasshopper spermatocytes¹⁵⁹. A variation of this experiment, at a molecular level was done in *D. melanogaster* cells by overexpression of chromokinesin (a plus end motor that localises on chromosome arms)²⁶⁵. In cells that have monopolar spindles, chromokinesin overexpression leads to increase in polar ejection forces generating tension on syntelic attachments that stabilises them.

1.7.3 Correcting Merotelic Attachments

In case of merotelics the MTs attached to the wrong pole can impeded the motion of KTs towards the correct pole forming lagging chromosomes. Such aberrant chromosomes are not detected by SAC, suggesting that the cell copes with these frequent errors by normal cell division mechanisms. The direct application of trial and error based “on” and “off” tension states (like in case of syntelics) is problematic for merotelics as they generate tension. Thus, cycles of complete attachment and detachment of MTs until a correct configuration is found like in case of syntelics is not applicable for merotelics. There are broadly two main frame works for merotelic EC, tension dependant and independent or basic model (fig. 1.10)¹²¹.

1.7.3.1 Tension Dependant Error Correction

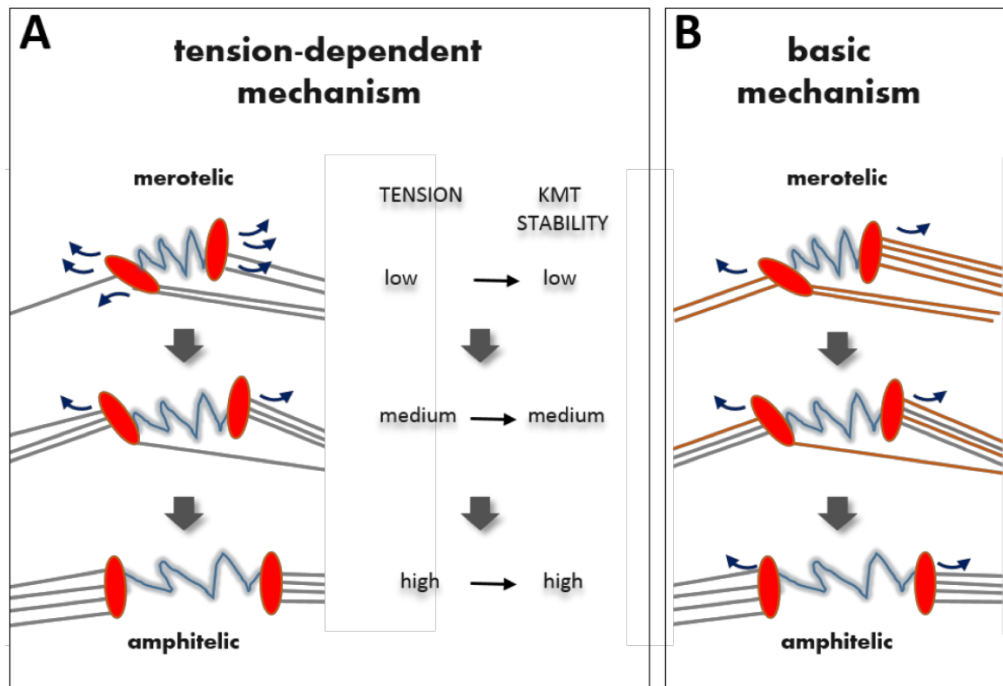


Figure 1.10. Tension and basic error correction mechanism

(A) Tension dependant mechanism, the rate of KMT turnover depends on tension between KT's (red) shown by stretch of spring (blue). As KT bind and loose MTs probing for correct/amphitelic/highest tension generating configuration that is assumed to produce higher stability and hence slower detachment. This process would eventually lead to complete amphitelic configuration. In the non-discriminate version, shown here, at a single KT pair level all the KMT attachments (correct and incorrect) are considered equally stable and hence are equally destabilised. However, in the discriminate version discussed in text, the lifetime of merotelics are shorter than amphitelics. (B) Basic mechanism, the rate of KMT turnover does not depend on tension and is not selective as all older KMT (orange) are replaced with new ones (grey) irrespective of correct or incorrect configuration. Picture and text adapted from: Mechanisms to Avoid and Correct Erroneous Kinetochore-Microtubule Attachments¹²¹

Tension dependant model relies on Darwinian selection of correct configuration over time (fig. 1.10). The model assumes that with increase in amphitelics the inter-KT tension rises, as a result of which the amphitelic detachments are slower than merotelics. The assumption is valid: a) if both correct and incorrect attachments generate pulling force capable to generate inter-KT tension thus acting as a readout b) if the number of amphitelics improve gradually from prometaphase to metaphase, tension should also modulate the stability of KMT attachments enabling gradual correct configuration. However, the mechanism at level of a single KT pair is indiscriminate i.e. both correct and incorrect MT attachments are equally stable when bound to KT.

However, the above discussed tension dependant model raises some interesting questions. If tension is the main modulator then are the two-sisters effected equally? i.e. if one of the sisters has acquired correct configuration then destabilisation of its attachments would be a counterproductive process to form amphitelic attachments. Additionally, destabilisation of amphitelic attached KT that has reduced number of MTs compared to its sister would also be counterproductive²⁶⁶. The sensitivity of such destabilisation raises concern if it is feasible to discriminate and correct a single merotelic attachment. A single merotelic attachment causes less tension imbalance (~5% if 1 of the 20 KMT attachment is merotelic) than directional forces of metaphase oscillations. Also, the overall speed of the tension-based model, if applied to all KMT of the sister KT pair would not be able to attain correct configuration within mitotic window to attain correct configuration^{267–269}. Additionally, if EC is solely based on magnitude of inter-KT tension it needs to reconcile with the force balance that can produce metaphase aligned chromosome oscillatory movements and accompanied by change in distance between KT sisters (also known as breathing)^{105,270–273}. Polar ejection forces and KT localised regulators dampen metaphase oscillations via MT growth suppression^{274,275}. These can also have an effect on inter-KT tension but the reason for them not effecting EC needs further investigation.

1.7.3.2 Tension Independent Error Correction

Tension-independent or basic framework relies on geometric changes and KMT turnover for EC^{117,121,276–279} (fig. 1.10). This is in contrast to tension-dependant model where geometry plays an initial role of reducing wrong attachments rather correcting the already formed merotelic²⁶⁴. In early mitosis, the priority is to capture the MTs, irrespective of configuration, although their EC is a rate limiting step for bi-orientation^{115,280–282}. In early mitosis, KT has enlarged coronas and curvature while less than ~10% KTs have back to back geometry, allowing for quick capture of MT although with higher errors^{117,276,277,283,284}. Without the

compromise the traditional search and capture mechanism, even with increased MT dynamics, is inefficient and can lead to mitotic delay^{279,285}. Towards metaphase, KTs are favourably (back-to-back) positioned and all older MTs are gradually released due to KMT turnover. Their replacements would have correct amphitelic attachments due to KT geometric constraints²⁷⁶. The back-to-back geometry is most favourable when KT are midway between the poles which is possible as chromosomes can congress without bi-orientation by an alternate mechanism^{277,286}. However, new incorrect attachments still form on the congressed chromosomes but with a low error rate. The cell reaches a steady state in which the rate of erroneous attachments is balanced by the KMT turnover. With steady state reached before AO which has stringent geometric constraints the final KMT configuration is favourable, unless AO starts before steady state²⁷⁶. The average KMT turnover time (2-6 mins) fits in well within the human mitotic clock (i.e. duration of metaphase 10-20 min)¹²¹. However, increasing the duration of mitosis does not significantly improve EC²⁵⁷. The success of this model is independent of the assumption that incorrect KMT attachments are less stable than correct KMT and can account for normal segregation of 45 out of 46 chromosomes provided small number of merotelics are tolerated and geometric constraints taken into account²⁷⁶.

As mitosis progresses from prometaphase to metaphase, the half-life time of MT increases, KMT turn over slows and KMT attachment stability increases^{268,269,276,287}. As per tension framework this happens as more amphitelic attachments are formed that are more stable while the basic mechanism does not require the KMT turn over to slow down but happens as a result of cell physiology. Moreover, the slowing of KMT turn over happens due to an independent mitotic clock that does not rely on getting amphitelic attachments right^{268,288}. Faster turnover suggests increased rate of EC which is required and observed during early mitosis^{246,287,289}, while the requirement of lowered KMT turnover can be explained to build robust k-fibers²⁷⁶.

1.7.4 Molecular Mechanism of Tension Dependant Kinetochore-Microtubule Stabilisation

1.7.4.1 Regulation of Microtubule Dynamics

Tension has been suggested to play a role in MT dynamics. Pulling force applied to depolymerising MT ends slows down its shortening and promotes MT rescue^{290,291}. Additionally, few piconewtons of tension applied to yeast KT's using optical trap increases the life time of KMT attachments, though a further increase in force decreases its stability²⁹². This behaviour reflects the MT property to polymerise under pulling force. In yeast as KT's bind more strongly to growing plus ends of MT's compared to shortening ones, the force leads to an increase in the lifetime of attachment to dynamic MT ends. This increase is enabled by promoting a longer duration for MT polymerisation state. If the tension sensitive stabilisation is contributed by this mechanism, then amphitelic KMT plus ends should spend more in polymerisation state compared to other kinds of attachments. Although, the hypothesis is not tested, bi-oriented KT's of budding yeast oscillate in absence of MT flux. In this case, MT polymerisation at one KT is balanced by depolymerisation at its sister KT and both sisters are not in the strongly attached state. In some organisms, the time spent in polymerisation state is increased as a result of poleward flux. However, the flux velocity in mammalian cells is lower than the rate of chromosome oscillations, hence at metaphase equal time is spent in polymerisation and depolymerisation states by KT-bound MT ends. These alternating states are confirmed by visualising EB (end-binding) protein that binds to polymerising MT ends and shows intermittent localisation at oscillating KT's^{293,294}.

1.7.4.2 Aurora-B Mediated Mechanisms

AurB plays a vital role in regulating KMT interactions. Phosphorylation of AurB KT substrates regulate MT binding, KMT attachment turnover and chromosome segregation. Tension and phosphorylation level of AurB substrates are inversely proportional^{254,295–299}. In absence of tension AurB phosphorylation levels are high and hence AurB destabilises incorrect KMT attachments^{263,300}. However in a study performed, Ndc80 was not completely rephosphorylated after the already formed KMT attachments were disrupted by nocodazole²⁹⁹. AurB can destabilise KMT attachments in two ways. First by promoting detachment of KMT attachments as observed invitro^{254,255,299,301,302}. Consistent with this observation is that when KT targeting of AurB in mitotic cells is increased stable KMT attachments cannot be observed²⁹⁵. Phosphorylation of KT AurB substrates like Ndc80C reduces the MT binding affinity and fewer KMTs are observed in phosphomimetic mutants of Ndc80³⁰³. Perturbing other AurB substrates like Dam1 (in yeast) and Ska complex (in higher eukaryotes) also destabilises KMT attachments²⁶³. Secondly, AurB can promote catastrophe and depolymerisation as per in vitro observation when expressing phosphomimetic mutants of Ndc80 and Dam1^{208,255}. Live cell imaging studies have reported that AurB promoted depolymerisation rather than instantaneous detachments of syntelic attachments. Syntelics are first pulled towards the pole followed by detachment as a result of enriched polar AurA activity^{251–253}.

1.7.4.3 Aurora-B Propagation Models

Two different models have been proposed on how AurB activity at the KT changes in response to interkinetochore tension. They are discussed below:

The spatial separation model that evolved from budding yeast experiments suggests that tension sensing is directly proportional to the distance from enriched inner centromeric pool of AurB and its outer KT substrates that regulate MT binding^{304,305}. In case of amphitelic attachment tension pulls the bi-oriented sister KTs away from the enriched inner centromeric regions. This stretch separates the kinase from its substrates leading to reduced substrate phosphorylation. Furthermore, positioning AurB to close proximity to KT substrates leads to increase phosphorylation and destabilisation of KMT attachments³⁰⁵. Consistent with the spatial separation model, perturbing AurB centromeric localisation disturbs normal regulation of KMT attachments. Phosphorylated histone (H3 and H2A) play a vital role in AurB centromeric localisation^{306–309}. Mutation of these histone phosphosites, in fission yeast, prevents CPC targeting and aberrant biorientation. Restoring normal CPC localisation via fusing Survivin (Sur), CPC component, to chromodomain that binds pericentromeric heterochromatin, rescued biorientation defect³⁰⁸. In humans, inhibition of Haspin kinase (responsible for H3 phosphorylation) or Sur mutation prevents normal AurB localisation leading to defective EC^{310,311}. Furthermore, mutating Cdk phosphosites on CPC prevents its centromeric targeting in both human and fission yeast leading to aberrant biorientation³¹². This can be rescued via fusion rescue as discussed earlier. These studies strongly support the role of inner centromeric pool of AurB in EC. As per the model, centromeric AurB emits a gradient while the tension regulates the change in the position of its KT substrates that modulate substrate phosphorylation^{254,298}. The ability of centromeric AurB to generate and maintain a steep gradient, that is located nanometres away from its substrates, can be explained by coupled kinase-phosphatase system that gives rise to bistable and non-linear behaviour³¹³. This centromeric pool of AurB is central for EC mechanism and bi-orientation^{307,312,314}.

Unlike in fission yeast and mammalian cells, budding yeast disruption of centromeric pool of AurB does not lead to major CS error or mitotic progression³¹⁵. This difference may be as the EC in budding yeast does not need to address merotelics as there is a single MT per KT. In alternate class of models, no specific regulatory role is attached to centromeric AurB pool³¹⁵. For tension-dependant EC, a specialised pool of KT localised kinase that is in close proximity to outer KT targets is invoked by some models. One such model is where the intra-KT stretch produced separates AurB from its substrates. Incenp acts as a flexible arm to determine the reach of AurB kinase from its KT binding site^{263,316}. This model can explain selective stabilisation of KMT attachments provided the intra-KT stretch relates to bi-oriented sister KT tension. However, the correlation between intra-KT stretch and tension on bi-orientated sister KTs is questioned^{157,317,318}. Another model proposes, tension sensitive AurB substrate binding sites that get inaccessible under tension and stabilise the attachments^{300,315}.

1.7.4.4 Selectivity of Aurora-B Mediated Error-Correction Mechanism

Both the tension dependant and independent EC mechanism are considered in the framework of the simplest hypothesis that the regulatory mechanisms are indiscriminate i.e. at a single KT they effect the stability of incorrect and correct KMTs equally¹²¹. However, most AurB EC models often assume that the stability modulation is selective and it applied only to incorrect KMTs while the correctly attachment KMT are either not regulated or positively regulated¹²². The precise mechanism for such discrimination is not very clear and it can be different for syntelic and merotelics. In yeast, AurB destabilises end on syntelics while lateral attachments are immune to AurB EC, thus to allow probing of right tension producing configuration³¹⁹. As amphitelic attachments in yeast are highly stable and do not turn over, and hence AurB destabilisation may not be needed³²⁰. Merotelics are more common in organisms with multiple KMTs. Selectivity solely based on tension-based mechanism is difficult to

explain as the tension is shared by all attachments on the KT. Hence, additional parameters need to be considered, some of which are (a) Directionality, as stability would be different for merotelic attachment if it is more lateral to KT plane than amphitelic attached³²¹ (b) Centromeric pool of kinesin-13 could destabilise KMT attachments if merotelics span between sister KTs³²² (c) Exposure of portion merotelic sister with incorrect attachments towards inner centromere, compared to the portion with correct attachments, exposes it to higher concentration of AurB EC activity^{122,257,260,267,323} (d) As MTs act as co-activators for AurB *in vitro*, potentially merotelic attachments contribute to AurB activity in way that could correct them^{324,325} (e) The kinase levels of AurB may in itself lead to EC as it accumulates at incorrectly aligned metaphase centromeres and merotelic attachments^{297,323}. An alternate view is that centromeric AurB prevents merotelics. In case of yeast, AurB mediates clamping of MT binding sites in a single KT via Monopolin and Condensin complexes inhibiting the chances of gaining merotelics^{326,327}. AurB regulates the localisation of condensin and its depletion gives rise to merotelics^{328–330}.

1.7.4.5 Aurora-B Dynamics

The success of cell division lies in the timely coordination of many events enabled by network of kinases and phosphatases. CPC, a four member complex, governs several key events in mitosis like error correction, formation and regulation of the contractile ring, etc.^{314,331}. It consists for 4 core subunits namely: Aurora-B (AurB), which is a Ser/Thr kinase and regulatory subunits Survivin (Sur), Borealin (Bor) and the scaffold INCENP (Icp). CPC is widely conserved in fungi and animalia³³². Sur, Bor and N-terminus (NT) of Icp forms a triple helical localisation module³³³. The kinase module is composed of Icp IN-box bound to AurB³³⁴

CPC initially accumulates on the pericentromeric heterochromatin during late S phase enabled by heterochromatin protein 1 (HP1) binding to Icp^{335,336}. A fraction of HP1 interacts

with human Mis14 (member of Mis12C) during interphase³³⁷ that can enable CPC recruitment to inner centromeric region prior to mitosis. H3-S10 phosphorylation by AurB dissociates HP1 binding from neighbouring H3K9me3 (Trimethylated Lys9 of Histone H3) enabling a switch from interphase mode to mitotic one^{338,339}. POGZ is required for H3-S10 phosphorylation and also for removal of CPC and HP1 from chromosome arms, thus promoting enrichment at inner centromeric region³⁴⁰. CPC enrichment on the inner centromere is enabled by interaction of Sur and Bor with centromeric histone marks created by mitotic kinases. Sur binds H3-T3 created by Haspin kinase while Cdk1 phosphorylated Bor binds H2A-T120 created by Bub1 kinase via Sgo as its centromeric adapter protein^{306,309,312,341}. As the histone marks overlap at the centromeric region they enable CPC enrichment³⁰⁸. PP1/Repo-Man phosphatase antagonises Haspin by dephosphorylating H3-T3 at chromosome arms^{143,342}. Centromeric region is not antagonised as AurB phosphorylates S893 of Repo-Man preventing PP1/Repo-Man recruitment to centromeres. PP2A in mammalian cells antagonises inhibitory S893 phosphorylation of Repo-Man leading to reversal of phosphorylation of H3T3 and H2AT120 that suppresses CPC recruitment at chromosome arms and release of centromeric CPC³⁴³. Bor targeting to centromere is also antagonised by down regulation of Cdk1 at AO³¹². However, removal of CPC from centromeric region is not a prerequisite for CPC localisation at cell equator, thus suggesting that major relocation to cell equator happens from chromosome arms^{344,345}.

Active removal of CPC from chromosomes arms is promoted by Culin3-based E3 ligase that ubiquitinates AurB and subsequently enables its removal by AAA+ ATPase Cdc48/p97 and its adaptor proteins Ufd1-Npl4^{346–349}. This process has been suggested to determine the level and distribution of CPC on chromosome arms prior to mitotic exit and chromosome decondensation and nuclear envelope reformation after the exit³⁴⁸. The relocation of CPC after its active removal from the chromosome arms depends on Icp and AurB

interaction with motor kinesin Mklp2^{350–353}. The interaction of CPC with Mklp2 happens only during MAT when the inhibitory Cdk1 phosphorylation of Icp (T59) and Mklp2 (at multiple sites) is removed via PP1/PP2A^{354,355}. Dephosphorylation of Icp (T59) also increases its microtubule binding affinity³⁵⁴. Cdk1 phosphorylation of Mklp2 is required for its timely recruitment to the anaphase chromosomes to modulate the spindle dynamics³⁵⁵. In budding yeast (where Mklp2 is absent) CPC spindle midzone localisation depends on Bim1 (homologue of EB1) while in *Dictyostelium discoideum* it depends on Icp interaction with Kif12 (kinesin 6 related central spindle binding protein)³⁵⁶. Prior to AO, Cdk1 phosphoinhibits AurB to interact with EB1³⁵⁷. In *S. cerevisiae*, AurB relocation also depends on Cdc14 phosphatase. Cdc14 dephosphorylates several Cdk1 and AurB locations mainly in the microtubule binding sites of Icp as phosphorylation of these sites prevents microtubule binding of Icp prior to AO^{358–360}. The conservation of this mechanism in mammalian cells is unclear³⁶¹. Failure to relocate CPC from centromere at AO reactivates SAC in yeast and mammalian cells as observed by Mad1, BubR1 and Bub1 KT recruitment^{358,362}. However, this does not create a functional SAC that inhibits APC^{Cdc20} and hence the segregation of sister chromatids and CycB degradation happen with normal kinetics.

The centromeric localisation of CPC is controlled mainly by three feedback loops: 1) Haspin and AurB 2) Bub1 and AurB 3) Plk1 and AurB discussed below:

Feedback loop 1 (Haspin and AurB): H3-T3 phosphorylation by Haspin, is recognised by Sur (BIR domain) enabling AurB recruitment which further phosphoactivates Haspin^{307–309,311,363,364}. In *X. laevis* (XL), Plk1 and not AurB is vital for H3-T3 phosphorylation. On mitotic entry, Cdk1 phosphorylates Haspin, enabling Plk1 recruitment that in turn phosphorylates Haspin. Both kinases Plk1 and AurB are required for Haspin phosphorylation leading to AurB centromeric enrichment^{364–366}. Hence, the feedback loop as described in fig.

1.11,1a. *In vitro*, methylation of H3-K4 that neighbours H3-T3 inhibits its phosphorylation by Haspin³⁶⁷. Additionally, prometaphase and metaphase dephosphorylation of H3T3 by PP1γ-Repo-Man is antagonised by AurB phosphorylation of Repo-Man disabling its chromosome targeting^{342,343,368}. Hence, the feedback loop as described in fig. 1.11, 1b.

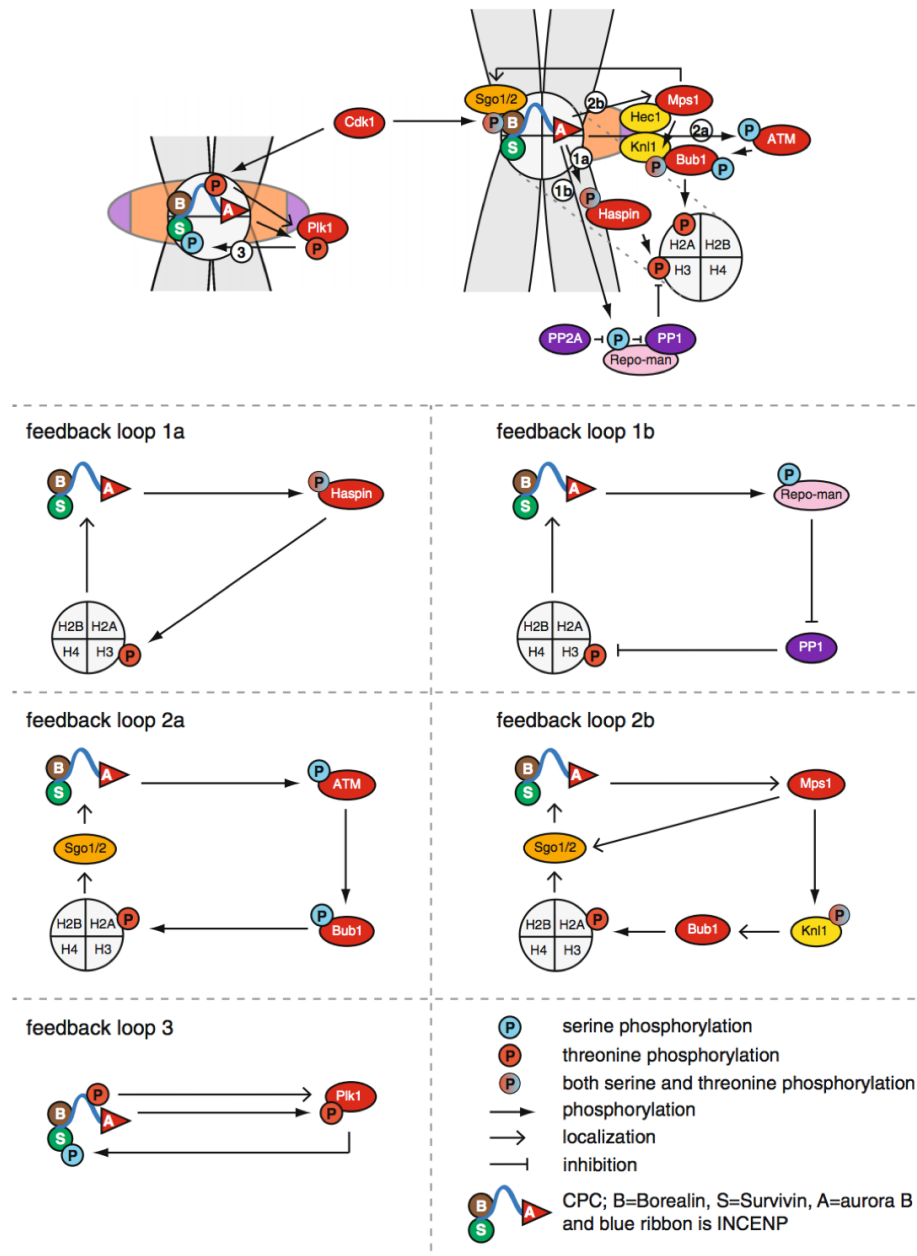


Figure 1.11. Feedback loops governing chromosomal passenger complex localisation
Picture and text adapted from: Cell division: control of the chromosomal passenger complex³⁶⁹

Feedback loop 2 (Bub1 and AurB): DNA damage ATM kinase phosphoactivates Bub1 that promotes H2A-T120 phosphorylation and stabilises Sgo (CPC centromeric adapter)

binding to nucleosomes^{306,308,370–374}. While, ATM itself is activated by AurB phosphorylation³⁷⁵. Hence, the feedback loop (fig 1.11 2a). In addition to the kinase activity of Bub1 its localisation is also required for H2A phosphorylation^{306,308,370}. Bub1 localisation depends on Mps1 activity in both human and yeast cells^{241,376–379}. In case of yeast, Mps1 phosphorylates Spc105/Knl1 that enables Bub1 binding. In mammalian cells, Bub3 may also contribute to localisation of Bub1 to the KTs^{380,381}. Hence, Mps1 inhibition delays recruitment of CPC to the KTs as Bub1 recruitment depends on Mps1. Additionally, Mps1 also plays a role in a more direct pathway where it enables Sgo localisation required for CPC centromeric recruitment that is downstream of Bub1 kinase³⁷⁸. As AurB is required for optimal Mps1 and Bub1 recruitment and phosphorylation of H2A, the positive feedback loop^{362,378,382–384} as shown in fig. 1.11 2b.

Feedback loop 3 (Plk1 and AurB): In diploid RPE1 and fetal fibroblasts, AurB centromeric enrichment on misaligned chromosome depends on Plk1 activity in addition to its own, although inhibiting Plk1 does not change H2A-T120 and H3-T3 phosphorylation levels²⁹⁷. Plk1 phosphorylated Sur (S20P) promotes AurB activation while Cdk1 mediated Icp phosphorylation (T388p) enables Plk1 centromeric localisation^{352,385}. In DM early mitosis, AurB phosphorylates centromeric Polo in its activation loop (T210 in humans and T182 in DM), while AurB and Incenp depleted cells show a reduction in Plk1-T210 phosphorylation levels³⁸⁶. Hence a feedback loop involving Plk1 and CPC (fig. 1.11 loop 3).

1.8 Spindle Assembly Checkpoint

The singularity of mitosis is to produce two genetically identical cells governed by mechanochemical feedback surveillance mechanism referred as SAC^{124,387}. SAC performs 3 key activities^{161,162}: a) senses any spindle defects, unaligned chromosomes, non-optimal

tension as a result of unattached or unstable stable KMT attachments b) inhibits AO by preventing CycB degradation c) inhibits sister chromatid separation by preventing Separase activation. SAC halts the cell cycle progression to perform its function by generating diffusible wait signal called Mitotic Checkpoint Complex (MCC)¹²⁶ that modulates APC activity which governs MAT³⁸⁸. KMN network proteins along with AurB and Mps1 play a vital role in enabling SAC functions³⁸⁹. For e.g. AurB phosphorylated Ndc80C is key to recruit Mps1 which phosphorylates Spc105 generating a cascade to recruit SAC and regulatory proteins including Mad2^{384,390–396}

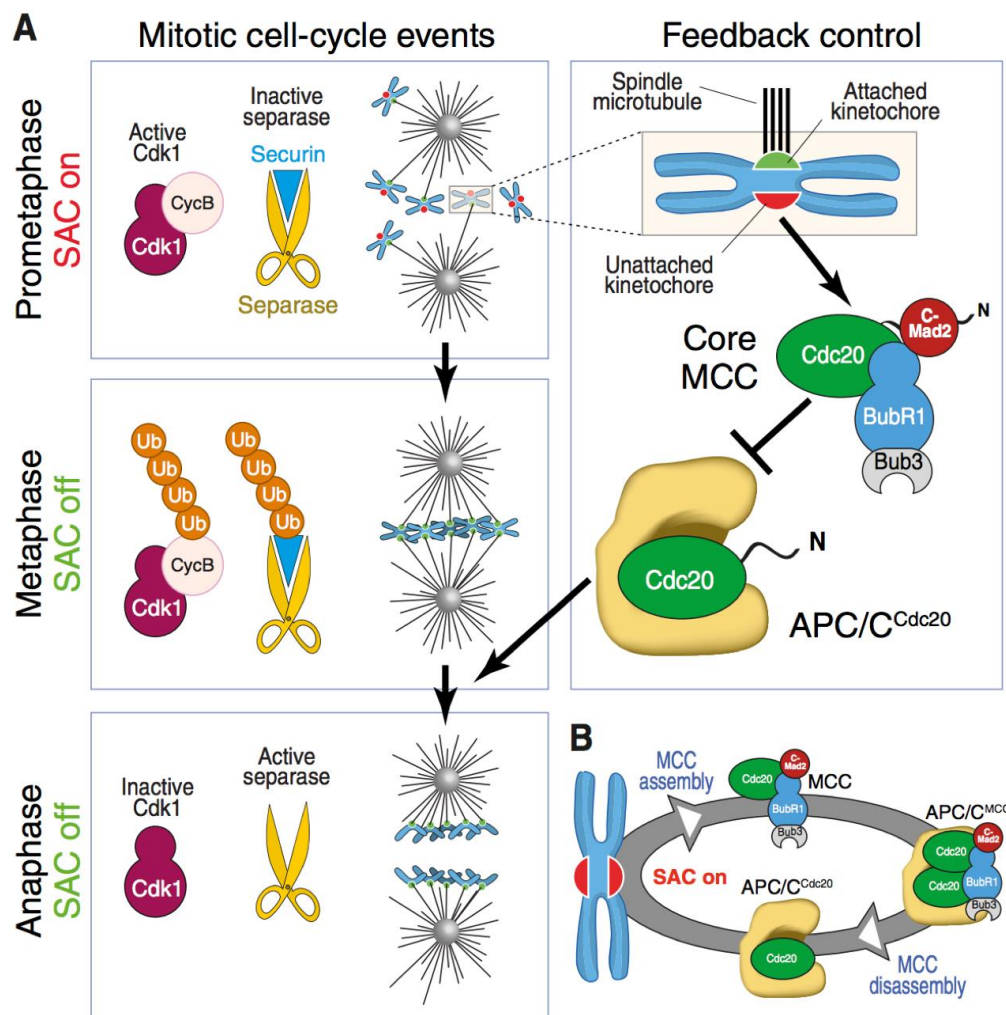


Figure 1.12. Spindle assembly checkpoint

Picture and text adapted from: The Molecular Biology of Spindle Assembly Checkpoint Signaling Dynamics¹²⁴

The wait signal generated by SAC prevents AO by sequestering APC activating subunit Cdc20^{397,398}. Biochemically, MCC is composed of four members: C-Mad2 (close form of Mad2), Bub3, BubR1 and Cdc20 and is primarily generated at the KT with maximum potency in mitosis³⁹⁹. However, partial low potency complex called as BBC (made of Bub3, BubR1 and Cdc20) contributes to timely entry into mitosis¹²⁶. The open (O) to close (C) conformation change is driven by Mad1-CMad2 complex that's is bound to unattached or unstably attached KTs^{400,401}. Cdc20 has dual role^{109,399,402-404} a) by being a part of MCC b) by activating APC i.e. forming APC^{Cdc20}.

MCC generates a potent diffusible wait signal by inhibiting APC activity even from a single unattached or unstably attached KT^{110,405,406}. Inhibiting APC activity not only arrests the cell in mitosis with high Cdk1-CycB but also prevents sister chromatid separation by preventing cleavage of Cohesin ring¹¹². By sequestering cytoplasmic and APC bound Cdc20, MCC not only inhibits any new APC activity but also inhibits already activated APC (APC^{Cdc20})⁴⁰⁷, preventing premature CS. Once the SAC requirements are satisfied, KTs stop producing MCC and the already matured MCC disassembles, enabling APC activation.

The four core subunits of the Cohesin complex are Scc1/Mcd1/Rad21, Scc3/Mcd3, Smc1 and Smc3^{53,408}. Cohesin associated proteins Pds5 and Wpl1 that regulate its function bind Scc3 and to the Scc2/4 Cohesin loader^{409,410}. At AO, Separase removes Cohesin mediated sister chromatid linkages by cleaving Mcd1 subunit⁴¹¹. Separase is inhibited by Securin during early mitosis, which is activated during metaphase via regulated degradation of Securin by APC-Cdc20⁴¹²⁻⁴¹⁵. Securin is essential in budding yeast however, in fission yeast and metazoans Securin mutants are viable undergo Cohesin cleavage during mitosis⁴¹⁶⁻⁴²⁰. The observations suggest additional mechanisms for regulating Separase activity. In vertebrates, Separase activity is inhibited by Cdk1 phosphorylation and binding and this regulation works

redundantly with Securin inhibition of Separase^{421,422}. Human Separase activity is also shown to be regulated positively and negatively via PP2A interaction^{423,424}. Although, down regulation of Cdk1-CycB activity (via APC^{Cdc20}) is an required for mitotic progression, in budding yeast complete degradation of CycB activity was not observed to be necessary for sister chromatid separation⁴²⁵. Preventing complete CycB degradation can render the MAT reversible by reactivating SAC and the wait signal as observed in mouse oocytes¹⁰⁹.

A single KT must generate enough MCC to avert AO and sister chromatid separation. However, if MCC scales linearly with the number of non-amphitelic KMT attachments it would generate excessive MCC, delaying AO after formation of amphitelic ones⁴²⁶. Generally, the kinetics of MCC generation and its steady-state concentration suggests that it falls short of requirement to completely inhibit APC⁴²⁷. The residual activity of APC can degrade CycB along with drop in activity of additional proteins leading to mitotic slippage with unattached KTs^{19,428,429}. An inverse relation exists between CycB and MCC^{430,431}. SAC inactivation or silencing stops generation the new MCC and rapid inactivation already formed MCC¹¹³.

SAC works on a feedback mechanism that is dependent on error monitoring, error correction machinery and enforcement/effector module along with signals from mitotic spindle, kinases and phosphatases control system¹²⁴. APC^{Cdc20} is the SAC target while MCC is the SAC effector. The error monitoring and correction module looks for any unattached and or poorly attached KMT attachments, which then serve as site for generation of MCC³¹⁴.

1.9 Anaphase Promoting Complex or Cyclosome

1.9.1 Structure

Vertebrate APC is made of ~15 different subunits (~1.22 MDa). It is organised into mainly 3 structural domains as shown in fig1.13: (a) platform, that joins the subunits (b) the catalytic core, together with E2 provides catalytic activity (c) Tertatricopeptide repeat, (TPR or arc lamp) lobe, provides vital scaffolding functions^{23,60,432}.

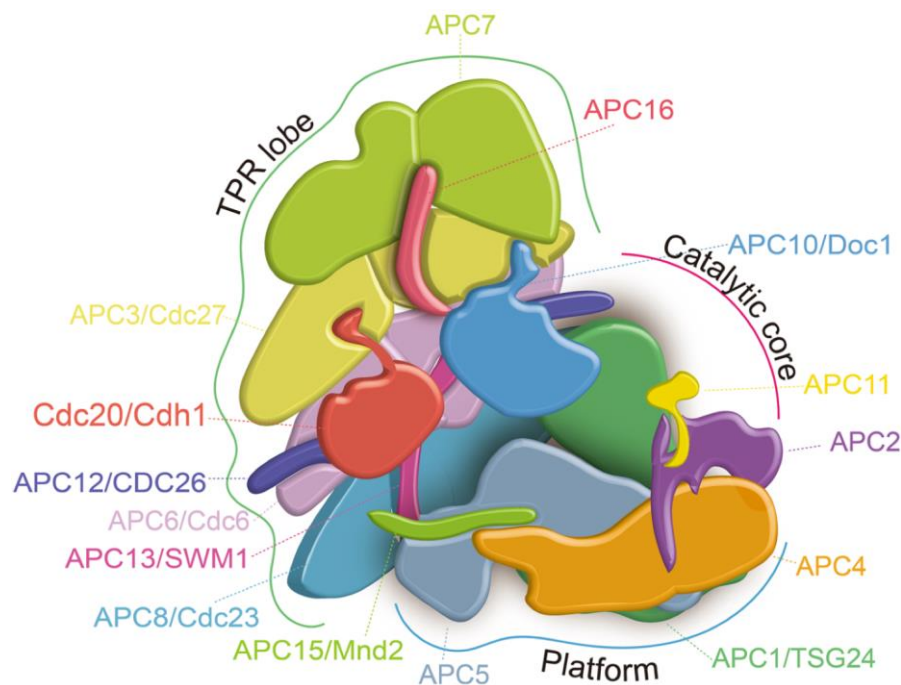


Figure 1.13. Schema of APC subunit arrangement

Picture and text adapted from adapted from: Insights into APC/C: from cellular function to diseases and therapeutics²³

1.9.2 Co-Activators, Substrate Recognition and Turnover

APC activation and enhancement of its ubiquitination activity is enabled by conformational changes brought due to binding of its coactivators (Cdc20 or Cdh1 in a 1:1 stoichiometry)^{433–435}. Cdc20 associates with phosphorylated APC enabling mitotic substrate degradation while Cdh1 replaces Cdc20 during late mitosis till G1. Cdc20 is degraded by Apc^{Cdh1} that potentially has a greater substrate recognition^{433,436}. APC substrate specificity is

determined by Cdc20 and Cdh1 recognising the destruction box (D-box) and KEN-Box (e.g. CycB and Securin)^{437–441}, ABBA/Phe/A-box/IC20BD (e.g. CycA/Clb5, Acm1 which is a Cdh1 inhibitor in budding yeast, BubR1 and Bub1, A-Box is recognised by yeast Cdh1 and not human)^{442–449} and potentially other non-conserved motifs (e.g. CRY-box, O-box etc.)^{443,444,447,450–453} on the substrates that bind to WD40/ β -propeller domains of coactivators^{23,59}.

1.9.3 Regulation

Regulation of APC is a complex process governed by post-translational modifications like phosphorylation, acetylation, sumoylation and substrate specific factors. The activation of APC depends on regulation of its two co-activators i.e. Cdc20 and Cdh1. Additionally, other factors that govern APC modulation are the MCC, early mitotic inhibitor 1 (Emi1), etc. Some of the mechanisms are discussed below.

APC antagonist, Early mitotic inhibitor (Emi) 1 and 2 enable timely entry into mitosis^{454–456}. Emi1 via its ZBR domain and a D-box inhibits APC activity²³. ZBR domain directly suppresses E3 ligase activity of APC by binding to its core unit, while the D-box of Emi1 binds both of APC co-activators. Thus, Emi 1 works as a APC pseudo-substrate by preventing it from binding other substrates⁴⁵⁷. Emi1 ultimately switches from being a substrate to an inhibitor for APC-Cdh1⁴⁵⁸. Emi2 also blocks APC activity by competitively inhibiting association of Ube2S with APC10 (subunit of APC)⁴⁵⁹. On mitotic entry, Plk1 phosphorylates Emi1 and Emi2 thus marking them for degradation by SCF/ β -TrCP (β -transducin repeatcontaining protein)^{455,460–463}. Additionally, in mitosis Cdk1 can also phosphorylate Emi reducing its ability to bind and inhibit APC⁴⁶⁴. Some studies have challenged, Plk1 mediated Emi1 degradation and Emi1 degradation itself (using Emi1 non degradable mutant) as non-

essential requirement for mitotic entry but rather as a supportive function for timely entry^{465–468}.

Another mechanism by which APC is regulated is via Large tumour suppressor kinase (LATS) 1 and 2 serine/threonine kinases. They phosphorylate APC12/Cdc26 that modifies the interactions of the TPR subcomplex of APC ultimately regulating its activity⁴⁶⁹. Additionally, APC is regulated via deubiquitination (DUBS) that reverse the ubiquitination to their original form^{470–473}. Several DUBS have involved in counteracting APC activity, namely USP44, USP37, USP22, Ubp15 (budding yeast) etc.^{371,474–476}. The contrast between APC and DUBS mimics a switch like behaviour modulating cell cycle progression⁴⁷⁰.

Phosphorylation by Cdk1 and potentially Plk1 plays a major role in APC regulation. APC is phosphorylated at many sites on its various subunits that enhances the binding of its co-activator Cdc20^{402,477–482}. APC phosphorylation by Cdk1-CycB complex is with increased potency when the complex is bound to Cks^{477,478,483–485}. In addition, Plk1 has been suggested to activate APC in mitosis, although inhibiting Plk1 does not seem to block APC activation^{465,477}.

In early mitosis, APC phosphorylation (that relieves auto-inhibition) by Cdk1 and potentially by Plk1 increases its Cdc20 binding capacity^{402,477,482,484,486,487} while its Cdh1 interaction is restricted as Cdh1 is phospho-inhibited by Cdk1 and sequestered by Mad2^{482,488–490}. Cdc14 phosphatase antagonises inhibitory Cdk1 phosphorylation of Cdh1 while Mad2 is degraded, thus enabling its association with APC during later mitosis^{482,488–492}. APC^{Cdh1} ubiquitinates Cdc20, Aurora A and B, Plk1, Tpx2, etc^{493–498}. Although, the conventional view is that APC activator changes from Cdc20 to Cdh1 at AO, the process is more transient than like a switch⁴⁹⁹. Cdh1 is inactivated by degradation by SCF(β-TRCP and APC-Cdh1^{500–504}).

1.10 Mitotic Check Point Complex

1.10.1 Subunits

Tetrameric MCC is composed of C-Mad2-Cdc20-BubR1-Bub3 in 1:1:1:1, stoichiometry and the MCC can bind a second Cdc20 that is associated with APC^{126,399,407,505}. Bub3 is absent in fission yeast while BubR1 may have variations^{126,506,507}. MCC is referred as SAC effector as its assembly (enabled by unattached KT) is required to inhibit APC^{Cdc20} activity^{407,508}. The MCC assembly is a complex multi-step process enabled by regulatory and structural proteins, that may not be directly part of the MCC core^{113,125,388,389,509}. Mitotic MCC is primarily built on the structural KT proteins that forms the KMN network^{389,510}.

MCC subunit domains and its interactions has been well summarised by Liu and Zhang¹²⁶. BubR1 is the largest protein containing two sets of KEN (K1 and K2), ABBA (A1 and A2) and D (D1 and D2) boxes. BubR1 binds directly to C-Mad2 in the MCC via K1 and the three tetraco peptide repeating motifs (TPR). BubR1 interacts with Cdc20 in the MCC (hence forth called as Cdc20M) via K1, A2 and D2. The interaction with Cdc20 in the APC (henceforth called as Cdc20A) is via K2, A1 and D1. BubR1 is a pseudosubstrate that can inhibit APC^{Cdc20} as it has all the three degron sequences. BubR1 has additional motifs like KARD (required for its KT functions), etc. Bub3 is almost entirely made up of WD40 repeats forming a β -propeller with some key residues on the top forming recognition sites for GLEBS sequence of Bub1 and BubR1. Bub3 forms a cell cycle independent complex with Bub1 and BubR1 and targets them to the KTs. Cdc20 can recognise the KEN box and the D box as presented by the APC substrates. Cdc20 can also recognise the ABBA motif. The C-terminal of Cdc20 contains the WD40 domains forming a β -propeller. Cdc20 has three APC binding motifs: C-box, C-terminal isoleucine-arginine (IR) tail and the KILR/MIM motif. Cdc20M

uses the MIM motif to interact with Mad2 while Cdc20A when interacting with APC uses the C-box and MIM to interact with APC8 and IR motif to interact with APC3. Cdc20M interacts with BubR1 with its three degron sequences. Cdc20 also contains KEN and CRY box required for its own ubiquitylation by Cdh1. Mad2 is discussed along with the template model.

1.10.2 Template Model and Assembly

One of the rate limiting steps to form MCC is the conformational activation of Mad2, which exists in inactive/open (O-Mad2) and active/closed form (C-Mad2)^{113,511,512}. C-Mad2 binds several proteins like Mad1 and Cdc20 via its MIM/KLIR motif^{509,513–516}.

O and C Mad2 have the same stable core differing in their N and C terminals. When bound to a ligand, CT of C-Mad2 wraps around MIM (two β strands) forming a safety belt across the core, while O-Mad2 CT does not form a safety belt and blocks the ligand binding site^{511,515}. Unliganded O-Mad2 converts to thermodynamically stable unliganded C-Mad conformation, in which case the safety belt is formed without trapping a ligand^{517,518}.

The unattached KT leads to Mps1 enabled Mad2 activation enabling MCC formation via a series of recruitment and signalling events^{400,509,519}. The KMN network provides a platform for SAC proteins and microtubule attachments⁵¹⁰. On unattached KTs, AurB phosphorylates Ndc80C to recruit Mps1^{384,390,391} that generates a signalling cascade⁵¹⁹ and activation of Bub1-Mad1 scaffold⁵¹⁹. Mps1 recognises unattached KTs by direct binding to Ndc80C that serves as KMT docking site^{390,391}. Ndc80 bound to Mps1 phosphorylates MELT motifs of Knl1 providing a docking site for Bub1-Bub3^{520,521}. Bub1-Bub3 phosphorylated by Mps1 and Cdk1 recruits Mad1-C-Mad2 (constitutive complex) followed by BubR1-Bub3 and Cdc20^{239,445,448,522,523}. The BubR1-Bub3 complex can be recruited to the KT directly via Knl1 or via interaction with Bub1^{523,524}. Cdc20 interacts directly with Bub1 and BubR1 at the

KTs^{445,448,449}. The KT assembled and Mps1 phosphorylated, Mad1-C-Mad2 tetramer (2:2, stoichiometry) further recruits cytosolic O-Mad2, converting it to intermediate (I)-Mad2 at an accelerated rate^{113,509,519} (fig. 1.14). O and C Mad2 interact via dimerization that includes α CT-helix^{513,525}. I-Mad via a high energy transition unfolds its CT to bind Cdc20 to form Cdc20-C-Mad2 that associates with BubR1-Bub3 to produce diffusible MCC^{509,526}.

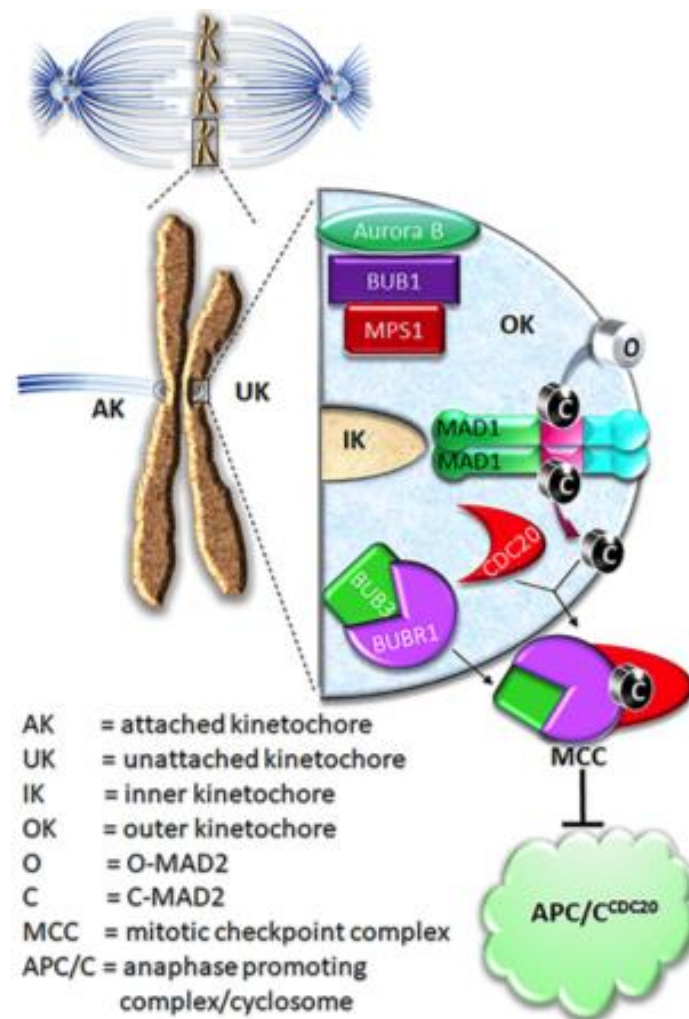


Figure 1.14. Template model for mitotic checkpoint complex formation and kinetochore assembly
 Picture and text adapted from adapted from: MAD1: Kinetochore Receptors and Catalytic Mechanisms⁴⁰⁰

KTs may not be involved in production of MCC during interphase as in metazoan KT's are not completely assembled and do not host all SAC proteins^{126,231,232,527}. Nuclear-pore dependant interphase MCC assembly has been reported^{399,528}. In this interphase MCC assembly mechanism, Cdc20 interacts with BubR1-Bub3 (cell cycle independent complex) forming

BubR1-Bub3-Cdc20 (BBC)⁵²⁹. Lower levels of C-Mad associate with BBC to form low potent MCC^{530–532}. Levels of C-Mad2 and Cdc20 increase during prometaphase and producing potent MCC^{401,533,534}. Thus, the main mechanism of potent MCC production is still during mitosis. The interphase assembled MCC inhibits low level of APC-Cdc20 regulating the time to metaphase that enables correction of merotelic attachments without overriding SAC⁵²⁸. BBC although a bonafied inhibitor of APC has lower potency compared to MCC¹²⁶.

Blocking E2 ligase or substrate recruitment or affecting their proximity can inhibit substrate ubiquitination¹²⁶. Binding of MCC with APC-Cdc20 reduces Ubch10 substrate association. MCC bound to active APC (APC^{Cdc20} or APC-Cdc20) comprises two molecules of Cdc20, one bound to APC (Cdc20A) and another as a part of four membered MCC (Cdc20M)^{407,535–537}. Structural studies of MCC-APC^{Cdc20} have provided insights into how APC is inhibited by MCC^{536,537}. Cdc20A binds tightly with MCC and the interaction shifts it away from the D-box co-receptor APC 10. Secondly, BubR1 with its two sets of D-box (D1 and D2), KEN-box (K1, K2) and ABBA motif (A1-A2) blocks all the three degron binding sites on Cdc20A and Cdc20M. Also, BubR1 TPR motif binds with APC2 creating a steric hinderance that prevents Ubch10 binding to the catalytic core, APC2-AP11. Thus, MCC blocks APC-Cdc20 access to substrates and Ubch10 inhibiting APC activity. However, binding of MCC does not prevent Ube2S binding with APC-Cdc20 and does not prevent ubiquitin chain elongation if the substrate is already ubiquitinated^{537,538}.

1.10.3 Disassembly and Silencing

On formation of amphitelic attachments, cells need to disassemble existing MCC (cytoplasmic and attached to APC^{Cdc20}) and silence KT SAC signalling for production of new MCC^{125,126}.

1.10.3.1 Disassembling Existing Mitotic Check Point Complex

The disassembly happens on two fronts: MCC bound to APC and cytosolic MCC^{113,539}. MCC bound to APC disassembly (fig. 1.15) relies on APC^{Cdc20} enabling the release of MCC via autoubiquitination of Cdc20M which was antagonised by DUB, UPS44^{24,474,535,540}. Further Cdc20M degradation releases BubR1 and Mad2^{541,542}. *In vivo* p31^{comet} promotes Cdc20M ubiquitination^{543–546}. SAC mediated autoubiquitination of Cdc20M also depends on Mad2 and BubR1 suggesting it's ubiquitination occurs in context of APC^{Cdc20} bound to MCC⁵³⁷. Cdk1 phosphorylated CUEDC2 (Ub-reader) plays a role in detecting the Cdc20M ubiquitination state⁵⁴⁷.

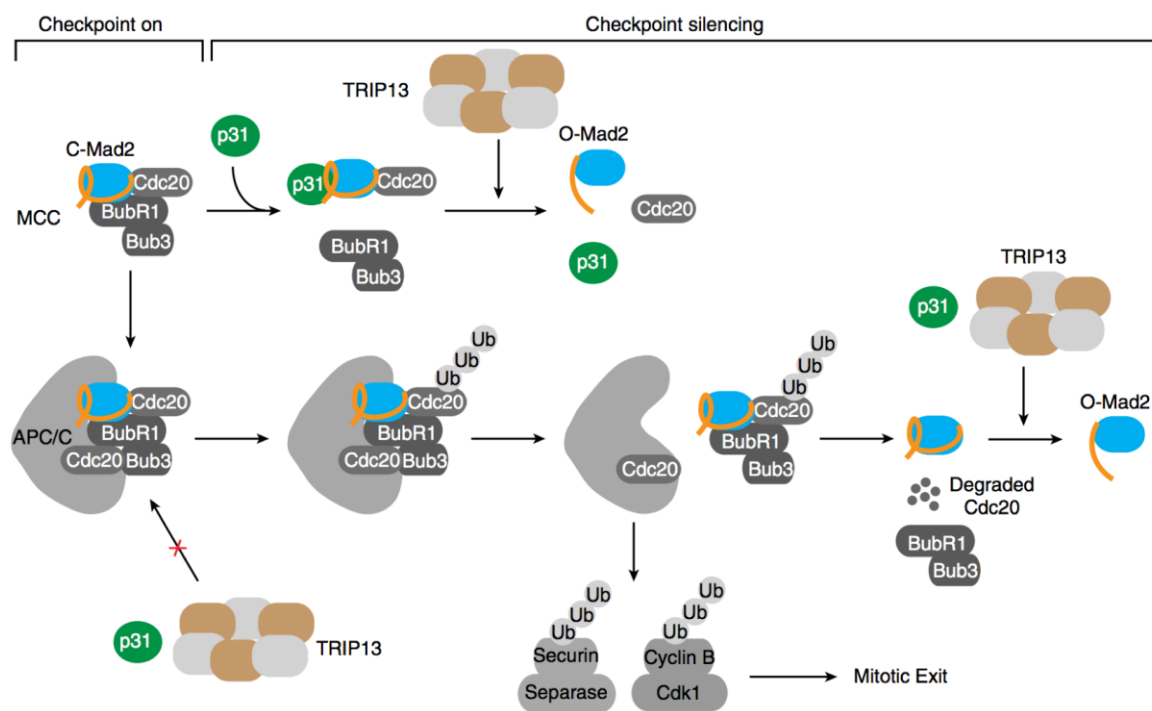


Figure 1.15. Mitotic checkpoint complex disassembly

Picture and text adapted from: Mechanistic insight into TRIP13-catalyzed Mad2 structural transition and spindle checkpoint silencing¹¹³

Cytosolic MCC disassembly (fig. 1.15) relies on p31^{comet} binding to C-Mad2 that is tethered to Mad1 at the KT's when the checkpoint is on and in the cytosol. This limits Mad2 conformation change from O to C form^{548–550}. p31^{comet} specifically binds C-Mad2 at the same interface BubR1 and C-Mad2, that disrupts MCC integrity and leads to displacement of

BubR1-Bub3¹¹³. Trip13-p31^{comet} (TP31C) only inactivates free MCC and cannot inactivate MCC bound to APC-Cdc20¹¹³. The p31^{comet}-C-Mad2-Cdc20 is recognised by Trip13 that catalyses the spontaneous conversion of C-Mad2 to O-Mad2 dissociating Cdc20 and leads to MCC disassembly^{113,551–555}. p31^{comet} acts as an adaptor to recruit C-Mad2 to TP31C and is phospho-modulated by Inhibitor of nuclear factor (IKK-β)^{556–558}. Additionally, in the mechanism discussed earlier i.e. MCC bound to APC disassembly, after the MCC dissociates from APC-Cdc20, Trip13-p31^{comet} can convert C-Mad2, which is released as a consequence of Cdc20M degradation, into O-Mad2. Absence of C-Mad2 conversion to O-Mad2 leads to aberrant C-Mad2 accumulation and O-Mad2 insufficiency in the system. O-Mad2 insufficiency leads to deficient I-Mad2 since O-Mad2 is required for the formation of I-Mad2 at the KT by Mad1-C-Mad2. Finally, it is the I-Mad that undergoes high energy transformation to C-Mad that is a part of mature MCC. Thus premature accumulation of C-Mad2 leads to spindle defects^{113,559}. Additionally, TRiC/CCT chaperonin binds and disassembles subcomplexes of MCC that lack Mad2⁵⁶⁰.

1.10.3.2 Silencing Formation of Mitotic Check Point Complex

This section discusses the contribution of several proteins in silencing SAC signalling that leads to formation of new MCC.

- a) The key checkpoint signalling mechanism at the KT is Mad1-C-Mad2, that needs to be removed, as artificial tethering of this complex activates SAC even after AO^{213,561,562}. RZZC (Rod/ZW10/Zwilch complex) localises cytoplasmic dynein minus end motor via adaptor protein Spindly, that removes Mad1-C-Mad2 from KTs once KMT attachments mature^{563–567}. Cenp-I protects Mad1-C-Mad2 premature removal till amphitelic KMT attachments are formed⁵⁶⁸. However, in yeast where RZZC is absent offloading Mad1-C-Mad2 depends on Bub1

removal⁵⁶⁹. The Mad1-C-Mad2 is also inhibited by capping of C-Mad2 by p31^{comet} once it is removed from the KT_s that prevents binding of O-Mad2⁵⁷⁰.

- b) Mps1 is essential protein required for SAC functioning and recruitment of key SAC components to the KT^{242,519,571}. Mps1 directly interacts with Ndc80 via its CH (Calponin Homology) domain and the same domain also establishes end on attachments^{215,390,391,396,572}. Hence it is postulated that progressive MT binding can potentially displace Mps1 from the KT_s and may lead to SAC inactivation^{390,391}. However, this hypothesis needs to be corroborated with evidence demonstrating that Mps1 is not only recruited on KT_s with robust MT fibres but is also present on attached KT_s in *S. cerevisiae*^{362,572}. Furthermore, cytosolic Mps1 can keep the SAC arrest caused by tethering Mad1 to the KT_s even after it has been removed from KT_s, suggesting its removal from the KT is insufficient for SAC silencing^{213,573}. This is in contrast to the reports suggesting that off-loading of Mps1 from the KT_s may be essential for SAC silencing and mitotic progression^{214,574}. However, since the experiments were conducted by tethering Mps1 to the KT_s it needs to be conclusively established that such an arrangement does not disturb the KMT attachment causing SAC activation. However, as a rebuttal to the above reservations, the following considerations have been raised¹⁶¹. The competition between Mps1 and MT_s for binding the CH domain of Ndc80 removed large portion of Mps1 but not all of it^{390,572}. Also, it has been shown that under certain conditions, KT_s with end on attachments recruit SAC proteins and retain Mps1^{112,362,390,572}. Furthermore, metaphase KT_s with end on attachments have Mps1 activity as it activates SAC if Mad1 is tethered to the KT_s^{213,561,562}. However, this raises the question of residual Mps1 not able to phosphorylate Knl1/Spc105, Although, an explanation for this hypothesis is that Ndc80 CH domains and Spc105/Knl1

phosphodomain act as two ends of a toggle switch^{161,430}. When the two terminals are together the SAC is on and when away the SAC is off. Only a stable amphitelic attachment can consistently and robustly keep the two ends apart. An alternative model suggests that as the distance between Mps1 and its substrates increases on KMT attachments and hence its ability to phosphorylate MELT motif of Spc105 diminishes^{430,572}. In absence of Spc105 phosphorylation SAC components are released or not recruited at KT's leading to SAC silencing⁵¹⁹. This model also supports the observation that intra KT distance is vital in SAC silencing and signalling^{157,158}. Additionally, this explanation corroborates with the finding that the ability of AurB to phosphorylate its substrates decreases as KMT attachments progress to bi-orientation^{254,295}. Hence this model potentially explains the release of Mps1, as its KT localisation is promoted by AurB, from the KT's as KMT attachments move towards amphitelic configuration^{382,384,395,396}.

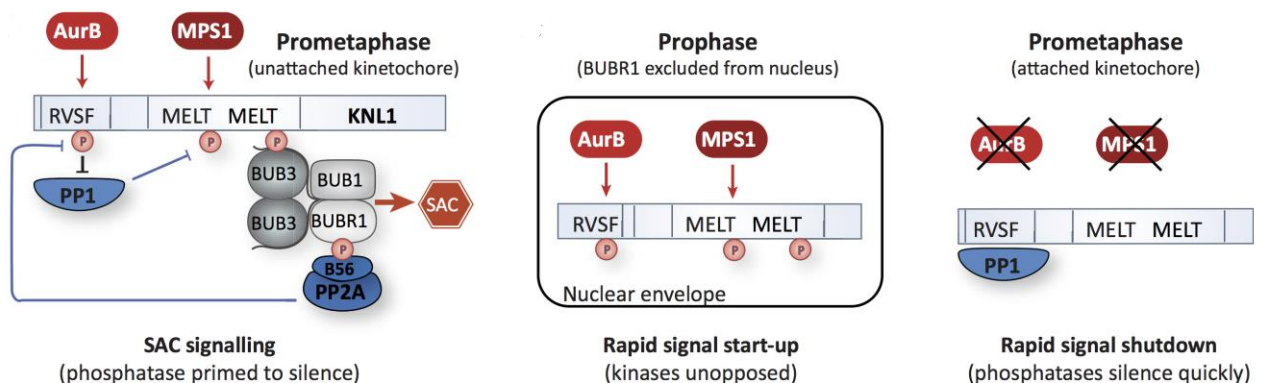


Figure 1.16. BubR1 and PP2-PP1 mediated silencing of spindle assembly checkpoint signalling
Picture and text adapted from adapted from: The Importance of Kinase–Phosphatase Integration: Lessons from Mitosis¹³⁵

- c) Distinct interactions of PP2A-B56 with BuR1 and Sgo regulates its KT and centromeric recruitment^{575–582} (fig. 1.16). Sgo is recruited to KT's by binding Bub1 phosphorylated H2A^{583,584}. Mps1 phosphorylated MELT of Knl1/Spc105 recruits most of SAC proteins including BubR1⁵¹⁹. PP2A interacts with Cdk1 and Plk1

phosphorylated BubR1 (KARD domain) and interference with this interaction leads prevents KMT stabilisation as it leads to increased AurB activity^{578,579,585–588}. PP2A dephosphorylates PP1 (S893, that was initially phosphorylated by AurB) targeting PP1 to antagonise AurB phosphorylation of Knl1/Spc105 RVSF domain, enabling PP1 recruitment at Knl1/Spc105^{343,589–591}. PP2A mediated PP1 binding to RVSF motif of Knl1/Spc105 dephosphorylates MELT motif inhibiting Bub3 recognition that leads to removal of Bub1 and BubR1^{343,376,591–593}. One of the possible reasons that SAC signalling recruits a phosphatase, is that it may allow SAC signalling to start and stop rapidly (fig. 1.16). BubR1 is initially not present in nucleus, and hence kinase activity is unchallenged by phosphatases which changes after NEBD. PP2A-B56 is recruited to the centromeres through Sgo that dampens AurB activity (including reversal of MCAK) playing a role in KMT stabilisation and bi-orientation^{580,581}.

Chapter 2 Materials and methods

2.1 Stock Keeping

Two copies of transgenic *D. melanogaster* fly stock were kept. The working stock at room temperature (i.e. 25°C and backup one at 18°C. In order to transfer the flies to new bottles or vials (henceforth called as container), the older containers are gently tapped, keeping at an angle of 45°, on a rubber pad so that the flies are away from the mouth of the container. The angle prevented the flies from falling into the food at the bottom. The mouth of the container was unplugged, and the flies were transferred to a new container with fresh fly food. This process had to be done very quickly to avoid the flies from escaping. Some genotypes needed more flies in the container compared to others. specially if the hatching rate of the eggs was lower. Temperature plays an important role in the developmental cycle of *D. melanogaster*. The time from egg to adulthood at 25°C is approximately 11 days while at 18°C its around 19 days. The working stocks were transferred to new container every two weeks while the backup stocks were transferred every four weeks.

2.2 Virgin Collection

At room temperature, the female flies would be virgins up to 10 hours after hatching. However, the virgin flies were collected every 6-7 hours. In order to collect the virgins, container was tapped on a gas pad emitting an anaesthetic (CO₂) so that the flies would fall on

the pad. Another approach is to use an injector to inject CO₂ gas in the container kept at an angle to prevent the flies falling into the food. An injector has a narrow pipe like a syringe which can be put in the container via its mouth without removing the cotton plug to prevent the flies from escaping. Once the flies were anaesthetised, they would be transferred to the gas pad. The females were selected and put in a new container. The old container was kept at 25°C for 3 to 4 days and checked if the eggs hatched. If the eggs did not hatch, then collected virgins would be used for experiments otherwise they would be discarded. This was one of the best ways to ensure that the collected flies are virgins.

2.3 Setting Up Crosses

For setting up crosses the ratio of males to females was kept at 1:3. The number of females were always higher than males. Healthy flies that were able to fly and not too aged were selected for setting up the crosses. I used α -tubulin-GAL4-VP16 driver^{232,594} to express fusion proteins (GFP or mRFP or mCherry tagged to the gene of interest) in syncytial embryos. The males from the driver lines were crossed to virgin female responder flies. The responder lines contained the coding sequence for the gene of interest that was fused to a fluorescent tag under the control of UAS promoter⁵⁹⁵. I only used one copy of the driver and the UASp transgene²³² as overexpression of certain genes was lethal, can affect the analysis of imaging data and lead to identification false positive interactions if used for AP-MS experiments. After setting up the crosses at 25°C the males mated with the females for two days before collecting the embryos for imaging purpose.

2.4 Syncytial Embryo Collection and Preparation

For large scale embryo collection, required for affinity purification, stable lines expressing proteins were multiplied. The flies were grown in suitable container for embryo collection depending on the number of embryos needed. In order to multiply the stocks, the flies must be transferred to new container containing fresh cornmeal fly media every 2 days. The flies were kept at 25°C during stock multiplication and embryo collection process. Once the flies have multiplied, they can be transferred to fly cages at 25°C. A small cage can take a few hundred flies while a large one can take tens of thousands. The flies that were used in cages were not aged. The flies must be well fed on yeast paste applied to pre-warmed grape juice agar plates 1-2 prior to embryo collection. On the day of embryo collection, the overnight grape juice agar plate was changed in the morning with a fresh grape juice plate and yeast. The first 0-90-minute collection of embryos was discarded. Again, the old grape juice plate was replaced by a new one with fresh yeast. 0-90 minutes embryo collection was done successively for affinity purification experiments and a total of 1.5 grams of embryos were collected. For imaging purpose 0-60-minute embryo were collected as the process to get them ready for imaging required time. A paintbrush dipped in water was used to remove the embryos some of which may be mixed with yeast on the plate. The embryos were put in a sieve dipped in distilled water such that the yeast passed through, but the embryos were trapped. Sometimes two sieves were used one over the other. The top sieve had a larger pore size than the one below. After this process the embryos were washed three times in distilled water to completely remove the yeast. To remove the chorion from the embryos they were dipped in 50% bleach and gently moved around in the sieve using a paint brush for 2 to 4 minutes. Following which the embryos were washed 3 times in distilled water to remove the bleach. Each wash was for 2-3 minutes

and the embryos were rotated clockwise and anti-clockwise using a paint brush. For smaller collection (usually for imaging purpose) a 6 well plate was used. If the embryos were used for affinity purification, then they were transferred using a paint brush to a 1.5 ml microfuge tube. This tube was then dipped in liquid nitrogen to flash freeze the embryos and then stored in -80°C freezer. To use the embryos for imaging purpose a cover slip was prepared. The coverslip had a vertical line of Sellotape glue applied to it using a pipette. The embryos were then aligned in horizontal orientation on vertical line of glue on the coverslip such that all the embryos had the same orientation. The cover slip with the embryos was then placed on a specially prepared metal slide. The embryos were then covered by Voltalef 10 oil obtained from VWR international limited. This oil prevents the embryos from drying while allowing the oxygen to pass. The slide with the cover slip was then placed on the temperature-controlled microscope stage set at room temperature (RT) for imaging.

2.5 Live Imaging of Syncytial Embryos

As mentioned earlier, the metal slide with embryos was placed on a temperature-controlled microscope stage at RT. The temperature of the stage effects the speed of mitotic cycles. Live imaging was performed using Zesis Axiovert 200M microscope that had the Inject Man (Eppendorf) micromanipulator and CellTram Oil (Eppendorf) manual microinjector fitted to it. Mitotic cycle 12 was used for all imaging experiments as the nuclei were close to the surface of embryo making it ideal for imaging and the embryo had enough number of nuclei available for quantification. For measuring the intensity of fluorescent tagged KT proteins in syncytial embryo the top and the bottom of embryo were focused and a step size of 0.75 micron was selected. This gave around 10 to 13 slices in a Z stack for each time

point in each channel (green and red) depending on the thickness of the embryo. The time interval between each successive time points was 18s during which the green channel (EGFP) was excited using 488 nm laser and if need be the red channel (for mRFP or mCherry) was excited using 568nm laser of Perkin Elmer Ultra VIEW RS III spinning disk confocal system that had a Zesis 100x/1.4 oil immersion objective. Hamamatsu 1394 ORCA-ERA camera was used to capture the images with a bin size 1×1 ²³².

2.6 Quantification of Live Imaging Data

Once the images were captured the raw data was converted into stack of tiff using Velocity and then exported to ImageJ for analysis. Each time point was examined to remove those slices from the Z stack that had no GFP signal or high noise or dust to get correct quantification of the dynamics. The cleaned Z stack from each time point and channel was merged to produce a single tiff image using the maximum intensity projection tool in ImageJ. This merged Z stack at each time point for each channel was used for analysis. Anaphase onset (AO) was defined as time point 0s i.e. the time point when the KT split. As the images were taken at 18s interval those before time point 0s were labelled as -18s, -36s and so on. The image taken after AO (i.e. 0s) were labelled as 18s, 36s and so on. A ROI was drawn over the region of interest covering the KTs. The details of how the KT signal was measured by removing the background noise are covered in the next chapter. The photobleaching effect in this imaging set up was insignificant and discussed in following chapter. The intensities obtained were normalised to their maximum KT intensities for comparison and plotting (unless otherwise stated). Standard deviation calculated for each time point was plotted as error bars. Minimum 4 embryos and 4 nuclei were quantified. The number of samples varied from experiment to

experiment and the SDs are therefore for each individual experiment. More number of nuclei for quantification were used than that already accepted in publication of KMN network proteins⁵⁹⁶. Furthermore, there was not a significant difference in trends between individual embryos when averaged over all nuclei.

2.7 Purification of His Tagged UbcH10^{C114S} and Mad2^{L13Q} Under Native Condition

Take 50ml of Rosetta 2 cells and thaw on ice. Tap the microcentrifuge tube (MT) gently to mix and then incubate on ice for around 20 minutes. Heat shock the competent cells using a water bath at 42°C for 1 minute. Immediately put the MT tube having the competent cells mixed with the plasmid on ice. Mix the competent cells with 300ml of SOC medium (obtained from Sigma-Aldrich) and shake it in an incubator at 37°C for 1 hour. Plate the SOC on two LB plates with appropriate antibiotics in this case Ampicillin (100µg/ml) and Chloramphenicol (35µg/ml). On one plate add 75µl of SOC and on the other add 225µl of SOC and use the platter to spread the SOC on LB plate. Incubate both the plates at 37°C for 16 hours. Chose the plate that has separated colonies. Pick up a single colony and inoculate in the starter culture of 6 ml in 50ml falcon tube. The starter culture has appropriate antibiotics. Put the falcon tube in a shaker at 37°C overnight. The following day prepare 1.5 litres of LB with appropriate concentration of antibiotics. Divide the 1.5 litres of LB into 3 flasks of 1.5L (i.e. each flask has 500ml of LB). Add 2ml of starter culture in each 1.5L flask. Cover the flask and keep it in the incubator with a shaker at 37°C. Once the O.D reaches 0.6 induce it by adding 0.05 mM final concentration IPTG. After overnight induction at 16°C on a shaking incubator divide the LB from each flask into 50 ml falcon tubes. Spin the falcon tubes at 4000K RPM at

4°C for 20 minutes. Discard the supernatant and store the pellet at -80°C or start the lysis. If the pellet was frozen, then thaw the frozen pellet by keeping it on ice in presence of 30 ml Lysis buffer. Add lysozyme such that final concentration is 0.2mg/ml. From the stock solution of 10mg/ml add 0.6ml of lysozyme to 30ml of lysis buffer in which the pellet is suspended. Incubate on ice for 10-20 min. Add 1.125ml of 20%NP-40 (final conc. 0.5%), mix at 4°C for 15min on the rotator. Sonicate the lysate in 100ml glass beaker using a sonicator at 100% power. Sonicate 10 times (30-40 seconds sonication followed by 30-40 seconds pause). Remove the tip used for sonication between intervals. Centrifuge at 18000 rpm for 20 minutes at 4°C by using Beckmen rotor JA 25.50 (alternatively use Sorvall SS34) using the Beckmen centrifuge tube. The acceleration and deceleration are set to max i.e. 1. Pass the supernatant through a 0.45-micron filter to obtain the filtrate. Take 100ul from the supernatant and store it at 4°C. This is the Input (IN). Pre-chill 4L of PBS in cold room. Use 0.5ml of Ni-NTA agarose beads (ordered from Qiagen) for every 1L of bacterial culture. Prewash the beads in lysis buffer 4 times. Each wash must be with 10ml of lysis buffer in a 15ml falcon. Invert the falcon tube several times during the wash. Let the beads settle down by placing the falcon tube in the holder. Discard the supernatant. Rotate the filtrate at 20 rpm with the beads in 50 ml falcon for 2hrs at 4°C. Spin the tube at 2000 rpm for 3 minutes at 4°C. Then collect the supernatant in a new pre-chilled falcon tube. This is the flow through (FT). Do not discard the FT and store on ice till end of experiment. In case the binding has not taken place then the protein can still be obtained from the flow through. Run a gel with 1ul, 3ul, 6ul, 12ul, 18ul of supernatant followed by 6ul of input and 12ul of flow through. Stain the gel with Coomassie blue (obtained from BioRad). Compare to see if the band representing the protein has become less in flow through compared to input. This will tell about the binding efficiency of the protein to the beads. Wash

the beads 4 times with 10ml lysis buffer at 4°C using 15ml falcon tube. Prepare fresh 40ml elution buffer (EB). Wash the elution column (BioRad Poly-Prep Chromatography Columns Catalogue 731-1550 size: 0.8x4 cm) twice with 10 ml of 1x wash buffer. Pour the beads from the last wash in the elution column. Wash again with 10 ml of 1x wash buffer. Once very little wash buffer is left in the elution column add 1ml of EB and start collecting eluents in fractions. Collect 10 fractions each of 0.3ml. using pre-chilled 1.5ml pre-chilled MT tubes. Store the MTs that contain the eluents on ice. After eluting 10 fractions collect 3 more fractions each of 1 ml and store it on ice. This is to be safe in case the elution worked in the later fractions of 1ml each. Run 6µl from each sample on gel and stain with Coomassie blue to find out if the elution worked, to identify which fractions have the protein and the purity of the protein. After running the gel, it was found that most of the protein was present in fractions 2-5. Fractions 6-10 had less protein. Fractions 2-8 were pooled. Use dialysis cassette from (Thermo Scientific Catalogue Number LL54s618s) 3,500 MWCO (depends on the size of protein) of 0.5-3 ml capacity. Connect the cassette to the floater and hydrate it using a magnetic rotor at 2-2.5 RPM in pre-chilled PBS for 10 minutes. Use pre-chilled 1ml syringe to load the dialysis cassette with the protein. After loading the cassette suck the air out of the cassette using the syringe. Dialyse for 5 hours by keeping the cassette in pre-chilled PBS in a large glass beaker kept on a magnetic rotor. The dialysis cassette rotates as a result of the magnetic rotator at the bottom of the beaker. Change the buffer after 5 hours and dialyse further for 12 hours. Remove the purified protein out from the cassette using 1ml syringe and store in a 2 ml pre-chilled MT. Spin the sample at 14,000 RPM for 5 minutes at 4°C and collect the supernatant. Concentrate the supernatant using Vivaspın2 from Sartorius Stedim (Product number VS0201, Membrane 10,000 MWCO PES). This column membrane has sodium azide and glycerol and hence needs

to be washed. Put the column on a stand and fill the reservoir with 2ml PBS. Keep it at 4°C for 5 to 10 minutes and then spin down at 4000G for 7 minutes in the beckmen centrifuge using swing out rotor. Repeat this process once more and discard the PBS after the washes. Load the column with the purified protein sample and rotate for around 19 minutes using beckmen centrifuge swing out rotor at 4°C till the volume reaches near 130ul. This is the concentrate. Remove the concentrate in a fresh 1.5ml pre-chilled MT. Do not discard the flow through which is the retinate (RTN). Measure the OD using Bradford assay (BioRad). Warm the Bradford reagent to room temperature. Load 1 ml of Bradford reagent to cuvettes. Add 5ul BSA standards of 0 mg/ml, 0.2 mg/ml, 0.4 mg/ml, 0.6 mg/ml, 0.8 mg/ml and 1 mg/ml to each cuvette and mix by pipetting. From the concentrated sample take 1ul and add it to 5ul of PBS. From this diluted sample take 5ul and load it to the cuvette sample. Cuvettes for 0 mg/ml, 0.2 mg/ml, 0.4 mg/ml, 0.6 mg/ml, 0.8 mg/ml, 1 mg/ml BSA standards, retinate and the concentrate were prepared. Incubate the cuvettes at room temperature in dark for 20 minutes. Load the nanodrop program and choose protein Bradford assay. The instrument would calibrate by itself after which the blank sample is loaded i.e. cuvette with 1ml of Bradford reagent and 5ul of 0mg/ml BSA solution. Measure the OD's for all and load it in the Bradford excel sheet (pre-programmed excel sheet to calculate the concentration). Multiply the concentration by 6 as the samples were diluted 6 times. In this case the final concentration of UbCH10^{C114S} was 40 ug/ul and 35 ug/ul for Mad2^{L13Q}. Load a gel with 1ug and 2ug of BSA followed by 1, 2 and 10 ug of concentrate followed by 5ul and 10ul of retinate (this would tell if the proteins have not passed from the filter of the vivaspin column into the retinate) and then 5ul and 10ul of palette (to check if the precipitate has any proteins). This is an optional gel. Make 5ul aliquots of sample and flash freeze using liquid nitrogen.

2.8 Making Transgenic Flies Using Gateway Cloning

10 transgenic flies were made in total (gel images in appendix A). The first set of flies being C terminus GFP tagged Aurora B, INCENP, Borealin, Survivin and Polo. The second set of flies were expressing C terminus tagged Protein A (Pra) for Aurora B INCENP, Borealin, Survivin and Polo. All the genes were under UASp promoter. Order the cDNA of Aurora B INCENP, Borealin, Survivin and Polo from DGRC. Amplify the cDNA obtained from DGRC using PCR. I made use of AccuPrime Pfx DNA Polymerase for doing the PCR. I designed attB PCR primers (described in appendix A) as per Gateway Cloning Manual from Invitrogen. The PCR product was verified the sequence by Sanger Sequencing and also checked on agarose gel for the correct band size. After this a BP reaction was carried out as per the Gateway cloning protocol that enables the insertion of the PCR product into the entry clone via the donor vector (pDONOR221 from Invitrogen). The entry clone was purified and transformed using competent cell using DH5 α . Do a maxi prep from Qiagen protocol to obtain the entry clone. Check the entry clone sequence using M13 primers and gene specific. Perform LR reaction with the destination vector (UASp-Cterm-EGFP or PUASp-PrA-C-Term) to obtain the expression cone followed by a Qiagen maxi prep and check for sequencing using sanger sequencing method. The final concentration of the plasmid DNA should be around 1 μ g/ μ l and the O.D 260/280 should be 1.80-1.90. This plasmid was then given to Department of Genetics, University of Cambridge for microinjection service to produce the transgenic flies. Once the transgenic flies are obtained, they need to be balanced. Once the transgenic flies are obtained and balanced their expression of the protein by western blot using syncytial embryos (appendix A). Choose those lines for experiments in which the proteins are expressed to nearest to the endogenous levels

2.9 Reagents Obtained from Other Labs

GFP-BubR1, GFP-Mad2 and GFP-Fzy transgenic flies under endogenous promoter were obtained from Karess lab. GFP tagged single destruction box, double destruction box mutant flies and UASp-Polo-GFP transgenic flies were obtained from Kimata lab. Polo-GFP transgenic flies under endogenous promoter was already present in Glover lab. Purified p27 protein, His-UbcH10^{C114S} or His-Mad2^{L13Q} constructs, GFP-CID flies were obtained from Dr. Raquel Oliveira.

2.10 Implementation of DAPPER

For developing DAPPER, I collaborated with Dr. Abbas Haider Sayed from Department of Computing, University of Cambridge. I developed one of the most crucial algorithm and code of DAPPER required for parsing the XML mascot data file that was obtained from the mass spec facility after AP-MS. The code for the XML parser is present in the appendix D and developed in Perl. Dr. Abbas Haider Sayed integrated this parser code with the front end of DAPPER that he developed. The freely available Biomart warehouse system 0.7 was integrated with DAPPER and configured by Dr. Sayed. I defined the concept and build algorithms for DAPPER functions like intersection, intersection prime, distinct and sort. The algorithm for these functions were then converted into web enabled codes by Dr. Sayed as they had to be derived from the Biomart class. DAPPER resides on server at Department of Genetics, University of Cambridge. This server is maintained and configured by the departmental computing officer. The details about DAPPER are explained in later chapter.

Chapter 3 Image analysis

3.1 Introduction

This chapter compares several methods for image analysis and uses the most appropriate one for obtaining the dynamics of KT associated (including centromeric but henceforth covered by the term KT) proteins in *D. melanogaster* syncytial embryos. The obtained protein dynamics are used for investigations that are discussed in the following chapters. Image analysis is a complex task specially if the KT associated proteins have a background expression (i.e. nucleoplasm and/or cytoplasm). KTs being highly mobile adds to the complexity during measurement. The KTs can be spatially separated or clustered depending on the mitotic stage. Hence, a constant size region of interest (ROI) covering the KTs cannot be used for its signal measurement at every time point. To address this, if a variable size ROI is used then the total number of pixels within the ROI would vary impacting the measurement. Additionally, any potential photobleaching effect must be accounted for. Live imaging data from GFP tagged Polo and mCherry tagged AurB were used for comparing different methods of image analysis and defining an appropriate approach with error bars representing the standard deviation.

3.2 Signal Measurement of Kinetochore Protein with Background Expression

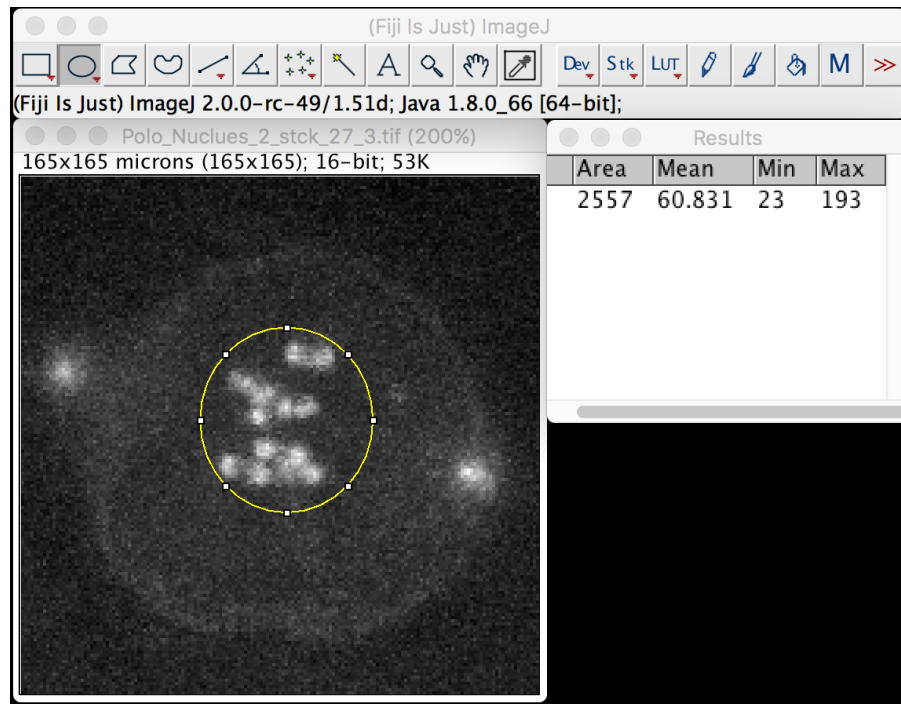


Figure 3.1. ROI covering GFP-Polo-KTs to measure its mean intensity
Using ImageJ, the mean and maximum intensities measured are 60.831 and 193 respectively.

The general approach to measure the KT signal in a cell is to draw an ROI around the KTs and measure its mean intensity (fig. 3.1). Polo in addition to the KTs is also expressed in the nucleoplasm and cytoplasm (fig. 3.1). Polo expression is always higher in the nucleoplasm compared to cytoplasm at any point during the mitotic cell cycle (fig. 3.9). Hence, the term background, unless otherwise specified, refers to nucleoplasmic background. As mentioned earlier, using a constant size ROI is not a viable option as the spatial distribution of the KTs changes during mitotic stage. Furthermore, using a variable size ROI to measure mean KT signal also does not give robust measurement. If a large ROI is used to measure the KT signal, the contribution of the KT signal to the mean of ROI goes down as it's an average of all the pixels in the ROI. This happens because in larger ROI there are more background pixels

compared to a smaller ROI. Fig. 3.1 and fig. 3.2 illustrates how changing the size of ROI at the same time point resulted in a significant shift of mean from 60.831 to 50.600 (i.e. 16.818% change).

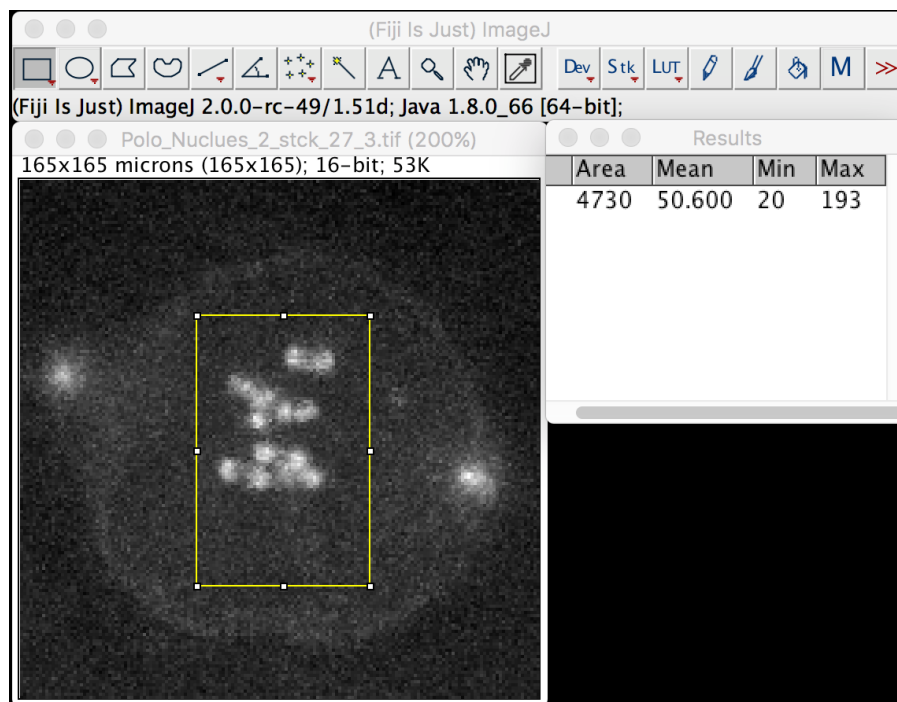


Figure 3.2. Changing the size of ROI effects, the mean and the not the maximum
Using ImageJ, the mean and maximum intensities measured are 50.6 and 193 respectively.

As observed in fig. 3.1 and 3.2, even when the size of ROI changed the maximum intensity was stable at 193. This was because the maximum intensity pixel was detected on the KT and not in the background. However, a single maximum intensity value to represent the KT signal instead of mean, would not be a reliable measure as errors may arise due to an imaging artefact. After discussion with Dr. Raquel Oliveira, I took the approach of calculating the mean of top 10 maximum intensity pixels in the ROI to represent the KT signal (fig. 3.3). This number was empirically determined as the top 10 maximum intensity pixels were always on the KT in the imaging set up used. I had written an ImageJ plugin to find the mean of top N

(where N is variable that was set to 10 for measurements) maximum intensity pixels in the ROI. The code for this plugin is present in appendix B. Using this method, the mean of the top 10 maximum intensity pixels shifted from 193 to 182. (i.e. correction of 5.69%).

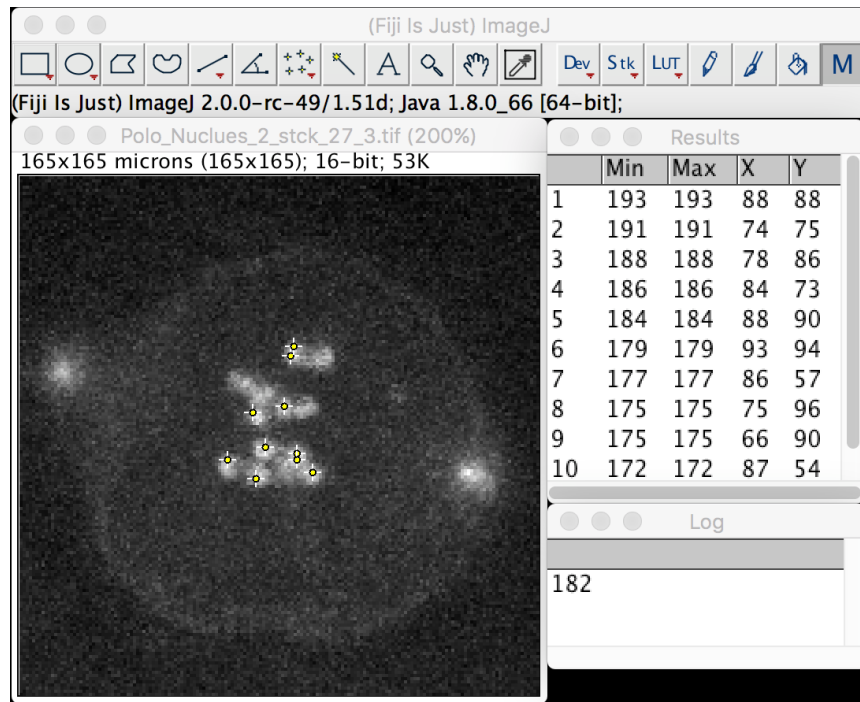


Figure 3.3. An ImageJ plugin that calculates the mean of the top 10 maximum intensity pixels in the ROI
The mean obtained using ImageJ plugin, that calculates the mean from top 10 maximum intensity pixels, was 182. A shift of 5.69% if a single maximum intensity pixel was used to represent the KT intensity.

I wanted to investigate if using higher number of pixels instead of 10 would influence the trend in Polo dynamics. Fig. 3.4 shows that there was no significant change in Polo dynamics if mean from top 25 (2.5X higher) intensity values were used instead of 10 at each time point. As anticipated the change was minimal as the selected pixels were on the KTs. However, by increasing the number of pixels to obtain the dynamics caused the peaks and the trends to become blunt and hard to detect which can influence the outcome of the mathematical analysis in the following chapter.

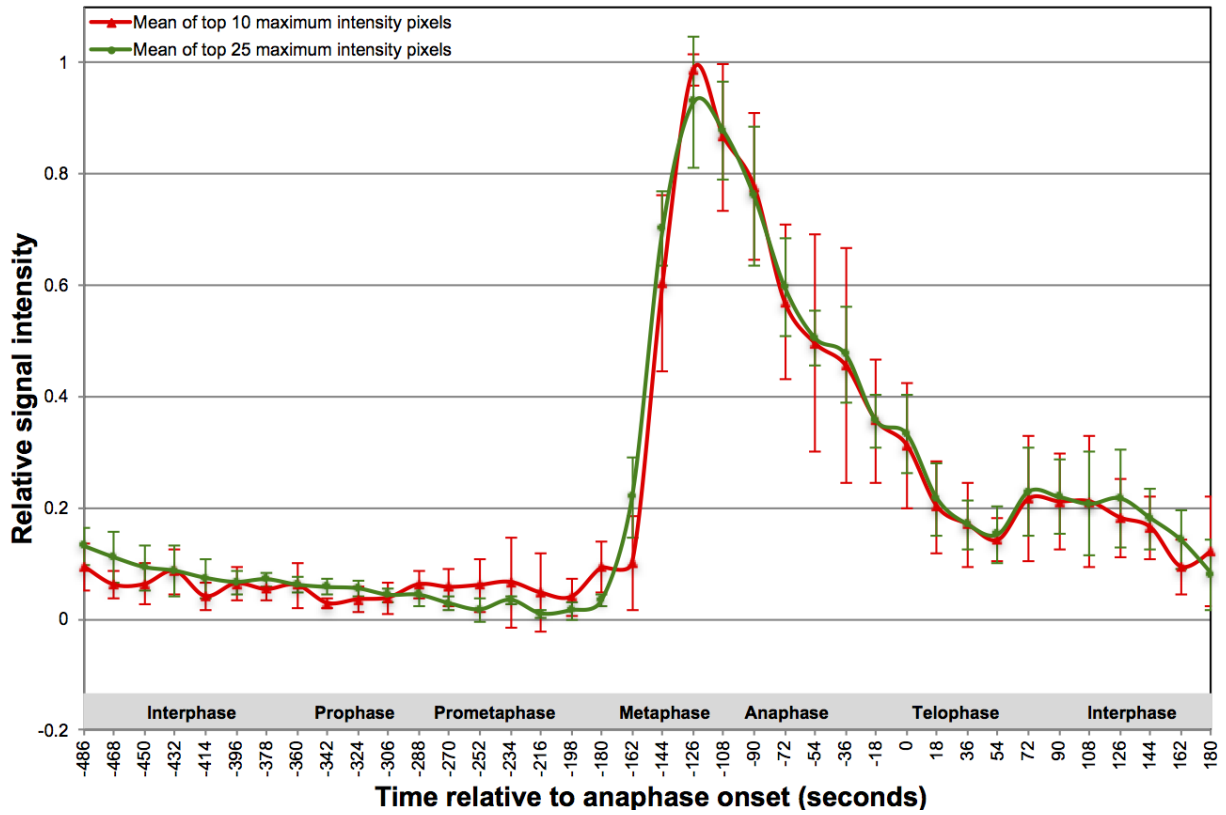


Figure 3.4. Plot showing normalised dynamics of GFP-Polo KT obtained using top 10 and 25 maximum intensity pixels
 Red and green curve were obtained using top 10 and 25 maximum intensity pixels for background correction respectively.

3.3 Background Correction

KT's are themselves embedded in the nucleoplasm and hence their true intensity is masked. True or unmasked KT intensity can be obtained by subtracting the masking nucleoplasm background from the total KT signal (KT signal plus background) at every time point. I have investigated three different methods to identify the masking background. These methods either use constant size ROIs, four (fig. 3.5) empirically chosen, or a single variable size ROI (without covering KTs).

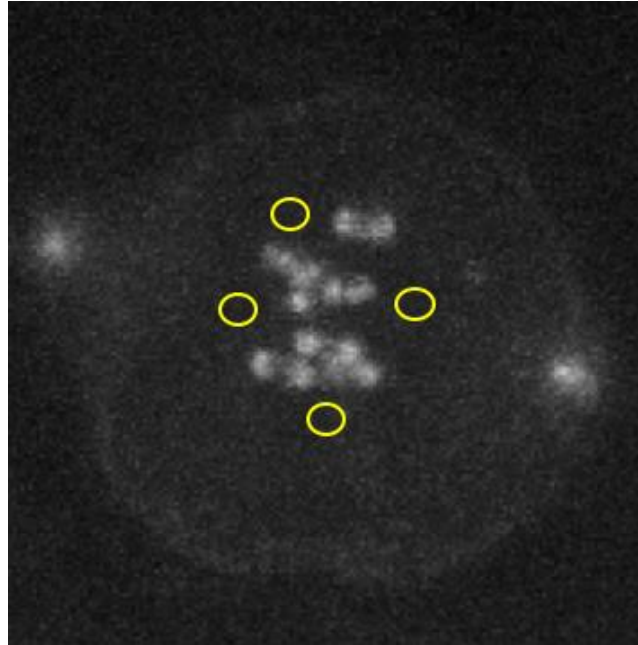


Figure 3.5. Four constant size ROI used to calculate the nucleoplasmic background at each time interval

In the method 1, background intensity was the mean of four constant size ROI for every time point. The background value was then subtracted from the corresponding total KT intensity for every time point to obtain unmasked normalised KT intensity (fig. 3.6 green curve). I used four constant size ROI to reduce the pixel selection bias and to represent background from all directions in the nucleoplasm. Using constant size and same number of ROI at each time point, ensures that the mean is calculated from the same number of pixels at each time point. In method 2, a single variable size ROI was used, and its mean was calculated at each time point from top 10 maximum intensity pixels (as opposed to all pixels in ROI as in method 1) to represent the background signal. It was this background signal which was subtracted from the total KT intensity at every corresponding time point to obtain unmasked normalised KT intensity (fig. 3.6. blue curve). Method 3 also used a single variable size ROI and the mean of that ROI was obtained as mentioned in method 2 for every timepoint. However, method 3 has an extra step to identify a single constant background value which was subtracted

from total KT intensity at every time point. This single background value is the maximum background value obtained between start till AO. The unmasked normalised KT intensity using method 3 is shown in fig. 3.6 by a red curve. The observations from fig. 3.6 show that no significant change in the trends of Polo dynamics irrespective of the method used for background correction.

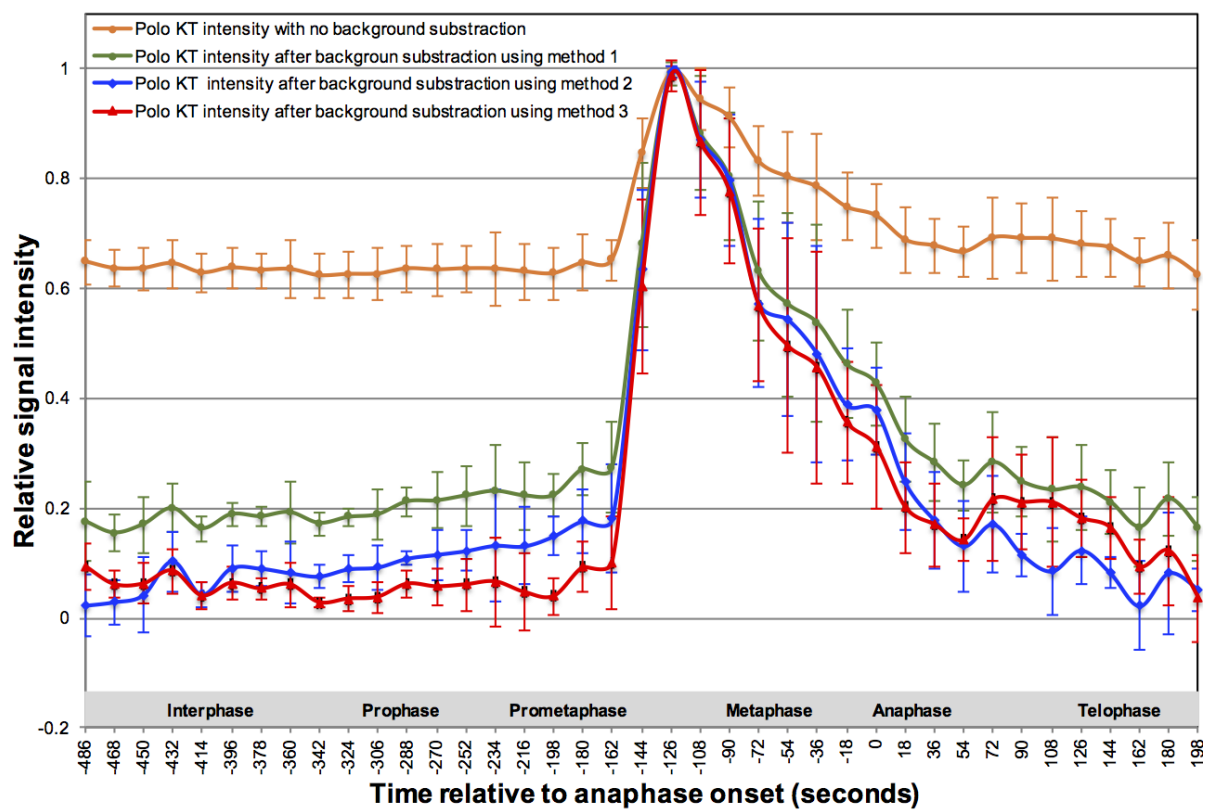


Figure 3.6. Comparison of normalised values of GFP-Polo with and without background correction
Orange represents Polo dynamics without background correction. Green, blue and red are Polo dynamics obtained after background correction using method 1, 2 and 3 respectively.

To compare levels of background obtained by method 1, 2 and 3 their absolute values were plotted as a function of time (fig. 3.7). Method 3 is the most efficient background correction followed by method 2 and then method 1 for the time interval between NEBD (-162s) and AO (0s). Method 3 was used for background correction to obtain protein dynamics.

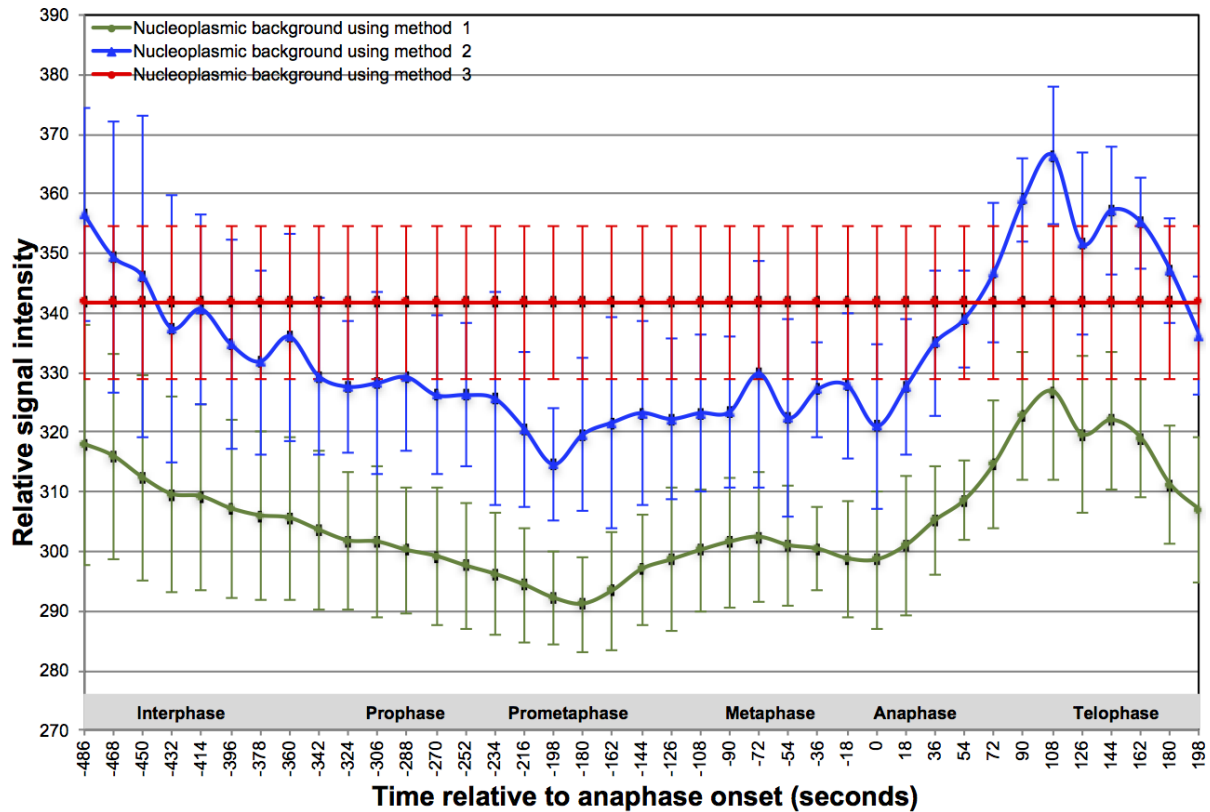


Figure 3.7. Comparison of nucleoplasmic background in GFP-Polo syncytial embryo obtained using three different methods

Green, blue and red curve shows background obtained using method 1, 2 and 3 respectively.

As an additionally check to see if similar Polo dynamics can be obtained using a more traditional alternate method, modal value of the ROI was multiplied by area of ROI and subtracted from integrated density of ROI (i.e. $KT\ signals = Integrated\ Density - Total\ Background$). Integrated Density is the summation of all the pixels in the ROI. Mode represents the background in the ROI, hence $Area \times Mode = Total\ Background$. Similar Polo dynamics were obtained using this alternate method and method 3 discussed earlier (fig. 3.8). This method was developed in consultation with Dr. Ian Dobbie from Department of Biochemistry, University of Oxford.

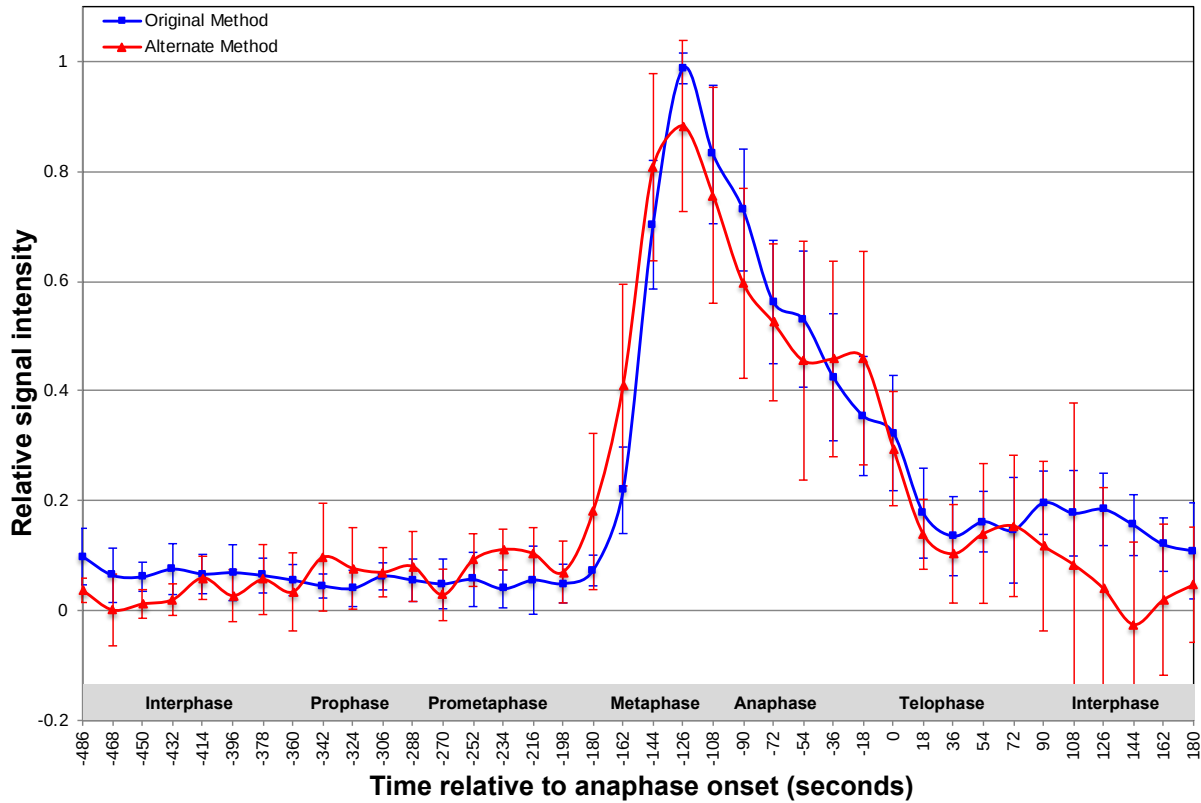


Figure 3.8. Comparison of Polo dynamics obtained using original and alternate methods
Red represents Polo dynamics using the new method while blue represents Polo dynamics obtained using an alternate method.

In order to compare the significance of the nucleoplasmic and cytoplasmic backgrounds, normalised dynamics were obtained from GFP tagged Polo and mCherry AurB syncytial embryos cycle 12 (fig. 3.9). Polo nucleoplasmic background was always higher than its own cytoplasmic background and AurB cytoplasmic background. AurB does not have a nucleoplasmic expression. Hence, Polo nucleoplasmic background was used for its background correction while in case of AurB (and other Chromosomal Passenger Complex Components) its cytoplasmic background was chosen for background correction.

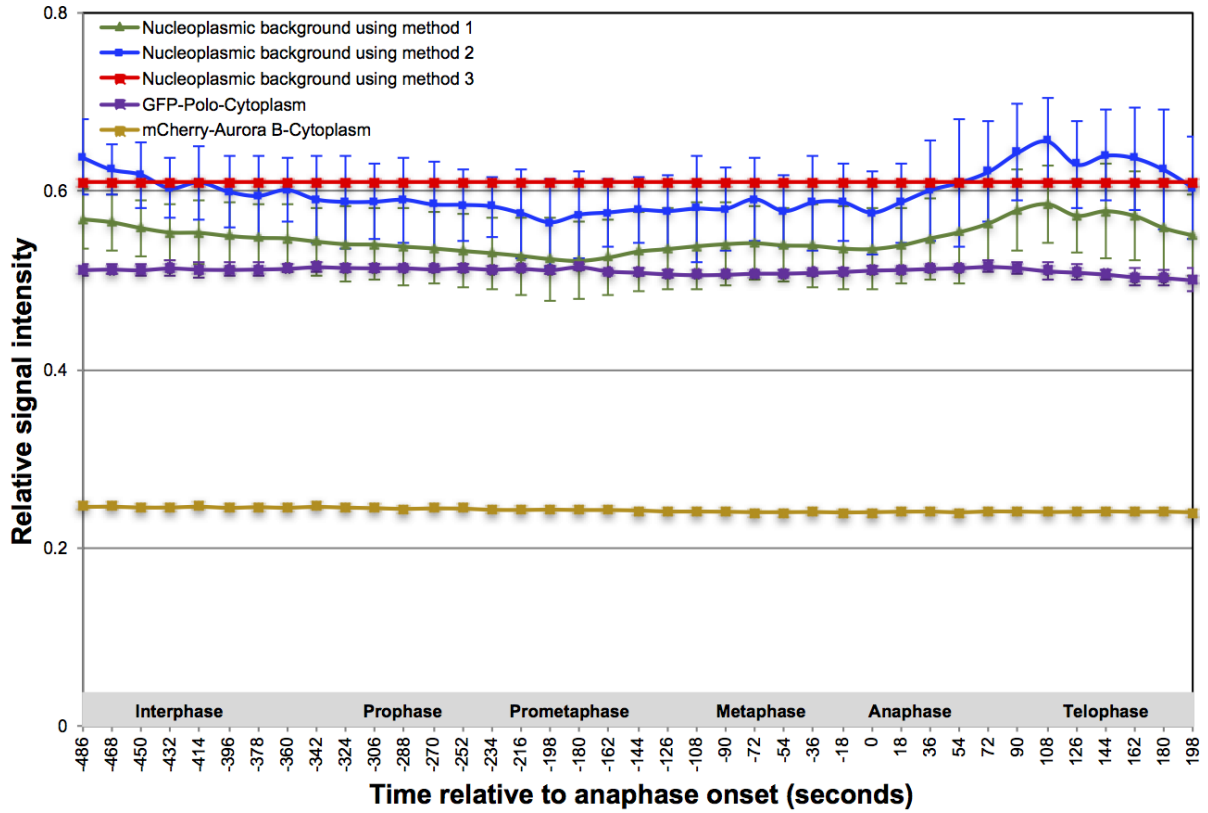


Figure 3.9. Comparison of normalised values of backgrounds in GFP-Polo and mCherry-Aurora B
Normalised dynamics of GFP-Polo-Nucleoplasm using method 1,2 and3, GFP-Polo-Cytoplasm and mCherry- are represented by green, blue, red, purple and brown respectively.

3.4 Photo bleaching

To establish if the trends in the dynamics of GFP or mCherry tagged fluorescent proteins are altered due to photobleaching in the imaging setup used, I measured the change in the cytoplasm fluorescence level of endogenously expressing GFP-Polo and mCherry-Aurora B transgenic fly. Cytoplasmic fluorescence of Polo and AurB should remain stable unless there is a significant photobleaching effect. I choose four constant size ROI in the cytoplasm of the of the embryo at each time point (fig. 3.10 and 3.11). Choosing four constant size ROI reduces the error that may be caused by the bias in choosing the location of ROI and ensures that the mean is calculated from the same number of pixels at every time point.

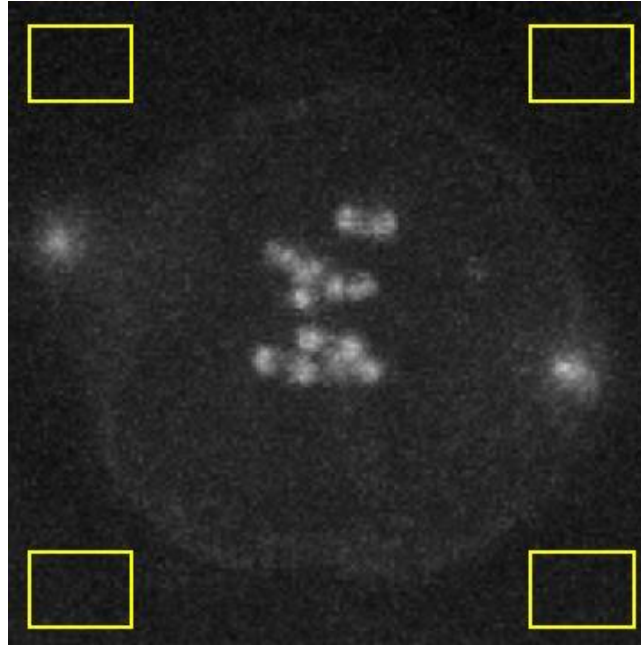


Figure 3.10. Four constant size ROI are chosen to find the mean GFP-Polo cytoplasmic intensity for every time point

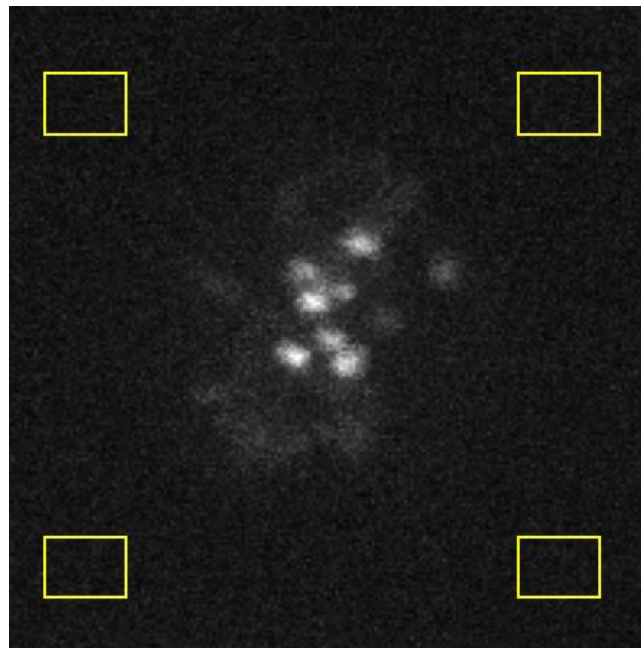


Figure 3.11. Four constant size ROI are chosen to find the mean mCherry-Aurora B cytoplasmic intensity for every time point

The average of the four mean intensities obtained from each ROI was used to represent the cytoplasmic background value for every time point. Their intensities were normalised to the maximum GFP-Polo-KT value to find out if the cytoplasmic values for GFP-Polo and

mCherry-Aurora B reduce over time. No significant drop in the cytoplasmic intensities of GFP-Polo and mCherry-Aurora B was observed over time (fig. 3.12). There was less than 1% change in the cytoplasmic value of GFP-Polo and mCherry Aurora B from the start till the end of the live imaging. The change in the GFP-Polo-KT values from NEBD till they are no longer distinguishable from the nucleoplasmic background just after AO was 81%. These observations show that there is no significant photobleaching effect to alter protein dynamics in both green and red channels in the imaging set up used.

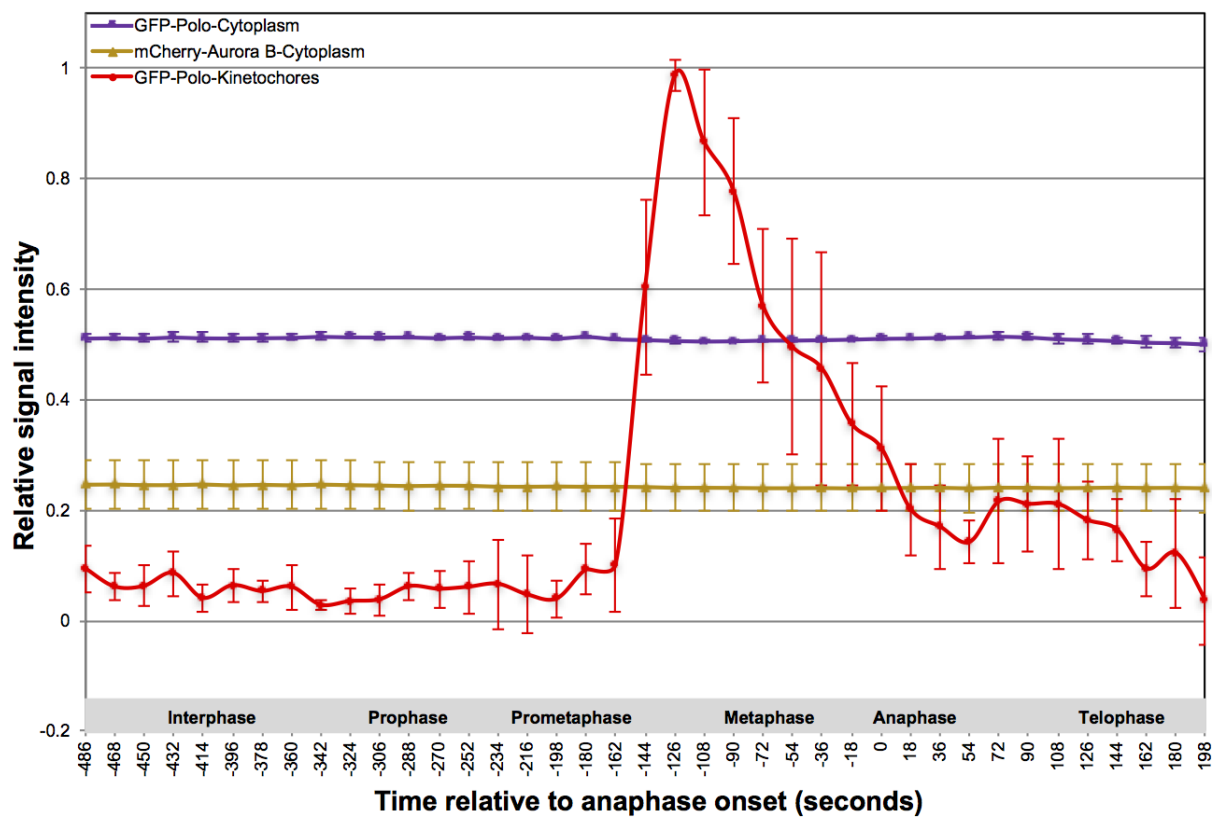


Figure 3.12. Comparison of normalised value of background corrected GFP-Polo KT, GFP-Polo and mCherry-Aurora B cytoplasm
Red, purple and brown represents dynamics of GFP-Polo KT, GFP-Polo cytoplasm and mCherry-Aurora B cytoplasm respectively.

In order to further ascertain that there is no photobleaching effect in the green channel (measured using GFP tagged Polo) and red channel (measured using mCherry tagged AurB) overall embryonic image (with multiple nuclei) was used to calculate normalised integrated intensity over the entire length of the movie. No significant change ($\sim 1.4\%$) was observed in both channels suggesting minimal photobleaching effect consistent with the earlier observation.

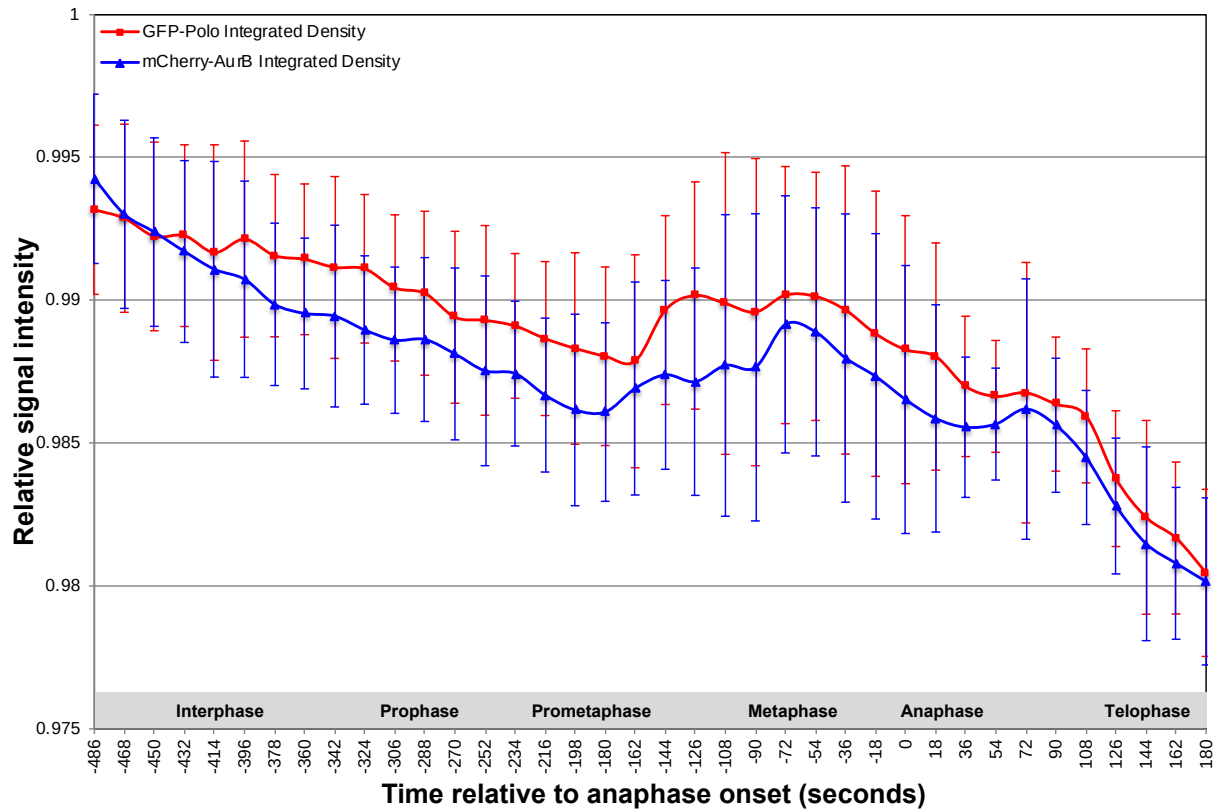


Figure 3.13. Comparing integrated density of GFP tagged Polo and mCherry tagged AurB

3.5 Assumptions and Limitations

Since the analysis was done on a merged Z stack image after removal of the background slices it was assumed this would be robust enough. However, not every KT at each time point is in the same plane. For more accurate measurement, each KT must be traced in the Z stack

and the background slices (below and above the KT thickness) must be removed. This would require tracking and segmentation of individual KTs at every time point in the 3D Z stack rather than a merged image. Background corrected individual KT intensities can then be averaged and normalised at every time point over the time course. Furthermore, it would be optimal to have the entire process automated via a custom written code. Additionally, having another marker like CID in another channel that does not have a background expression would be helpful to locate the KT signal once they disappear in the nucleoplasmic background after AO, if the KT dynamics post AO is the scope of research. With another marker and the single KT tracking the signals for background correction, the signals post AO can be better measured than the current approach.

3.6 Summary

In this chapter, I have compared several methods calculating protein dynamics using GFP-tagged Polo and mCherry tagged AurB and used the most efficient one for analysis. This method was verified by another supporting alternate method to ensure robustness. The image analysis of KT proteins can be a complex task given the KTs are highly mobile structures and some of the KT proteins have background expression (cytoplasm and/or nucleoplasm). Using a constant size ROI was not viable option due to distribution pattern of KTs nor was using variable size ROI as it did not give accurate and robust measurements. In order address these challenges, an ImageJ plugin was written to calculate the mean from top N maximum intensity pixels in the ROI to represent the KT signal. N was empirically set to 10 and the selected 10 pixels were always on the KT. Increasing N would give similar Polo dynamics, as far as the selected N pixels are on the KTs, but with lesser sharpness that can impact the mathematical

analysis in the following chapter. Irrespective of the approach used to measure the KT signal, there would be an influence of the nucleoplasm background signal as the KTs are embedded in it. Hence, the KT signal without background subtraction is referred as total KT intensity or masked KT signal. The true or unmasked KT signal refers to the one obtained by removing the background from total KT signal (True or unmasked KT signal = Total KT signal – Background signal). Three different background correction methods were compared to obtain Polo dynamics to ensure that the results are robust and reliable. All the three methods for background correction gave similar Polo dynamics and the last method i.e. method 3 was selected for background correction being the most efficient between NEBD and AO, which is the research focus. In order to identify any photobleaching effect two approaches (cytoplasmic mean from ROI and integrated density from the entire embryos) were used. Both methods showed no significant photobleaching change in green and red channels in the imaging set up used.

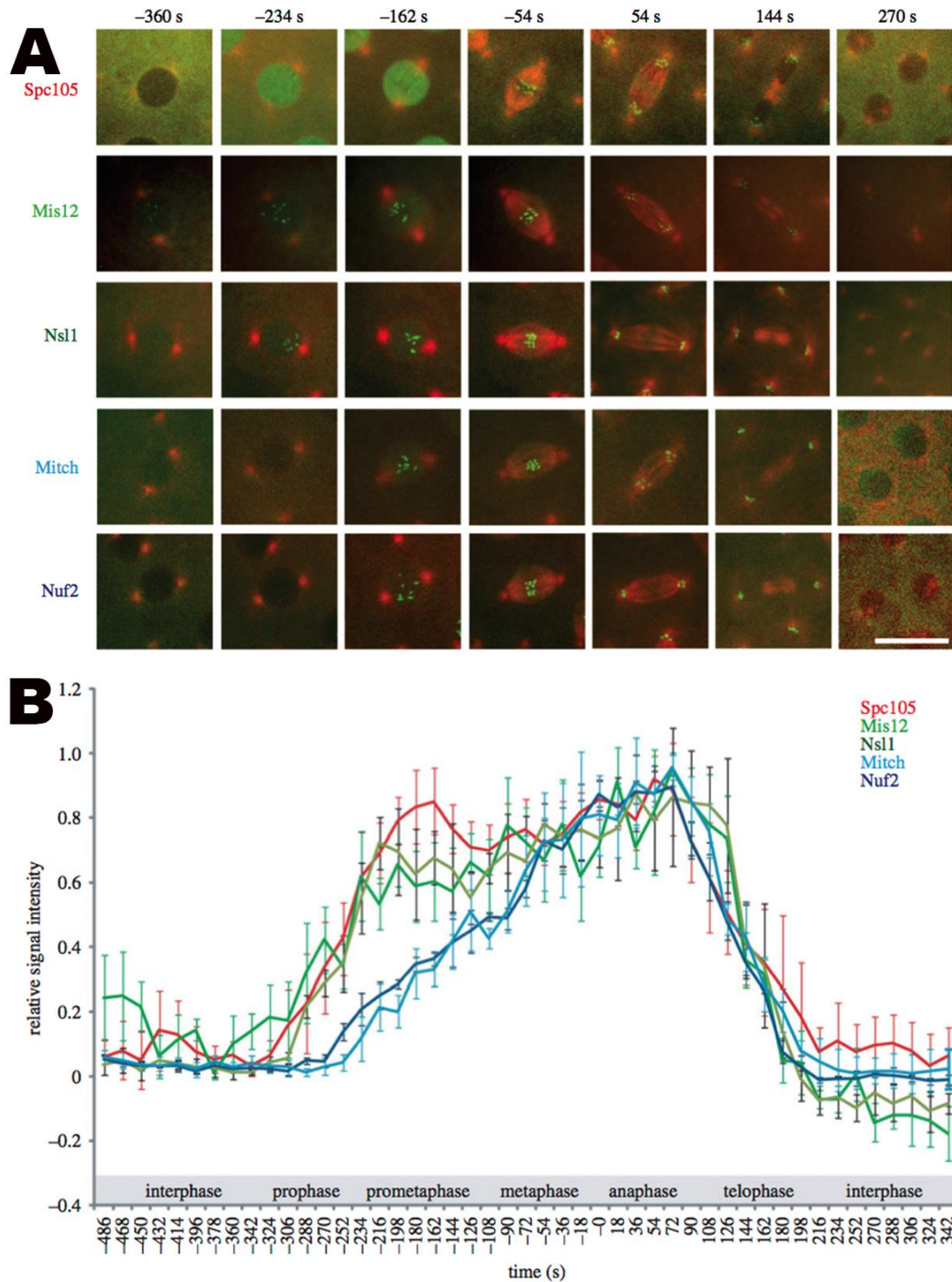
Chapter 4 Investigating the Dynamics of Kinetochore Associated Proteins

4.1 Introduction

In addition to building a global kinetic map of some of the key KT proteins, this chapter describes two novel computational approaches to analyse their dynamics. Synchronous mitosis in syncytial embryos of *D. melanogaster* make it an ideal system to characterise protein kinetics. The dynamics are obtained by analysing GFP tagged KT proteins using the method discussed in Chapter 3. The first approach investigates protein dynamics using differential equation analysis, to predict interdependencies in KMN network proteins which are experimental validated using RNAi²³². KMN network was chosen as it forms the structural component of KT and acts as KT interface to plus end MT attachment^{215,597}. The second technique estimates the number and the timing of biological events that shape the protein dynamics of Polo and BubR1 using rate kinetic analysis. The biological events are experimentally characterised (in Chapters 5 and 6). Polo was chosen as it is one of the master regulators of mitosis playing vital role in many functions e.g. centrosome duplication and maturation, spindle formation, chromosome alignment and cytokinesis while BubR1 was chosen as it plays vital role in SAC.

Imaging experiments and analysis were initially done under supervision of Dr. Zsolt Venkei. The computational techniques using differentially equation and rate kinetics were developed under supervision of Prof. Bela Novak

4.2 Mis12 Complex and Spc105 Show Co-dependant Recruitment Enabling Ndc80 Complex Loading



A. Stills from live imaging of KMN network proteins in cycle 12 syncytial embryos. Scale bar 10 μ m. B. Plot of normalised GFP signal variation over time. This figure has been taken and adapted from Venkei et al., 2012.

The computational approach to estimate and experimentally validate protein interdependencies from their dynamics is described in this section using KMN network proteins. Transgenic flies expressing GFP tagged proteins for Mis12, Nsl1, Mitch/Spc25, Nuf2 and Spc105 were analysed (fig. 4.1)²³². GFP tagged cycle 12 syncytial embryos were imaged using spinning disk confocal microscope. There was no aberrant phenotype observed during the mitosis except for Spc105. In the case of Spc105, 15% of embryos that expressed 3.5-fold-increased levels of GFP showed collapsing spindle phenotype. GFP intensities of tagged KT proteins were plotted as a function of time to produce a graph for KMN network protein dynamics (fig. 4.1). The gap between NEBD and AO was highly reproducible and hence could be used to align the dynamics.

Mis12-GFP showed a faint presence on the KT during the interphase (fig. 4.1). Both Mis12-GFP and Nsl1-GFP showed a sharp increase in KT loading in early prophase. This is followed by overlapping kinetics Mitch and Nuf2 that were not present in the nucleus during interphase and loaded after NEBD (fig. 4.1). Spc105 was not present in the nucleus during the interphase and imported during the NEBD. Spc105 associated with the KTs as soon as it was imported in the nucleus. The dynamics suggests that recruitment of Mis12 complex and Spc105 happens in a co-dependent manner and starts when Spc105 is imported in the nucleus. Ndc80 complex is soon recruited to the KT from NEBD and this loading happens in a Mis12/Spc105 dependent manner, investigated in detail later.

The mathematical analysis to establish KMN network protein interdependencies is discussed below²³². The reversible binding of one molecule of interest (Y) to the other (X) that is already present at the KT can be written as follows:

$$\frac{d XY}{dt} = k_{ass} \times X \times Y_{free} - k_{diss} \times XY = k'_{ass} \times (X_T - XY) - k_{diss} \times XY \text{ (Equation 3.1)}$$

In the above equation, XY is a kinetochore associated complex and is measured by the intensity of GFP tagged Y protein. Here, a two-step sequential binding process is described; in the first step, Mis12 and Spc105 are represented by X and Y respectively while in the second step, Mis12 and Ndc80 complex are represented by X and Y respectively. The right-hand side of the equation can be rearranged based on the following two considerations: a) The level of X that is not bound to Y can be expressed as difference between total X (X_T) minus the complex (XY) i.e. $X = X_T - XY$ b) Y_{free} is constant and abundantly in the system and hence can be coupled with the association rate constant (k_{ass}) to get first order association rate constant k'_{ass} (i.e. $k_{ass} \cdot Y_{free} = k'_{ass}$). As the molecules bind sequentially to the KTs, X_T is not a constant but a monotonically increasing function of time. Depending on the relative rate in the change of X_T and of Y binding to X there can be two possible cases. The possibility, if Y binding to X is fast compared to the change in X_T levels, then $dXY/dt = 0$ and hence equation 3.1 reduces to:

$$XY = \frac{X_T}{1 + \frac{k_{diss}}{k'_{ass}}} \text{ (Equation 3.2)}$$

In this case, the level of Y present at the kinetochore as a complex (XY) depends on the total levels of X i.e. X_T . The second possibility, is if the Y binding to X is slow as compared to the change in X_T , then for small values of XY equation 3.1 reduces to equation 3.3 i.e. rate of Y accumulation at KT would be proportional to X_T

$$\frac{d XY}{dt} \cong k'_{ass} \cdot X_T - k_{diss} \cdot XY \text{ (Equation 3.3)}$$

Firstly, by plotting the association of Nsl1 (a component of Mis12 complex) with Spc105 (fig. 4.2 A) a linear relationship was obtained, according to equation 3.2, with slope and intercept were not significantly different from 1 and 0 and $R^2 = 0.9687$ (fig. 4.2 A).

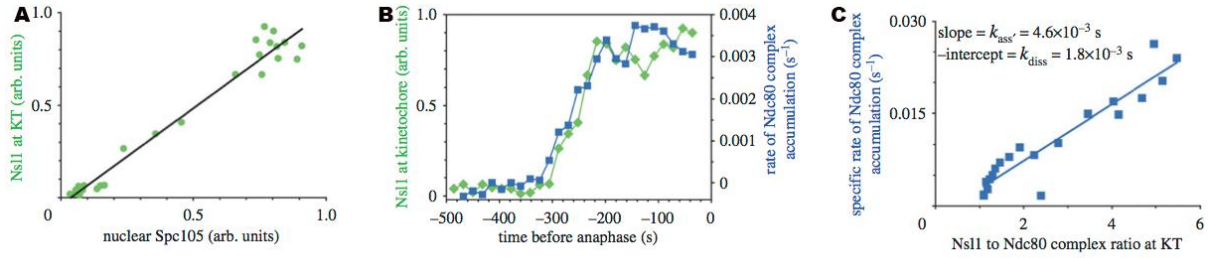


Figure 4.2. Association and dissociation dynamics of KMN network proteins

A. Plot of KT levels of Nsl1 as a function of nuclear Spc105 levels. This shows a linear relationship with slope and intercept not significantly different from 1 and 0 respectively. B. Comparison of Nsl1 recruitment at the KT and the rate of KT associated Ndc80 accumulation plotted against time with reference as AO. C. Rate of accumulation of KT associated Ndc80 complex plotted as a function of the KT associated Nsl1: Ndc80 complex ratio. This figure has been taken and adapted from Venkei et al., 2012.

This analysis suggests Mis12 complex and Spc105 bind very fast and hence $k_{diss} \ll k_{ass}'$. In contrast, when mean of Nuf2 and Mitch (components of Ndc80 complex) was plotted with Nsl1 (component of Mis12 complex) no linear relationship obtained (fig. 4.3).

However, strong correlation was found when the rate of KT recruitment of Ndc80 complex (dXY/dt) and Nsl1 (X_T) as a function of time was plotted (fig. 4.2B) as per equation 3.3. The results obtained from this analysis suggested that the binding of Ndc80 complex to Mis12 is a slow and reversible process. The rate constants (i.e. dissociation and first order association) of Ndc80 complex and Mis12 complex interaction was calculated by rearranging Equation 3.3 as both sides are divided with XY :

$$\frac{1}{XY} \frac{dXY}{dt} \cong k'_{ass} \cdot \frac{X_T}{XY} - k_{diss} \quad (\text{Equation 3.4})$$

By plotting the specific rate of Ndc80 accumulation ($1/XY \cdot dXY/dt$) as a function of Mis12:Ndc80 ratio gives a straight line that has a slope and intercept which corresponds to k_{ass} and k_{diss} values of $4.6 \cdot 10^{-3}$ and $1.8 \cdot 10^{-3} \text{ sec}^{-1}$. The analysis showed co-dependent recruitment of Mis12 complex and Spc105 that then provides a base upon which Ndc80 complex is recruited. These predictions of dependencies were then tested using RNAi²³².

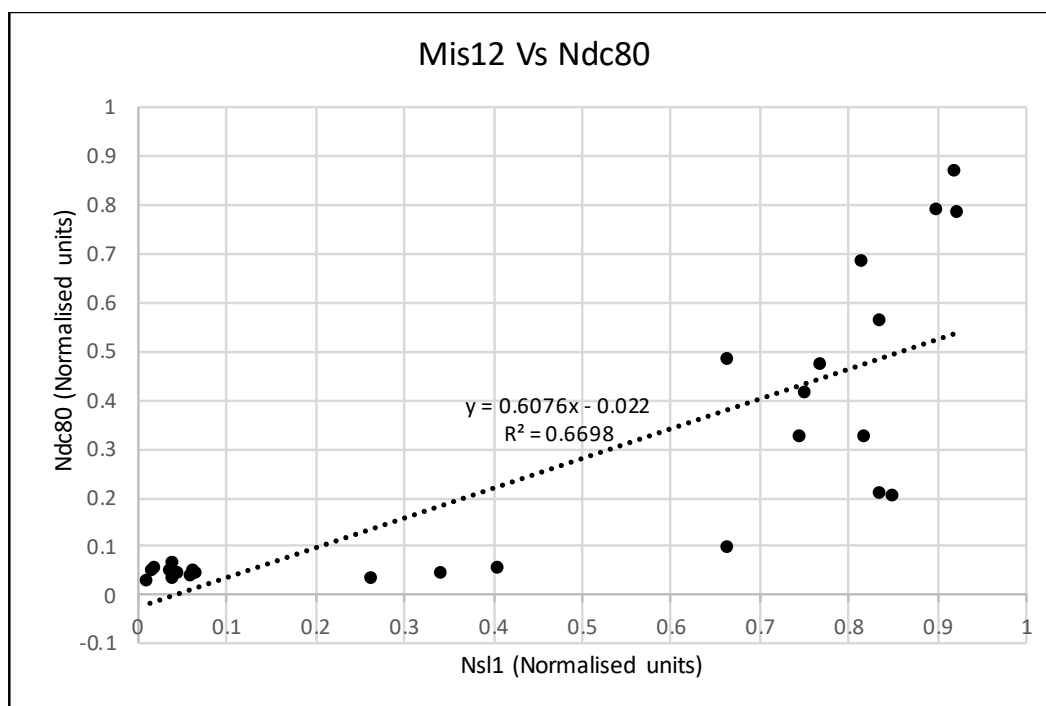


Figure 4.3. Mis12 and Ndc80 dynamics are not linear
The R^2 value is 0.6698 which suggesting a non-linear fit.

4.3 Monophasic Loading and Biphasic Unloading Dynamics of Polo

This section describes the second computational approach, using Polo and BubR1 dynamics, to estimate the number and the timing of biological events shaping their kinetics. Experimental validation and characterisation of the biological events are discussed in chapter 5 and 6.

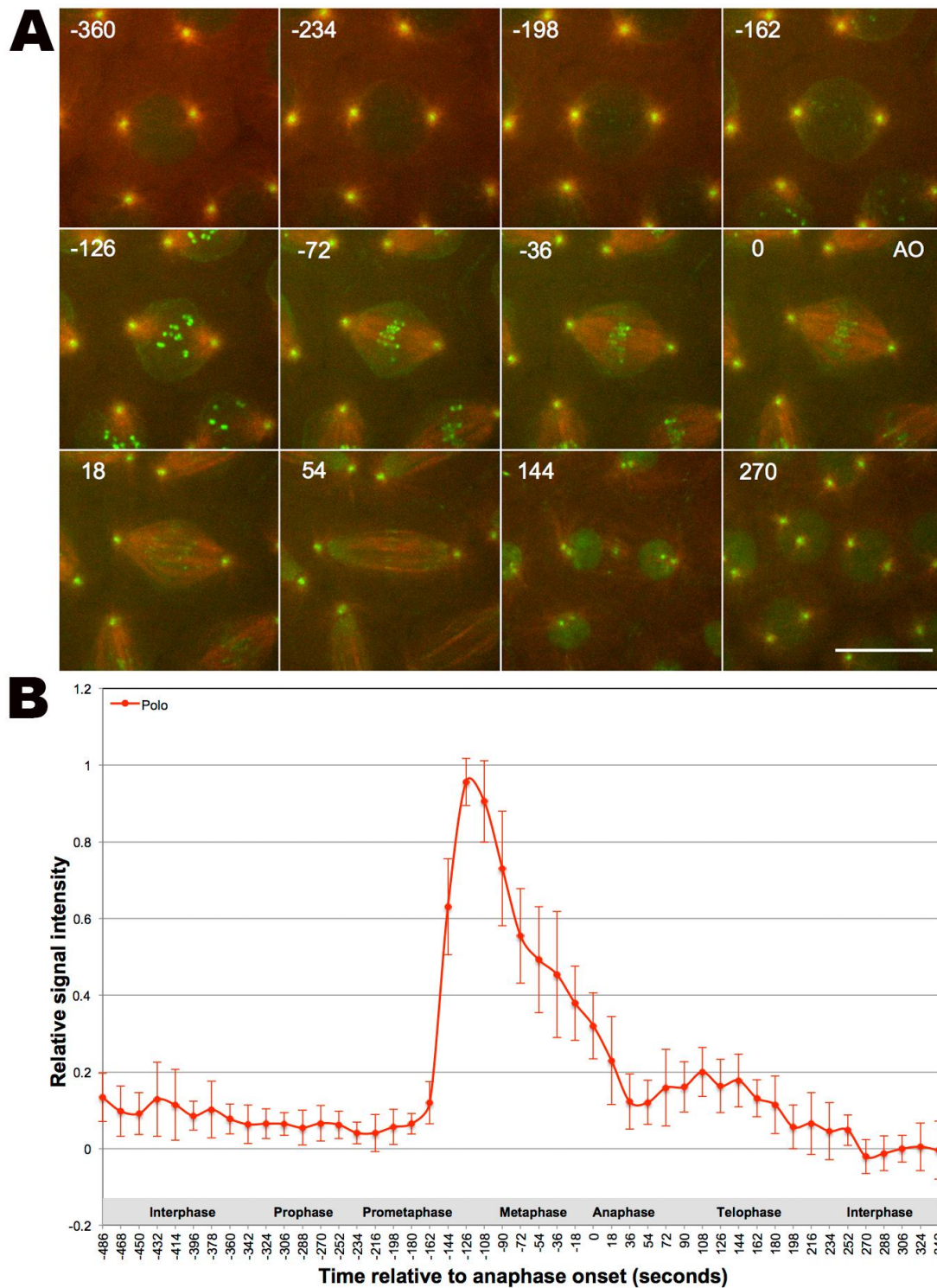


Figure 4.4. Polo-GFP localisation and dynamics in cycle 12 syncytial embryo

A. Stills from the live imaging of Polo-GFP syncytial embryos. The kinetochores are marked with Polo-GFP from time point -198s. Polo-GFP is present on the centrosomes throughout mitosis. At AO i.e. time point 0s, the

sister kinetochores split. At time point 54s the central spindle is marked with Polo-GFP. Finally, the midbody becomes visible at time point 144s. Scale bar 10 μ m. B. Plot of normalised Polo-GFP signal intensities over time

As Polo is one of the master regulators it shows a very dynamic localisation and has a tightly controlled expression^{598,599}. Transgenic flies expressing C-terminus (CT) GFP-tagged Polo in syncytial embryos under endogenous promoter were previously made in the Glover lab. In order to find out the loading and unloading dynamics of GFP-Polo-KT its normalised intensity was plotted as a function of time (fig. 4.4). The unloading phase of Polo beginning from -126s (when the drop starts) till AO (when the sister kinetochores split at time point 0s) was mathematically investigated using time derivative (fig. 4.5).

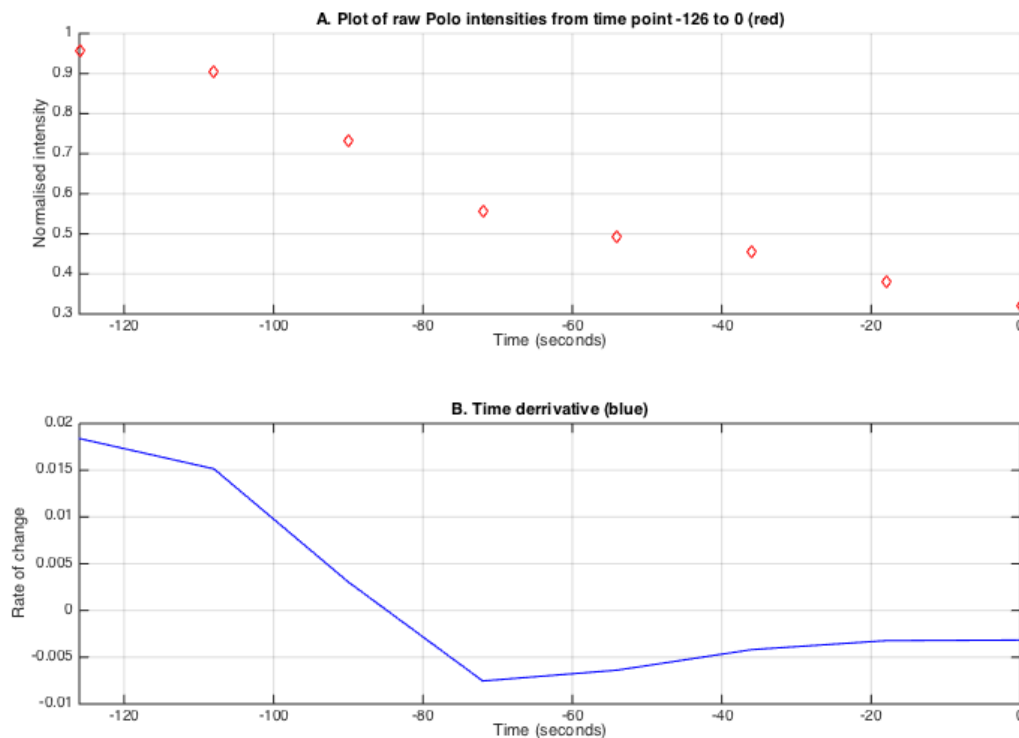


Figure 4.5. The unloading phase of Polo is biphasic

A. Polo intensities plotted from time point -126s to 0 (shown in red). B. Time derivative (shown in blue) suggests that there is a change in the dissociation rate of Polo i.e. in the first phase the i.e. time point -126s to -72s the dissociation is increasing while in the second half from time point -72s to 0s the dissociation rate becomes almost constant. This change in the rate at time point -72s may be triggered by a biological event, thus suggesting that two separate biological events govern the Polo drop.

The time derivative plotted (fig. 4.5B) shows that at time point -72s there is change in the rate of Polo unloading dynamics. This analysis suggests two separate biological events that shape the unloading Polo dynamics. The first event shapes the Polo drop from -126s to -72s or -54s and second event governs the Polo drop from -72s to 0s. The code for this analysis is present in appendix C.

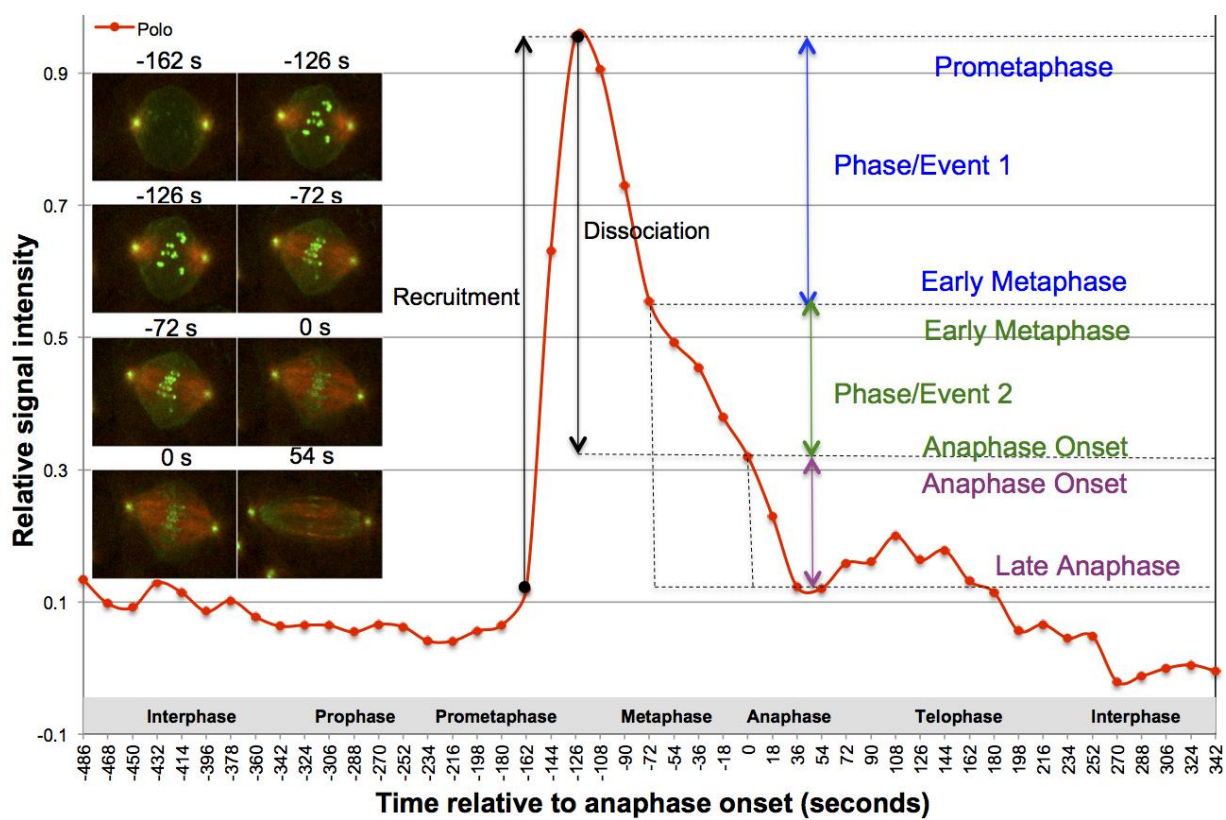


Figure 4.6. Kinetochore dynamics of Polo-GFP

Polo shows a very sharp recruitment phase from time point -162s to -126s. The unloading phase of Polo that starts at time point -126s till time point 0s seems to be biphasic. The first phase is from time point -126s till -72s or -54s and the second phase is from time point -72s or -54s till time point 0s. Both the first and the second drop of Polo seems to be governed by a biological event.

This biphasic nature in unloading phase of Polo is summarised in fig. 4.6. The estimated time point for change in event is approximate as there are many factors for noise in the system e.g. variation in the embryos being analysed, the time for which the laser was working, the background noise levels, the pixels chosen during image analysis etc. The offloading of Polo

is in harmony with the observation in human HeLa cells⁶⁰⁰ where it was shown that higher prometaphase level is required to stabilise KMT attachments while lower metaphase levels is required to satisfy SAC. The mathematical analysis of the recruitment phase revealed that only one biological event governs KT Polo loading. The biological events governing Polo loading and unloading are characterised in following chapters.

4.4 Investigating Dynamics of Chromosomal Passenger Complex

The global map of KT protein dynamics of *D. melanogaster* would be incomplete without CPC, as it is one of the key complexes regulating mitosis in many organisms ranging from yeast to mammals⁶⁰¹. Different subunits of the CPC function in a tightly controlled manner to regulate the diverse mitotic functions e.g. EC, cytokinesis etc.⁶⁰¹. In order to study the dynamics of the CPC, C-terminus GFP tagged transgenic flies of all the four CPC components were made. The constructs were placed downstream of a UASpGAL4 promoter to make the transgenic flies. The expression in syncytial embryos (gel images in appendix A) of GFP tagged transgenic flies was achieved when crossed with α -tubulin–GAL4–VP16 driver^{232,594}. There was no aberrant phenotype observed during the mitosis. In order to find out the loading and unloading dynamics of each CPC subunit, the normalised variation in intensities was plotted as a function of time (fig. 4.7 and 4.8).

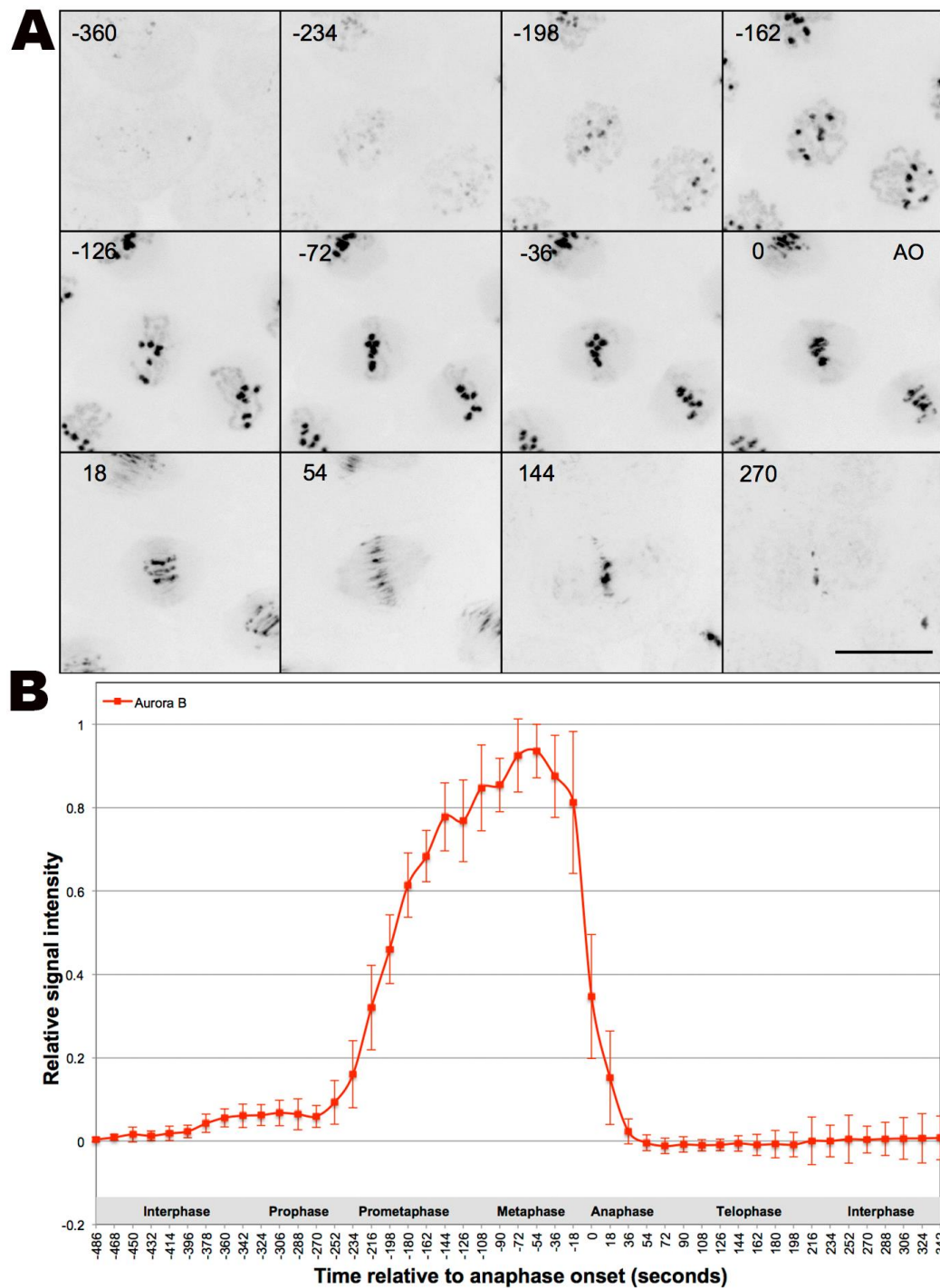


Figure 4.7. Aurora-B-GFP localisation and KT dynamics in cycle 12 syncytial embryo

A. Time series of Aurora-B-GFP syncytial embryo. At time point -234s Aurora B-GFP is present on the chromosomes. From time point -198s to 18s Aurora B-GFP is present on the KT. At time points 0s which is the AO, Aurora-B is present on the splitting KT. At time point 54s the central spindle is marked with AurB-GFP.

From time point 144s and 170s Aurora-B is present on the midbody which eventually disappears. B. Plot of normalised Aurora-B GFP signal intensity as a function of time. Scale bar 10 μ m

The CPC complex first localised on the chromosome arms during prophase. Towards end of prophase the CPC was found on the KT's in addition to the arms. At prometaphase and metaphase, it was enriched on the KT's. During the anaphase the CPC was present on the central spindle and in late anaphase and early telophase the CPC accumulated at the midbody, which eventually disappears. AurB localisation and the dynamics are shown in fig. 4.7 as a representative of CPC, as all subunits show the same localisation pattern (other image present in appendix A). The combined protein dynamics plot for all CPC subunits is shown in fig. 4.8.

As discussed in introduction, AurB recruitment is boosted by several feedback loops as a consequence of which it may be recruited slightly prior to other CPC members (fig. 4.8). All the subunits of the CPC are completely assembled to its peak values by late metaphase. As the drop in the CPC levels is associated with Cdk1 downregulation, it is likely that time point -72s or -54s denotes this point. Additionally, the changes in the AurB dynamics are related to its EC activity and its own recruitment. An increased level in early mitosis is required to destabilise erroneous attachments while reduction in its activity allows the formed correct KMT attachments to persist²⁹⁷. Furthermore, inhibiting Polo activity can lead to lowered EC increasing CS errors as in RPE cells AurB localisation partially depends on Polo activity²⁹⁷ while Polo activity partially depends on AurB phosphorylation in *D. melanogaster*³⁸⁶ (discussed in chapter 5).

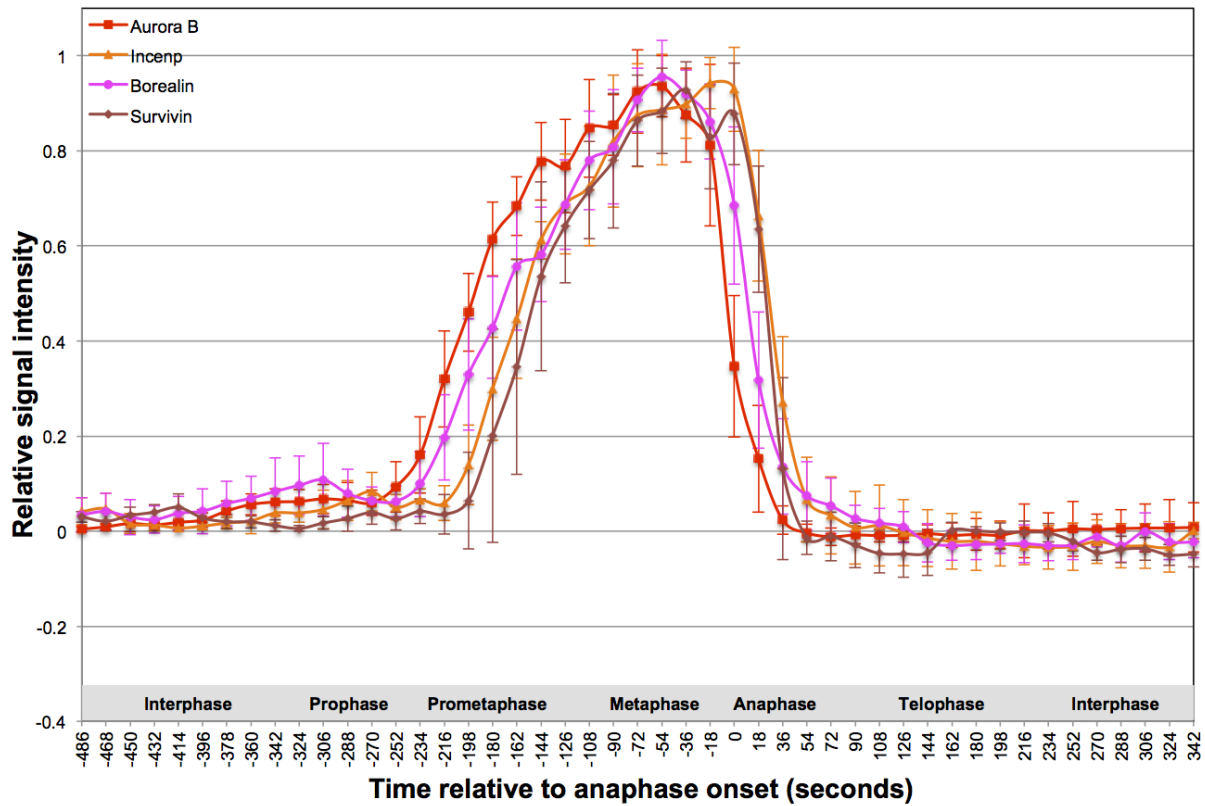


Figure 4.8. Normalised intensities of GFP tagged Aurora-B, INCENP, Borealin and Survivin

4.5 Characterising the Mitotic Check Point Complex Dynamics

MCC is composed of four core checkpoint proteins Mad2, BubR1, Cdc20 (Fzy) and Bub3^{448,486}. Transgenic flies expressing NT-GFP tagged Cdc20/Fzy, BubR1 and Mad2 under endogenous promoted were obtained from Karess lab. No aberrant phenotypes were observed during the mitosis except that cdc20 showed overexpression centrosome localisation. The normalised intensities were then plotted as a function of time to produce a graph of the loading and unloading dynamics (fig. 4.9 - 4.12). As Mad2 has a very high background plotting the dynamics was a bottleneck and hence the stills are used to show its localisation (fig. 4.11).

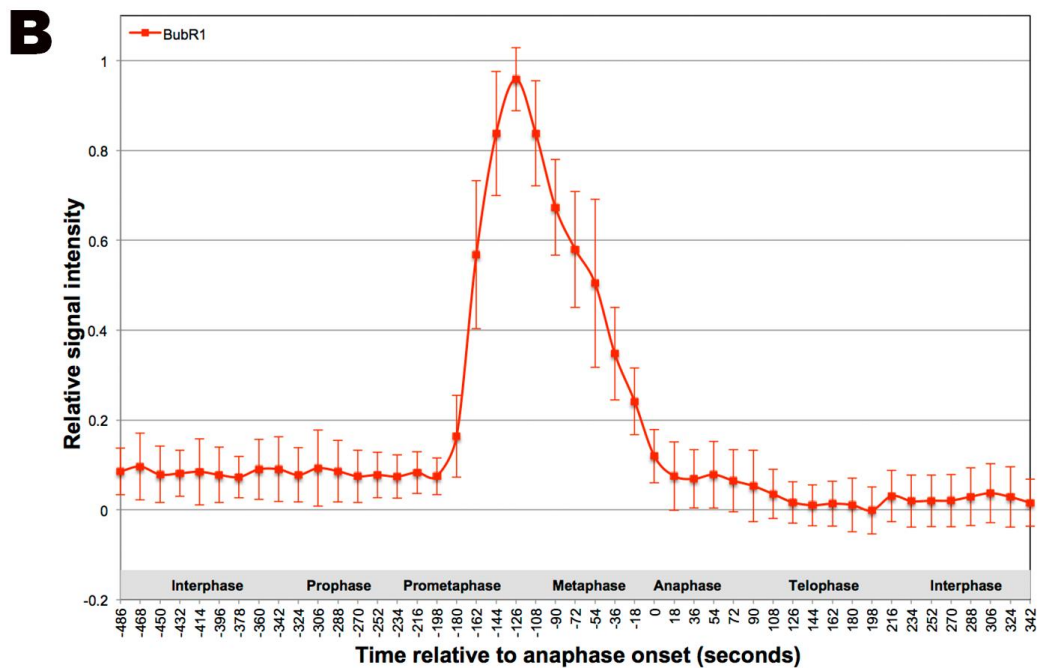
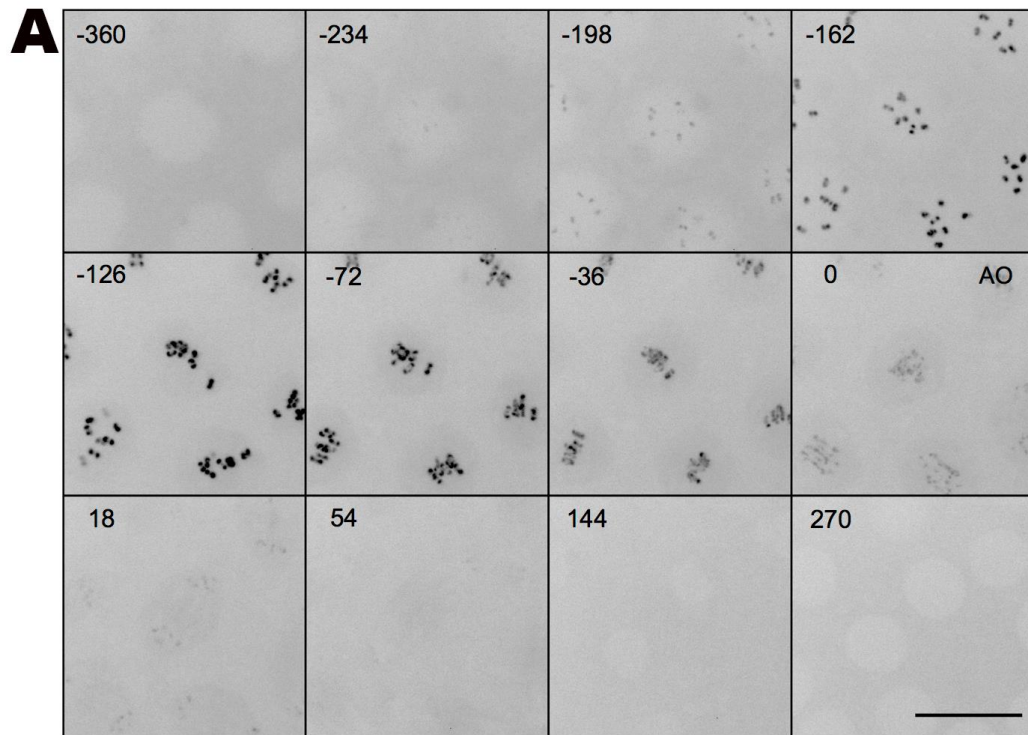


Figure 4.9. GFP-BubR1 localisation and dynamics in cycle 12 syncytial embryo

A. Time series of GFP-BubR1 syncytial embryo. At time point -234s, BubR1 appears on the KTs. At approx. time point -126s it reaches its peak value after which its values start decreasing. At time point 0s, GFP-BubR1 levels are significantly reduced and can be seen on the splitting KTs. At time point 144s, GFP BubR1

BubR1 disappears from the KT. Scale bar 10 μ m. B. Plot of normalised GFP-BubR1 signal intensity as a function of time.

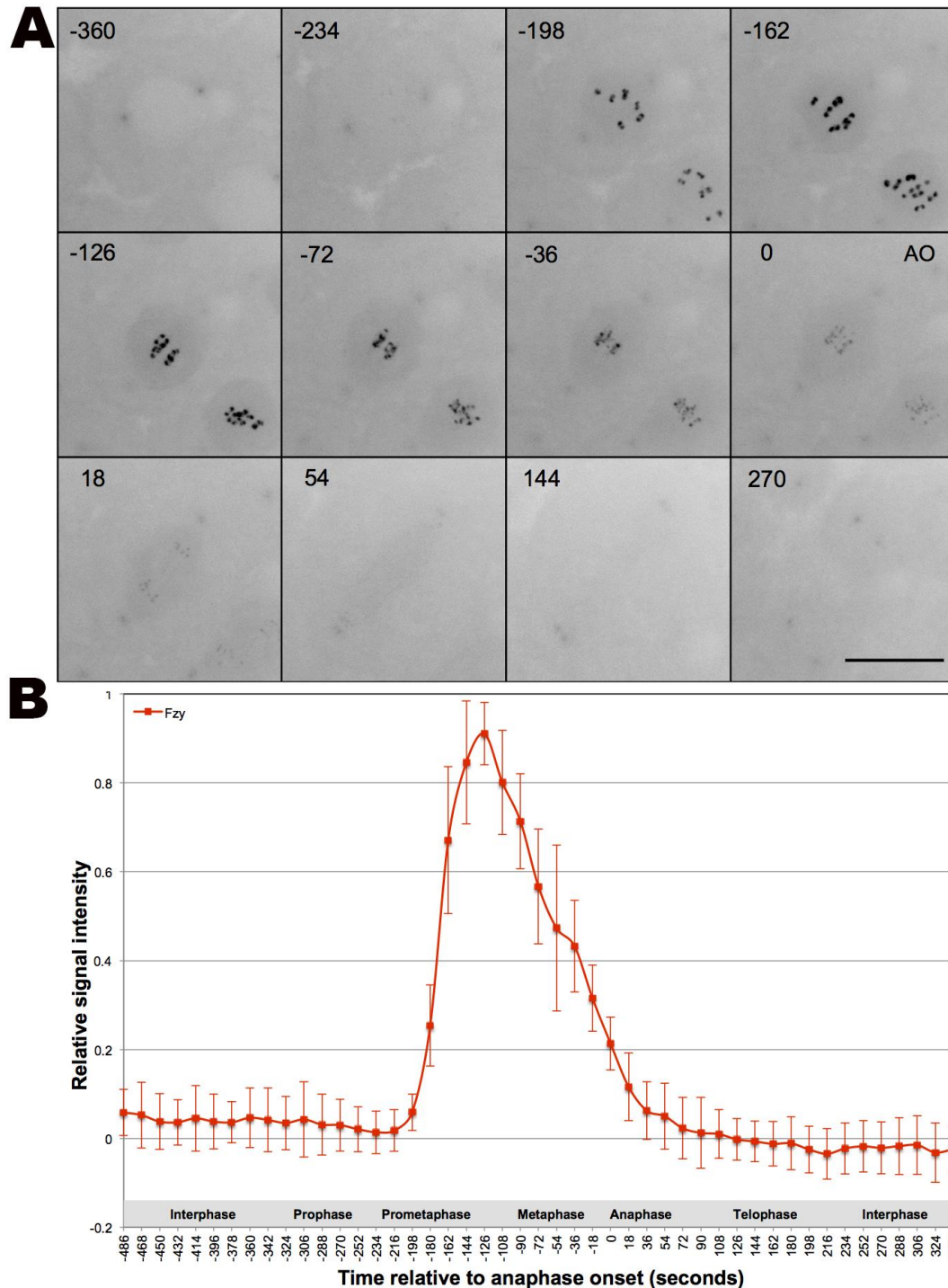


Figure 4.10. GFP-Fzy localisation and dynamics in cycle 12 syncytial embryo

A. Time series of GFP-BubR1 syncytial embryo. At time point -234s, BubR1 appears on the KT. At approx. time point -126s it reaches its peak value after which its values start decreasing. At time point 0s, GFP-BubR1 levels are significantly reduced and can be seen on the splitting KT. At time point

144s, GFP BubR1 BubR1 disappears from the KTs. Scale bar 10 μ m. B. Plot of normalised GFP-BubR1 signal intensity as a function of time

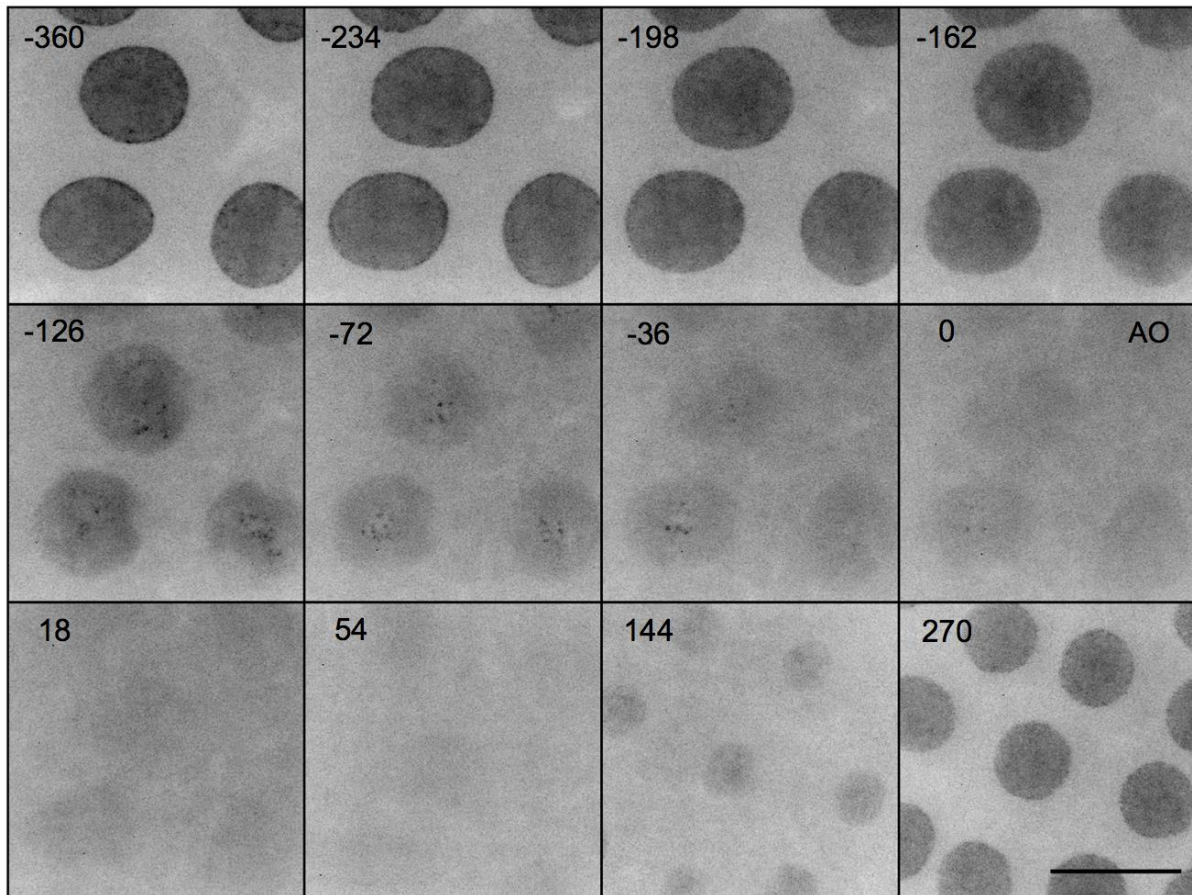


Figure 4.11. GFP-Mad2 localisation and dynamics in cycle 12 syncytial embryo

A. Time series of GFP-Mad2 syncytial embryo. At approx. time point -234s, GFP-Mad2 appears on the KTs. At approx. time point -126s it reaches its peak value after which its values start decreasing. Prior to time point 0s, GFP-Mad2 have disappeared from the KTs. Scale bar 10 μ m.

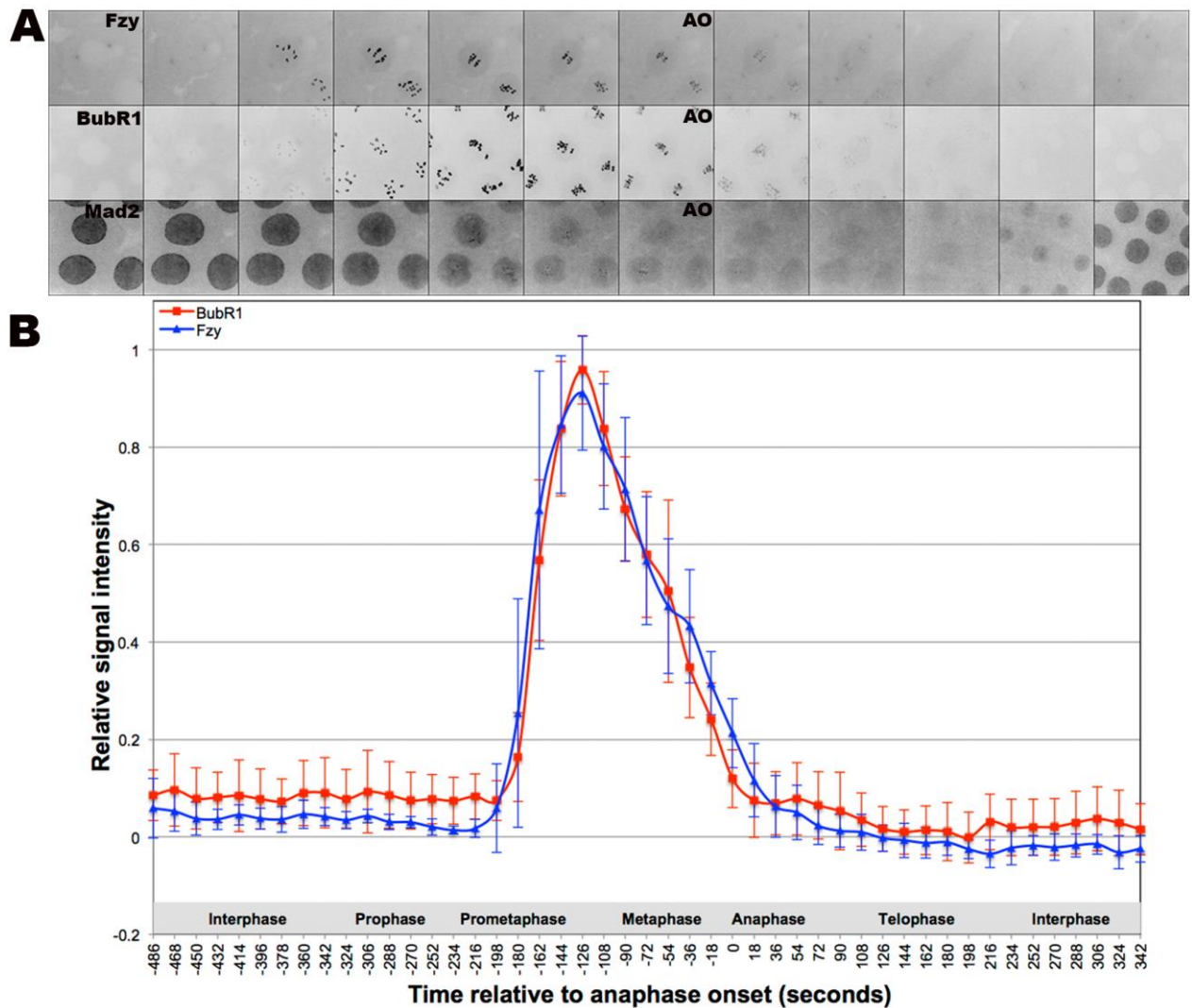


Figure 4.12. Comparing Fzy, BubR1 and Mad2

The data is aligned to AO and is represented by time point 0s. A. Still from live imaging of GFP tagged Fzy, BubR1 and Mad2. B. Normalised intensities of GFP-BubR1 and GFP-Fzy. Dynamics of Mad2 could not be accurately quantified due to high background.

The dynamics from fig. 4.12 suggest that both Fzy and BubR1 are recruited to the KTs with very similar temporal dynamics sharing a close resemblance with Polo. The normalised intensities and the time derivative of BubR1 from -126s to 0s are shown in fig. 4.13.

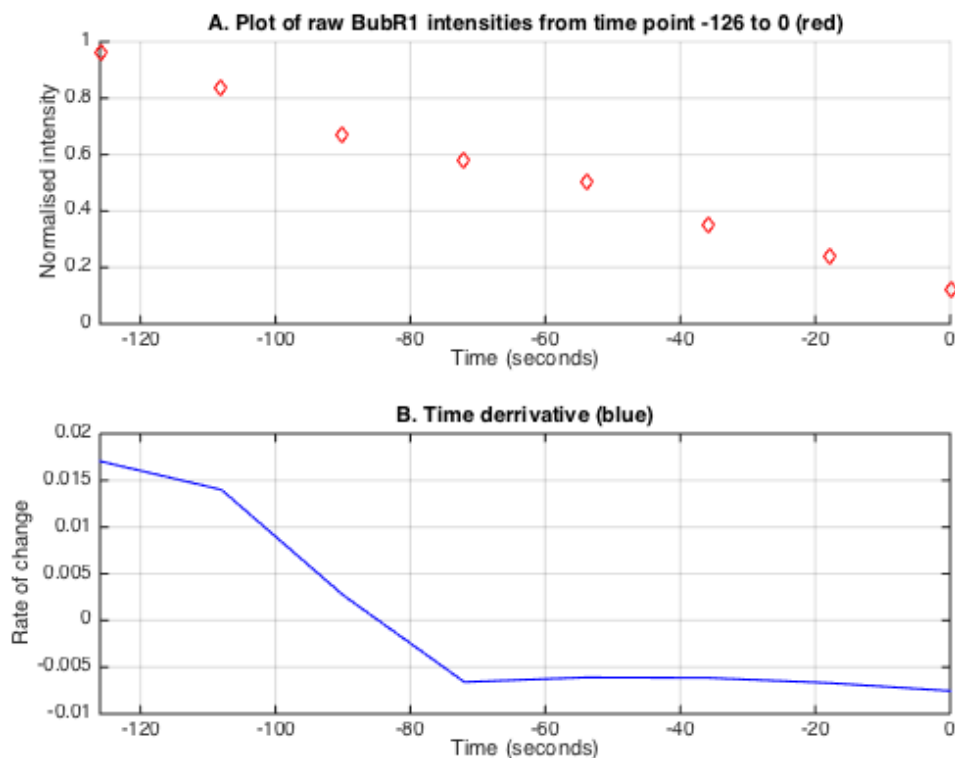


Figure 4.13. Potentially two biological events govern the BubR1 drop from time point -126s till 0s
 A. BubR1 intensities plotted from time point -126s to 0 (shown in red). B. Time derivative (shown in blue) suggests that there is a change in the dissociation rate of BubR1 i.e. in the first phase the i.e. time point -126s to -72s the dissociation is increasing while in the second half from time point -72s to 0s the dissociation rate becomes almost constant. This change in the rate at time point -72s may be triggered by a biological event, thus suggesting that two separate biological events govern the BubR1 drop.

Both Polo and BubR1 have a biphasic off-loading phase in addition to sharing very similar dynamics. The rate change in the unloading phase of Polo and BubR1 take place at the at approximately the same time point i.e. -72s. Based on kinetics it is likely that their dynamics are shaped by same biological events, which are experimentally investigated in later chapters.

4.6 Summary

One of the goals of thesis was to develop a global kinetic map of some of the key KT associated proteins and hence the dynamics of components of KMN network, CPC, MCC and Polo were established (fig. 4.14). The KMN network proteins are recruited much before the regulatory proteins like CPC, BubR1, Fzy and Polo. The structural proteins show a more stable kinetics as opposed to sharp dynamics of regulatory proteins. These observations fit well with the biological role that core structural proteins are recruited earlier to create a base that enables the loading of regulatory protein.

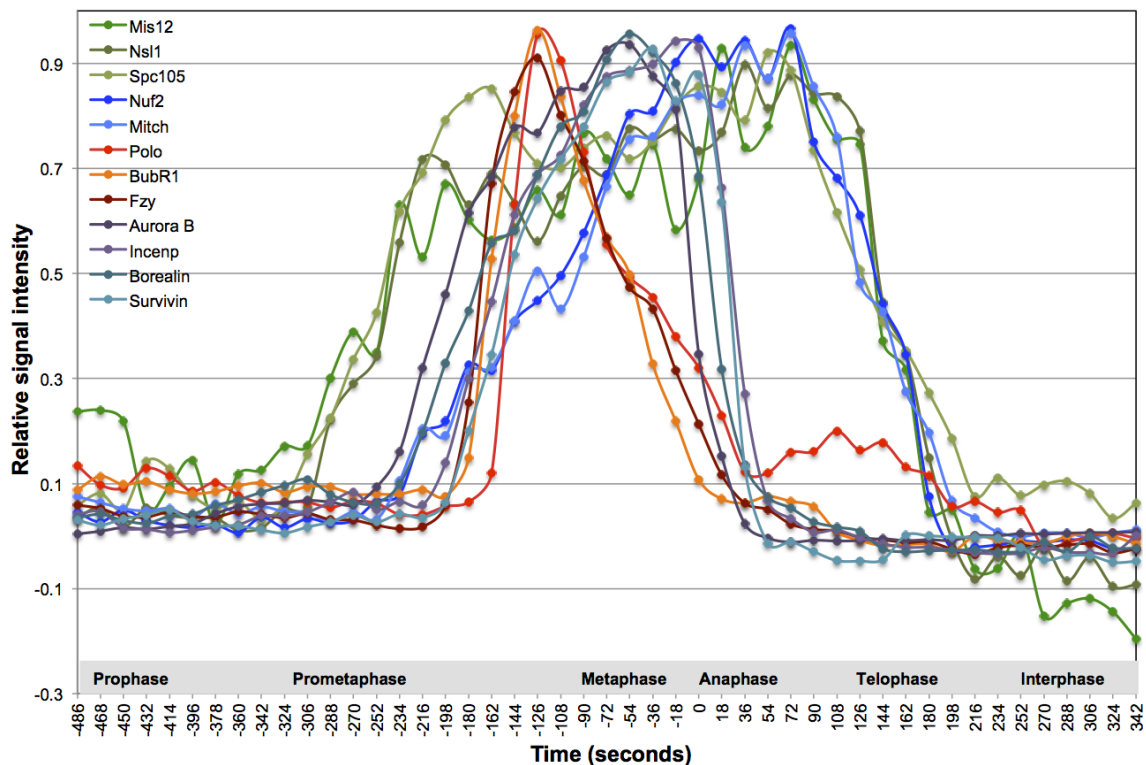


Figure 4.14. Quantitative dynamics of some of the key KT associated proteins

The second aim of thesis was to develop and experimentally validate two computational approaches that analyse protein dynamics a) to predict their interdependencies b) to predict the timing and number of biological events that shape the kinetics. The first method used

differential equation analysis to investigate KMN network dynamics to identify interdependencies that were experimentally verified using RNAi. Co-dependant recruitment of Mis12 complex and Spc105 was shown, which then provides a base to load Ndc80 complex²³². The second approach established the number and timing of biological events that govern protein dynamics of Polo and BubR1 use rate kinetic analysis. The analysis showed that the loading and unloading phase of Polo and BubR1 are monophasic and biphasic respectively. The monophasic loading phase (from NEBD till -126s) is suggested to be shaped by a single biological event. However, the biphasic unloading phase of Polo, is predicted to be shaped by two biological events: a) The first one from time point -126s till -72s b) The second one from -72s till AO (i.e. 0s). The following two chapters experimentally validates the outcomes of the mathematical analysis and characterise the biological events shaping Polo dynamics using BubR1 and Mad2 as control proteins.

Chapter 5 Investigating the Loading and Unloading Dynamics of Polo

5.1 Introduction

This chapter focuses on experimentally validating and characterising the biological events that shape Polo dynamics as hypothesised in previous chapter. Rapid Polo recruitment from nuclear envelope breakdown (NEBD) (-162s/-180s) till it reaches peak levels (-126s), was analysed to be monophasic. However, the analysis of the Polo unloading phase, from its peak levels (-126s) till AO (0s), was analysed to be biphasic where the rate change in the unloading dynamics happens at approximate time point of -72s. The offloading can thus be divided into the first drop from -126s to -72s and the second drop from -72s to AO (0s). During experimental validation Mad2 and BubR1 proteins were used as controls due to their established role in SAC.

The scope for validation and characterisation of the biological events covers the following: 1) What governs the optimal recruitment of KT Polo? For this question I specifically wanted to investigate if Polo and AurB activity play a role in its own recruitment. 2) What events cause to the first drop of Polo? For this question I specifically wanted to investigate if drop in the dynamics of Polo is dependent on: a) Polo degradation at the KTs in a RXXL destruction box dependent manner b) degradation of prometaphase and metaphase substrates of APC (i.e. APC^{Cdc20}) like CycB c) degradation of all APC (APC^{Cdc20} and APC^{Cdh1}) substrates covered by ubiquitin mediated destruction pathway d) process of SAC silencing e) any

combination of above does not depend on any of the above. 3) If the first Polo drop depends on SAC silencing process then does it depend on formation KMT attachment or obtaining maximum sister KT tension? For this question I specifically wanted to investigate if completion of KT occupancy by MTs and establishment of maximum tension on sister KTs happen simultaneously or sequentially. 4) Can Polo be used as a marker to sense the KMT attachments in a manner similar to Mad2? For this question I specifically wanted to investigate if Polo KT levels disappear like Mad2 on KT occupancy. 4) Can Polo be used as a marker to sense maximum tension on KTs? For this question I specifically wanted to investigate if levels of Polo are reduced from the KTs like BubR1. 5) Can Polo directly sense tension in presence of stable KMT attachments? For this I wanted to characterise the change in the level of KT Polo by comparing its levels under amphitelic attachment to its level when the tension is lost but the KMT attachments are not broken.

The term wild type in case of micro injection experiments refer to controls which have been injected with appropriate buffer, in other experiments wild type refers to non-injected embryos. The time-lapse imaging was started as soon as the microinjections were done in prophase of syncytial cycle 12. There were no visible phenotypes in any experiments that suggested impaired mitosis when injected with the buffer or vehicle (10% Dimethyl Sulfoxide i.e. DMSO or Phosphate Buffered Saline i.e. PBS unless otherwise stated). Additionally, there was no change in KT level and dynamics of GFP tagged proteins, when compared with non-injected GFP syncytial embryos. This suggests that any visible phenotype would be due to the effect of the injected compound or protein. After microinjecting the compound or protein or buffer a graph of absolute or normalised intensity of KT protein levels was plotted as a function of time. The analysis was done were done as per the methods section. Single and double

microinjection were initially done under supervision of Dr. Zsolt Venkei in addition to protein purification of UbcH10^{C114S} or Mad2^{L13Q}. The constructs for UbcH10^{C114S} and Mad2^{L13Q} and purified p27 protein were a gift from Dr. Raquel Oliveira. The Polo destruction box mutant and control flies namely UASp-GFP-RXXL-D-box (single destruction box), UASp-GFP-RXXL-DD-box (double destruction box) and UASp-GFP (control) flies were provided by Dr. Yuu Kimata.

5.2 Polo Loading depends on its own and Aurora-B Activity

Various studies have reported using compound called BI2536 (an effective Polo inhibitor in *Drosophila melanogaster*) to investigate role of Polo activity⁴⁶⁵. Inhibiting Polo activity in syncytial embryos using BI2536 at 1 μ M concentration for 30 minutes results in a similar phenotype as obtained by RNAi depletion of Polo in *Drosophila* DMel-2 cells. The BI2536 treatment leads to high mitotic index, delay in prometaphase to metaphase transition and spindle defects like incomplete or absent spindles, misaligned chromosomes and centrosome detachment. Furthermore, there are reports that AurB phosphorylates Polo at the centromeric region thereby activating it^{386,602}. Binucleine2 is the only known inhibitor of AurB to be functional in *D. melanogaster*^{386,602}. Inhibition of AurB activity using Binucleine2 in DMel-2 cells expressing GFP-AurB failed CS and showed two main phenotypes: a) chromosome aggregates b) lagging chromosome bridges⁶⁰³.

Based on the above reports, I wanted to test whether inhibiting the kinase activities of Polo or AurB would affect optimal Polo KT recruitment. Syncytial embryos expressing Polo-GFP were injected with 25 μ M BI2536. Inhibiting Polo kinase activity resulted in CS errors represented by chromosome aggregates and bridges (fig. 5.1 time points 144s and 288s) with

failed cell division. These observations are in alignment with the previously discussed phenotype in DMel-2 cells. This phenotype is due to spindle defects, as inhibition of Polo by BI2536 prevents formation of amphitelic attachments leading to failed division⁴⁶⁵. Although, Polo localisation to the centromeric region was not disturbed after injecting BI2536, a comparison of wild type (control) and BI2536 injected plot showed that Polo was not optimally recruited at the KTs (fig. 5.1 and 5.4). These observations suggest that kinase activity of Polo plays a role in its own KT recruitment.

Similarly, I wanted to test if activation of centromeric Polo by AurB phosphorylation plays a role in its optimal KT recruitment. AurB activity was inhibited by injecting 500 μ M Binucleine2 in double tagged Polo-GFP and Aurora B-mCherry syncytial embryos and levels of Polo were measured while observing AurB phenotype. Inhibiting AurB activity led to CS errors represented by chromosome aggregates and bridges with failed cell division (fig. 5.2 and fig. 5.3 at time points 144s and 288s). These observations are in alignment with the previously discussed phenotype in DMel-2 cells. One reason for this phenotype is because inhibiting AurB activity prevents the correction of erroneous KMT attachments leading to CS errors. Although, Polo localisation to the centromeric region was not impaired after injecting Binucleine2, a comparison of wild type (control) and Binucleine2 injected plot (fig. 5.2 and 5.4) showed that Polo cannot be completely recruited to the KTs. These observations suggest that AurB kinase activity plays a role in Polo KT recruitment.

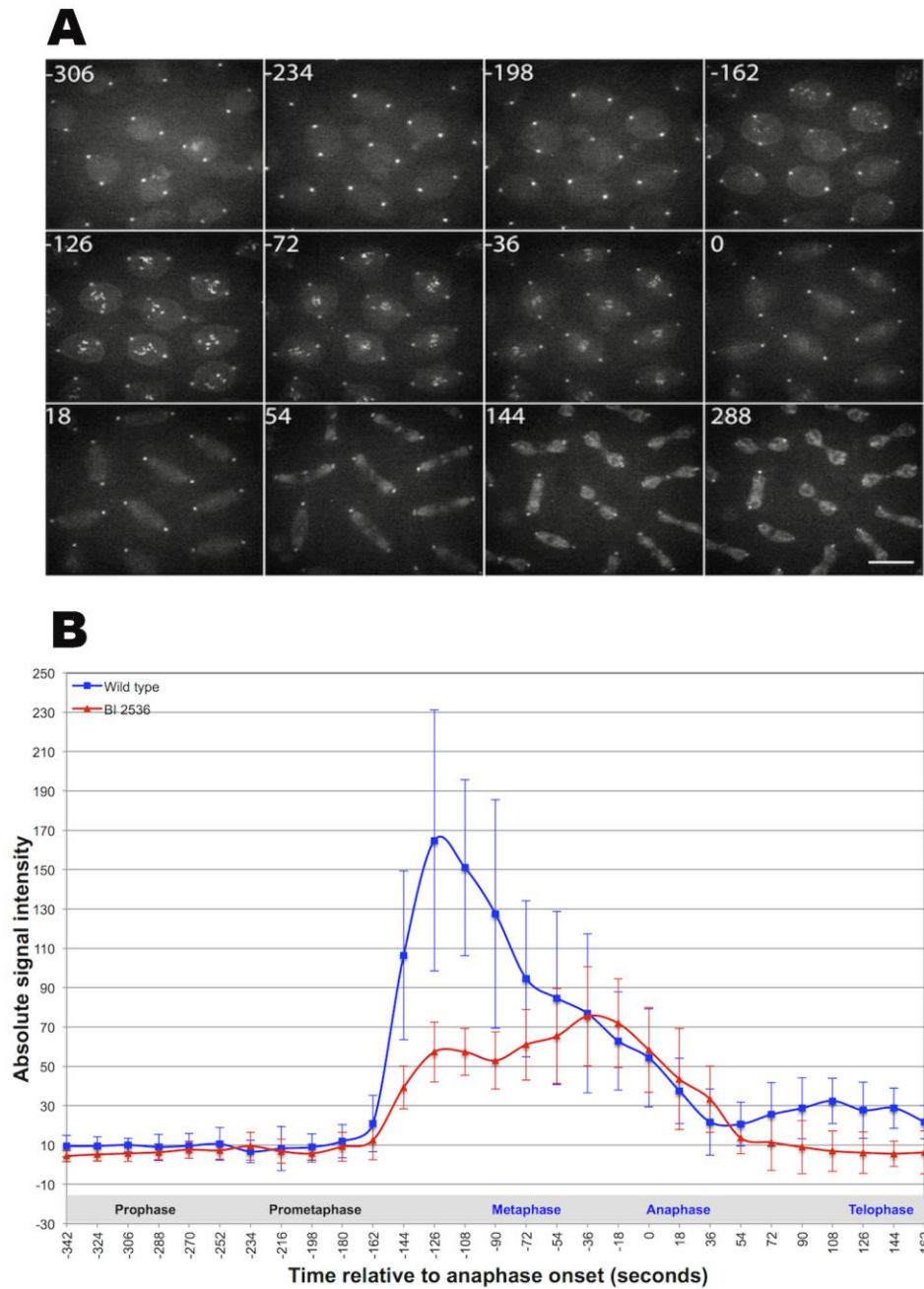


Figure 5.1. Inhibition of Polo kinase prevents it's optimal KT recruitment and leads to chromosomal segregation errors

A. Time series of Polo-GFP syncytial embryos after injecting 25 μ M BI2536. The chromosome bridges are visible from at point 144s. B. Plot of absolute Polo-GFP signal as a function of time. Dynamics of wild type Polo is shown in blue and Polo injected with BI2536 is shown in red. Scale bar 10 μ m.

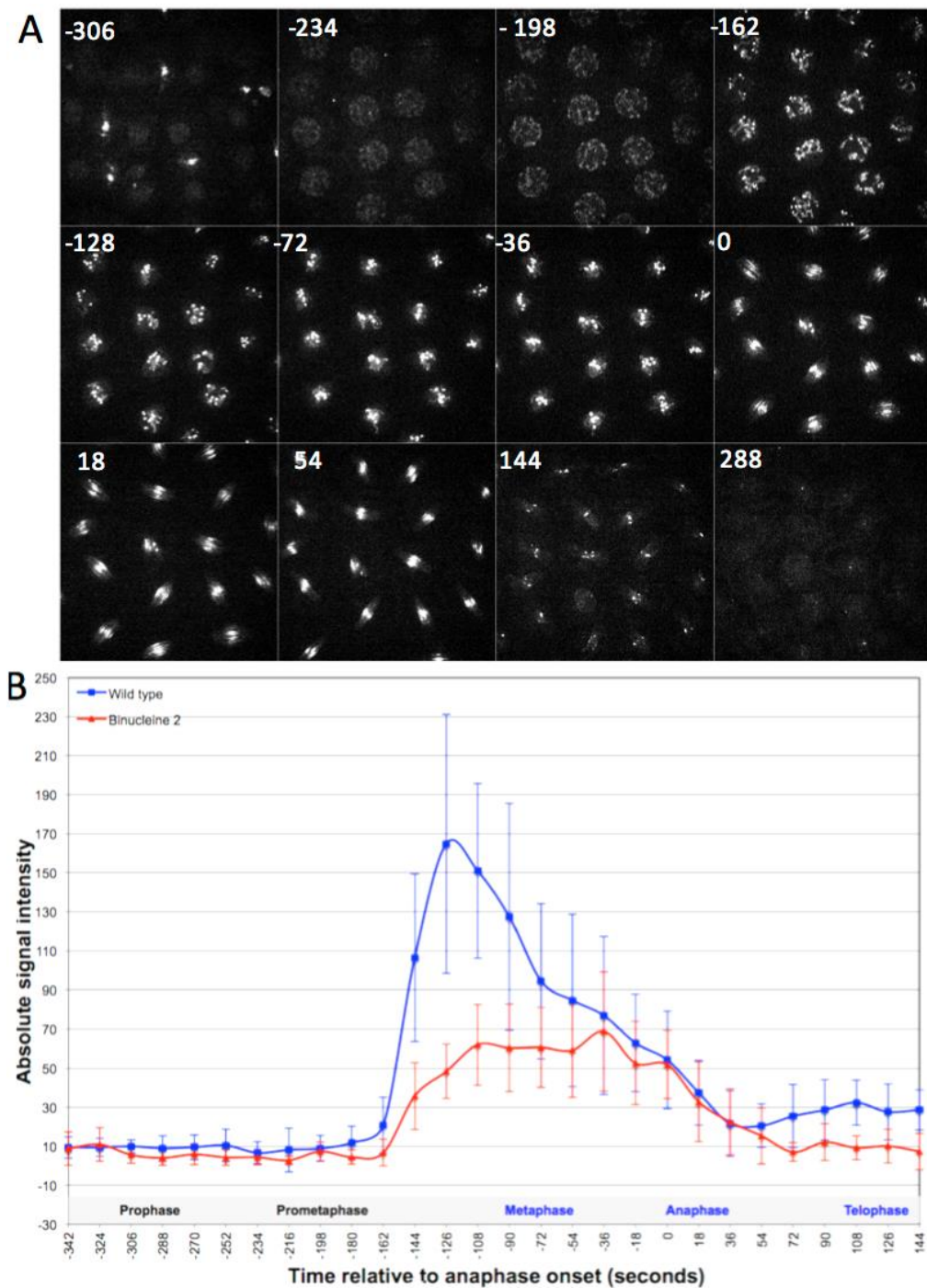


Figure 5.2. Inhibition of Aurora-B prevents optimal KT Polo recruitment and leads to chromosomal segregation errors

A. Time series of GFP-Polo syncytial embryos after injecting 500 μ M Binucleine2. The chromosome bridges are visible from time point 144s. B. Plot of absolute Polo-GFP signal intensity as a function of time. Dynamics of wild type Polo is shown in blue and Polo injected with Binucleine2 is shown in red. Scale bar 10 μ m.

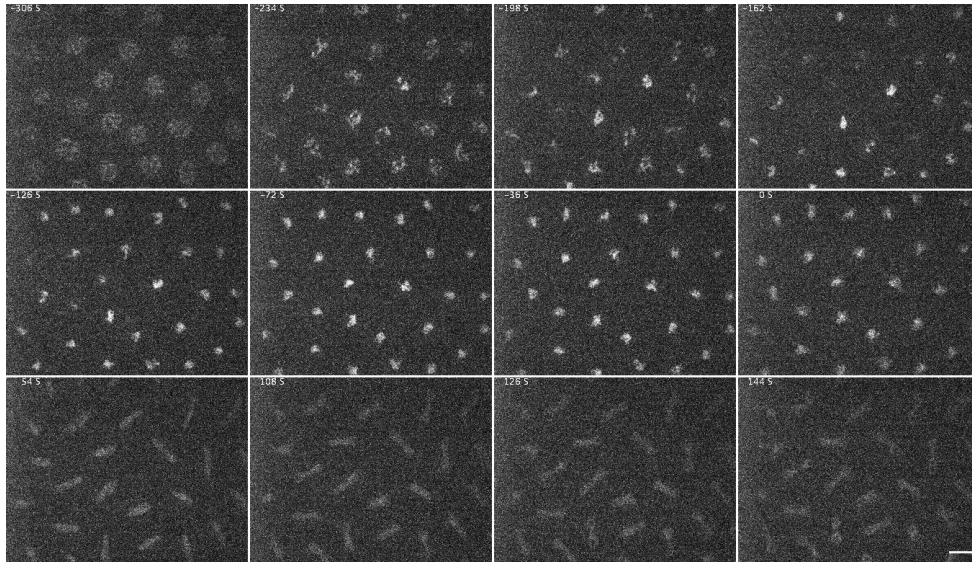


Figure 5.3. Inhibition of Aurora B leads to chromosomal segregation errors
Time series of Histone from RFP-Histone syncytial embryos after injecting 500µm Binucleine2. The chromosome bridges are visible from time point 54s. Scale bar 10µm

Combined plot (fig. 5.4) of wild type Polo and AurB along with BI2536 injected Polo and Binucleine2 injected AurB

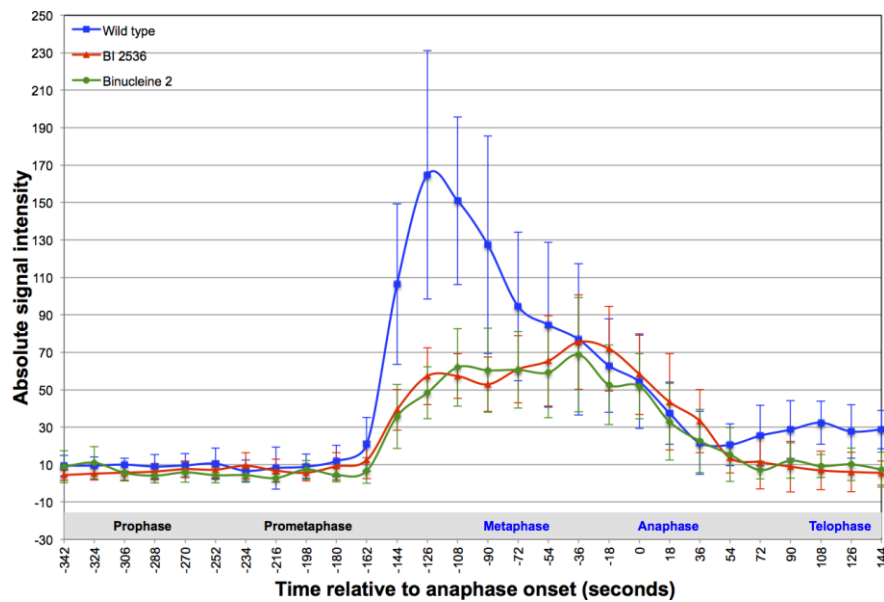


Figure 5.4. Inhibition of Polo and Aurora-B kinase leads to incomplete recruitment of KT Polo and chromosomal segregation errors
Plot of absolute Polo-GFP intensities as a function of time. Dynamics of wild type, Polo injected with BI2536 and Binucleine2 shown in blue, red and green respectively.

5.3 First Drop of Polo Does Not Depend on RXXL-Destruction-Box Degradation Mechanism

Mitotic human Plk1 degradation happens, when APC/C switches its binding from Cdc20 to Cdh1, in a RXXL-D-box dependent manner⁴⁹⁷. It is reported that APC/C^{Cdh1} has a larger pool of substrates than APC/C^{Cdc20} as it can recognise both RXXL-D-box and KEN-D-box signals⁴³³. Destruction of human Plk1 plays an important role in mitotic exit and cytokinesis⁴⁹⁷. In humans, Plk1 is an APC/C^{Cdh1} substrate and its degradation depends on the RXXL-D-box. APC specificity towards its substrate is governed by its activators, Cdc20 and Cdh1, via their NT⁴³³. Any mutation in the D-box of the substrate prevents its recognition by APC^{Cdc20} or APC^{Cdh1} preventing its degradation. *D. melanogaster* Cdc20/Fzy localises to centromeres and centrosomes in early mitosis and Cdh1/Fzr localises to the centrosomes throughout the cell cycle⁶⁰⁴. In syncytial embryos of *D. melanogaster* only Fzy/Cdc20 is present without Fzr/Cdh1⁶⁰⁴.

If the first drop of Polo is caused by its degradation in a RXXL-D-box manner, then mutating the D-box should prevent this drop. Polo has two RXXL-D-box sequences, any of which may be involved in Polo degradation. Single destruction box mutant flies have only one of the RXXL-D-box mutated as opposed to double destruction box mutant flies. Investigating Polo dynamics from both Dbox syncytial embryos would enable us to establish which of the two RXXL-D-box sequence plays a role in KT Polo degradation.

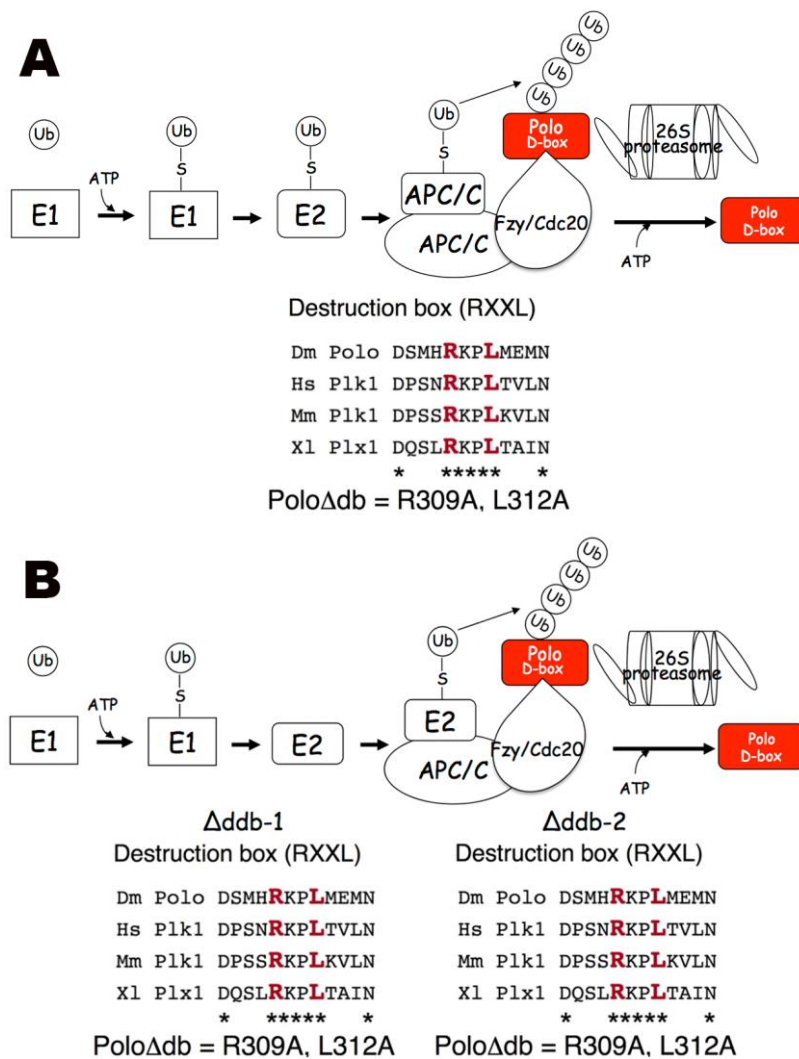


Figure 5.5. Construction of Polo destruction box mutants

This figure has been adapted from ³⁸⁶. A. Single destruction box Polo mutant where only one of the RXXL-D-box sequences is mutated B. Double destruction box mutant Polo where both the RXXL-D-box sequences are mutated.

Both the mutant versions of the flies had N-terminus (NT) of Polo protein tagged with GFP. Single RXXL-D-box transgenic flies are referred to as UASp-Polo-GFP Δ db and double RXXL-D-box transgenic flies are referred to as UASp-Polo-GFP Δ ddb. The expression in syncytial embryos of the transgenic flies was achieved when crossed with Act5C-GAL4 driver. Both, single (D-box) and double destruction (DD-box) box mutants went through mitosis without any impairment (fig. 5.6 and 5.7).

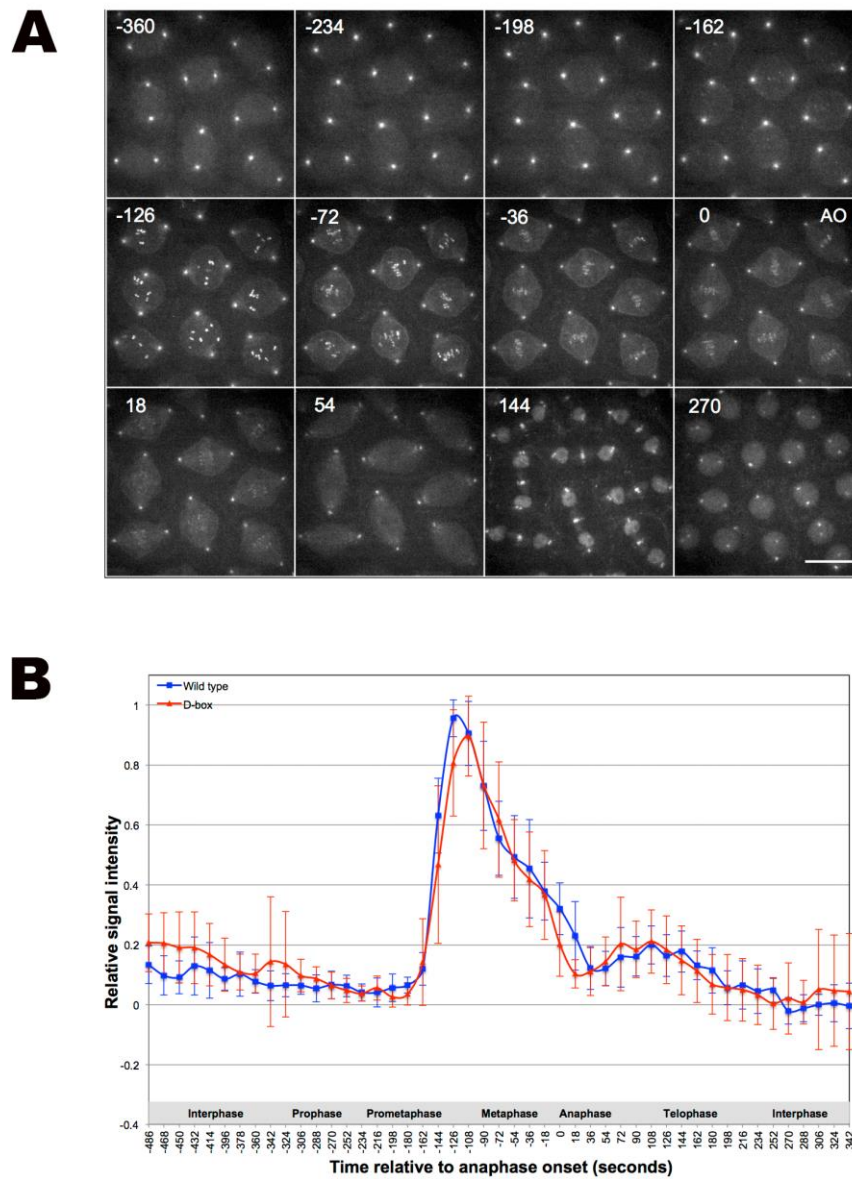


Figure 5.6. Mutating a single Dbox sequence of Polo does not cause any change in the dynamics and levels of KT Polo

A. Time series of Polo-GFP single destruction box mutant in cycle 12 syncytial embryos. B. Plot of normalised GFP signal intensity as a function of time. Dynamics of wild type Polo shown in blue and single destruction box mutant shown in red. Scale bar 10 μ m.

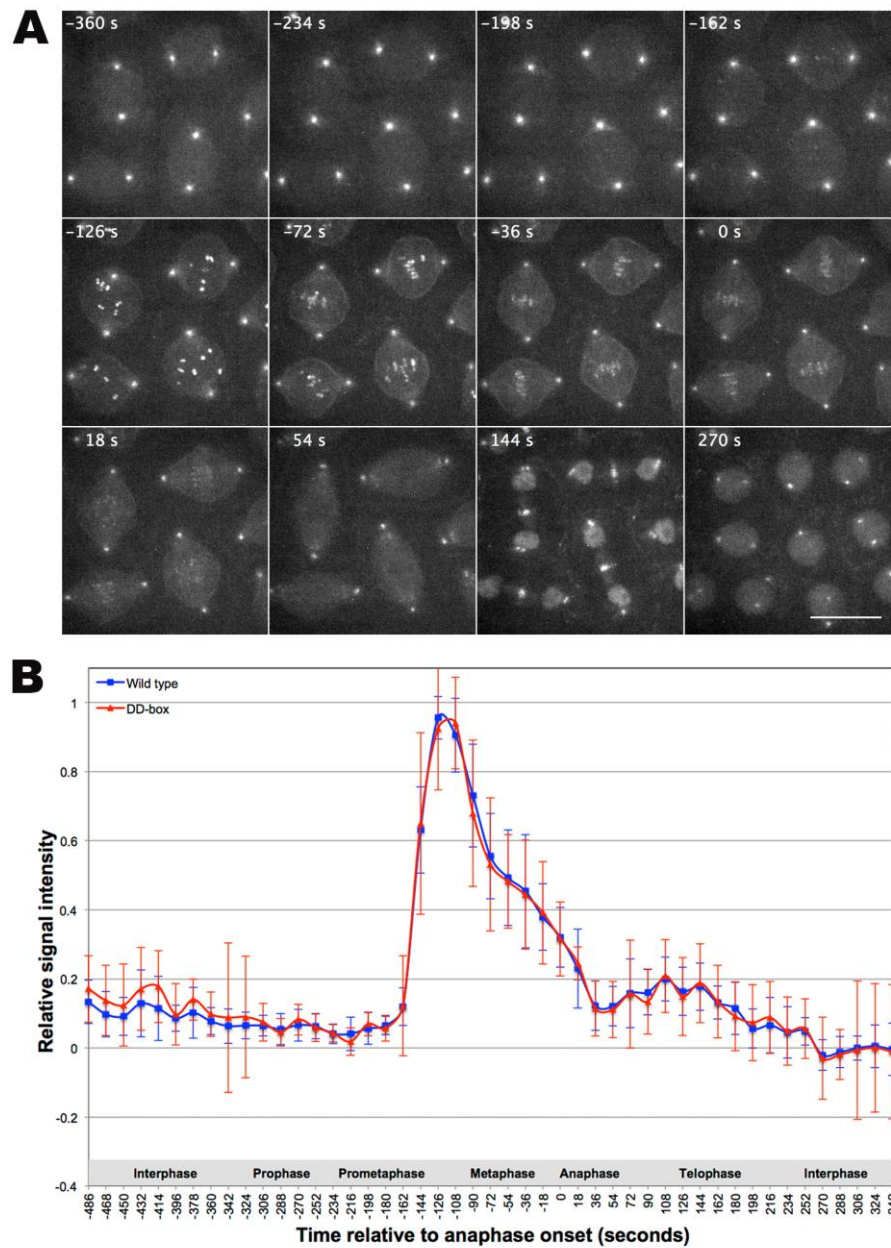


Figure 5.7. Mutating both Dbox sequences of Polo does not cause any change in the dynamics and levels of KT Polo

A. Time series of Polo-GFP double destruction box mutant in cycle 12 syncytial embryos. B. Plot of normalised GFP signal intensity as a function of time. Dynamics of wild type Polo shown in blue and double destruction box shown in red. Scale bar 10 μ m.

On comparing the D-box, DD-box and wild type plots (fig. 5.8) no significant difference in their dynamics were observed. These results showed that the first drop in KT Polo is not because of its degradation in a RXXL-D-box dependent manner.

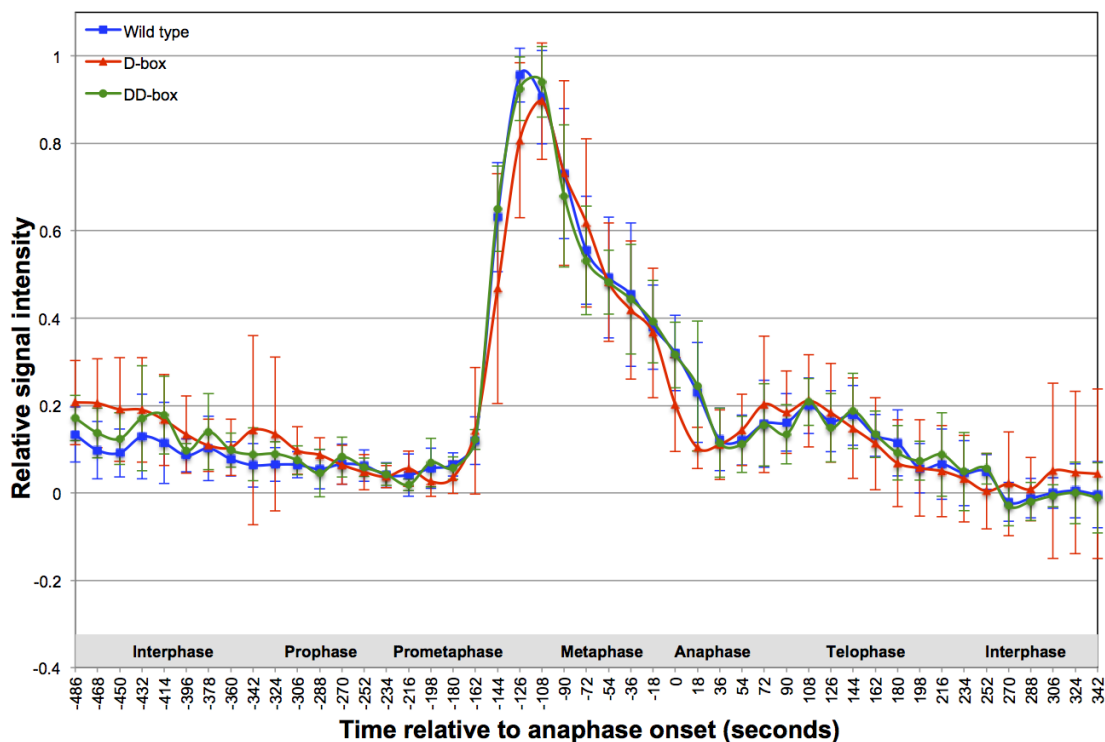


Figure 5.8. Normalised dynamics of wild type Polo, single destruction box (D-box) and double destruction box (DD-box) do not show significant difference

5.4 The First Polo Drop is Independent CyclinB activity and Ubiquitin Mediated Degradation Pathway

I wanted to investigate if the first Polo drop depends on degradation of a) prometaphase and metaphase substrates of APC (i.e. APC^{Cdc20}) like CycB b) any substrate of APC (i.e. APC^{Cdc20} and APC^{Cdh1}) covered by ubiquitin mediated degradation pathway. To address the former, I designed an experiment to sequester Cdc20 that would prevent activation of APC.

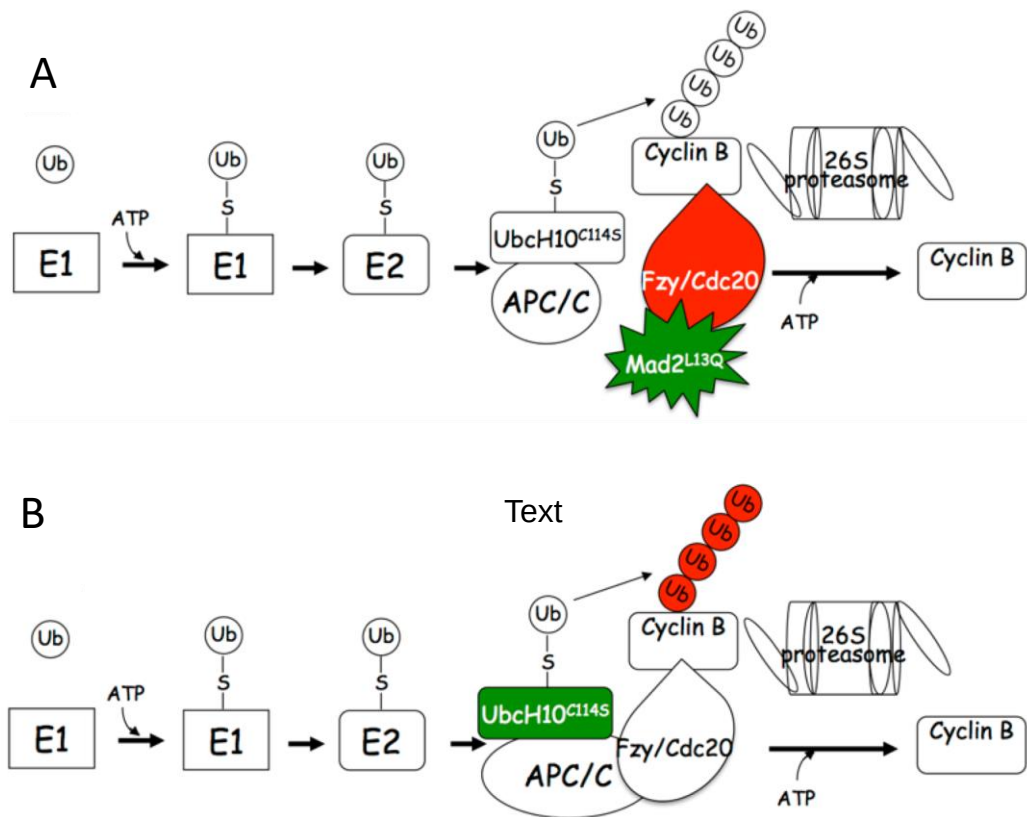


Figure 5.9. Injection of Mad2^{L13Q} or UbcH10^{C114S} prevents the normal functioning of APC/C to degrade Cdk1-CyclinB

A. Injection of Mad2^{L13Q} sequesters Cdc20 (APC/C activator) that prevents APC/C activation. Inactive APC/C is not able to degrade CyclinB. Injection of UbcH10^{C114S} prevents the ubiquitin chain formation on CyclinB and hence inhibits its destruction by 26S proteasome. This figure has been adapted from the figure by Dr. Yu Kimata.

This involved injecting Mad2^{L13Q} that sequesters Cdc20 and as a consequence stops degradation of APC^{Cdc20} substrates during prometaphase and metaphase like CycA, Nek2A, Securin, CycB^{23,112} (fig. 5.9). Mad2^{L13Q} is a modified and a more potent form of wild type Mad2 (Mad2^{WT}) that adopts a conformation to enhance the binding of Fzy/Cdc20¹¹². The second experiment involved investigating if the first Polo drop depends degradation of any APC (i.e. APC^{Cdc20} and APC^{Cdh1}) substrate covered by ubiquitin mediated degradation pathway. This involved injecting catalytic-dead version of human ubiquitin-conjugating

enzyme E2 UbcH10 or dominant negative (UbcH10^{C114S}) as it prevents ubiquitin chain formation on the substrates and hence they are not marked for degradation by 26S proteasome¹¹² (fig. 5.9). Injecting any of the two proteins prevents MAT as Cdk1-CycB activity is kept high that prevents Separase activation disabling cleavage of Cohesin rings¹¹².

Both UbcH10^{C114S} and Mad2^{L13Q} were diluted in PBS to their working concentrations of 30mg/ml and 25mg/ml respectively. Injection of Mad2^{L13Q} and UbcH10^{C114S} separately into syncytial embryos expressing Polo-GFP and mRFP-Tubulin that lead to a mitotic arrest. Comparison of the wild type (control) with Mad2^{L13Q} and UbcH10^{C114S} plots (fig. 5.10, 5.11 and 5.12) showed that the first drop in Polo-GFP still existed and is independent of a) CycB activity b) degradation of all APC (i.e. APC^{Cdc20} and AP^{Cdh1}) substrates covered by ubiquitin mediated destruction pathway.

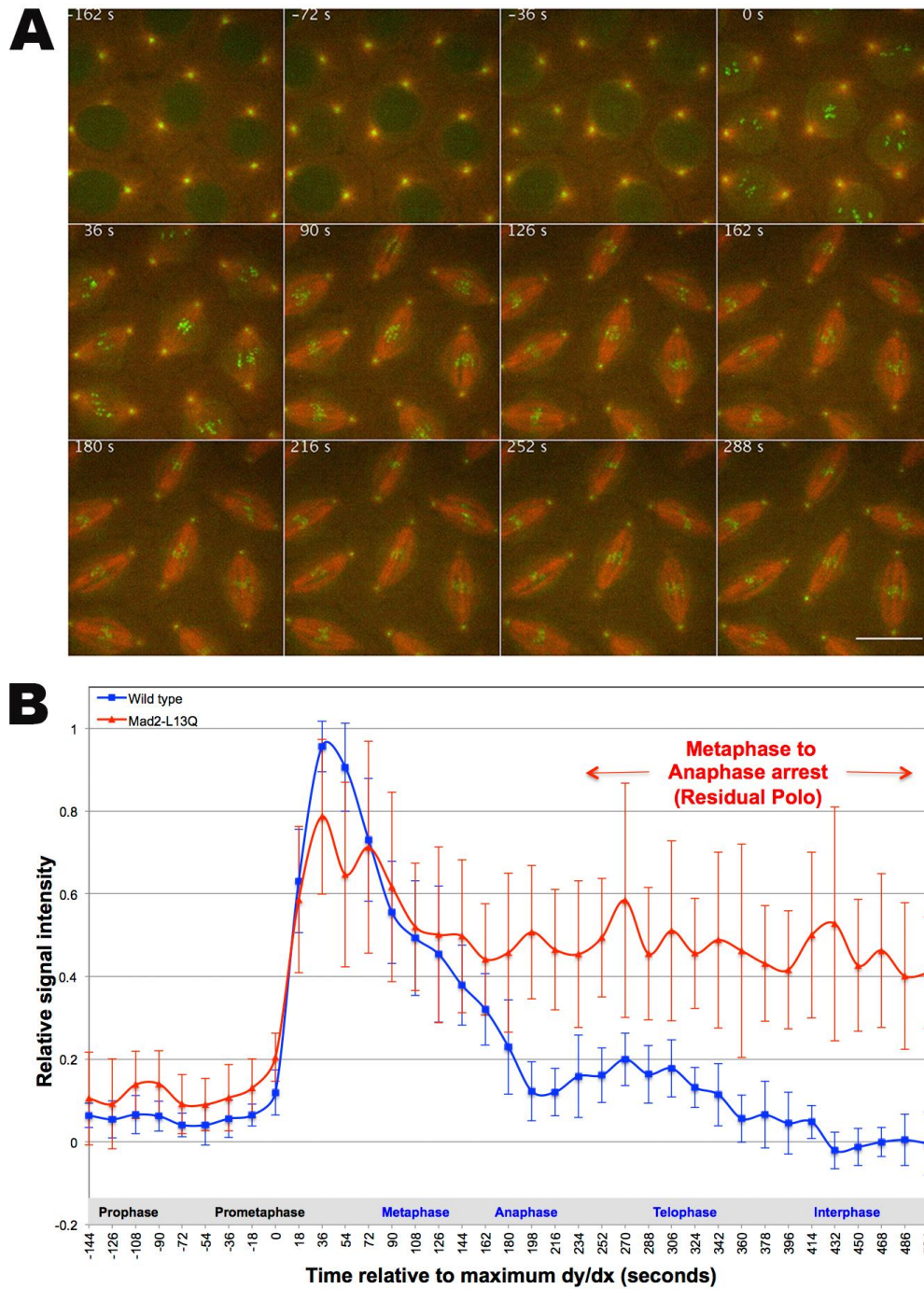


Figure 5.10. Injection of Mad2L13Q did not prevent the first Polo drop

A. Time series of Polo-GFP and mRFP-Tubulin syncytial embryos after injecting 25mg/ml of Mad2^{L13Q}. This led to an arrest in metaphase to anaphase transition state. B. Plot of normalised intensities of Polo-GFP signal as a function of time. Dynamics of wild type Polo shown in blue and Polo injected with Mad2^{L13Q} shown in red.

The data is aligned on the X-axis with reference to maximum change in slope between any two-time points i.e. maximum dy/dx . Scale bar $10\mu\text{m}$

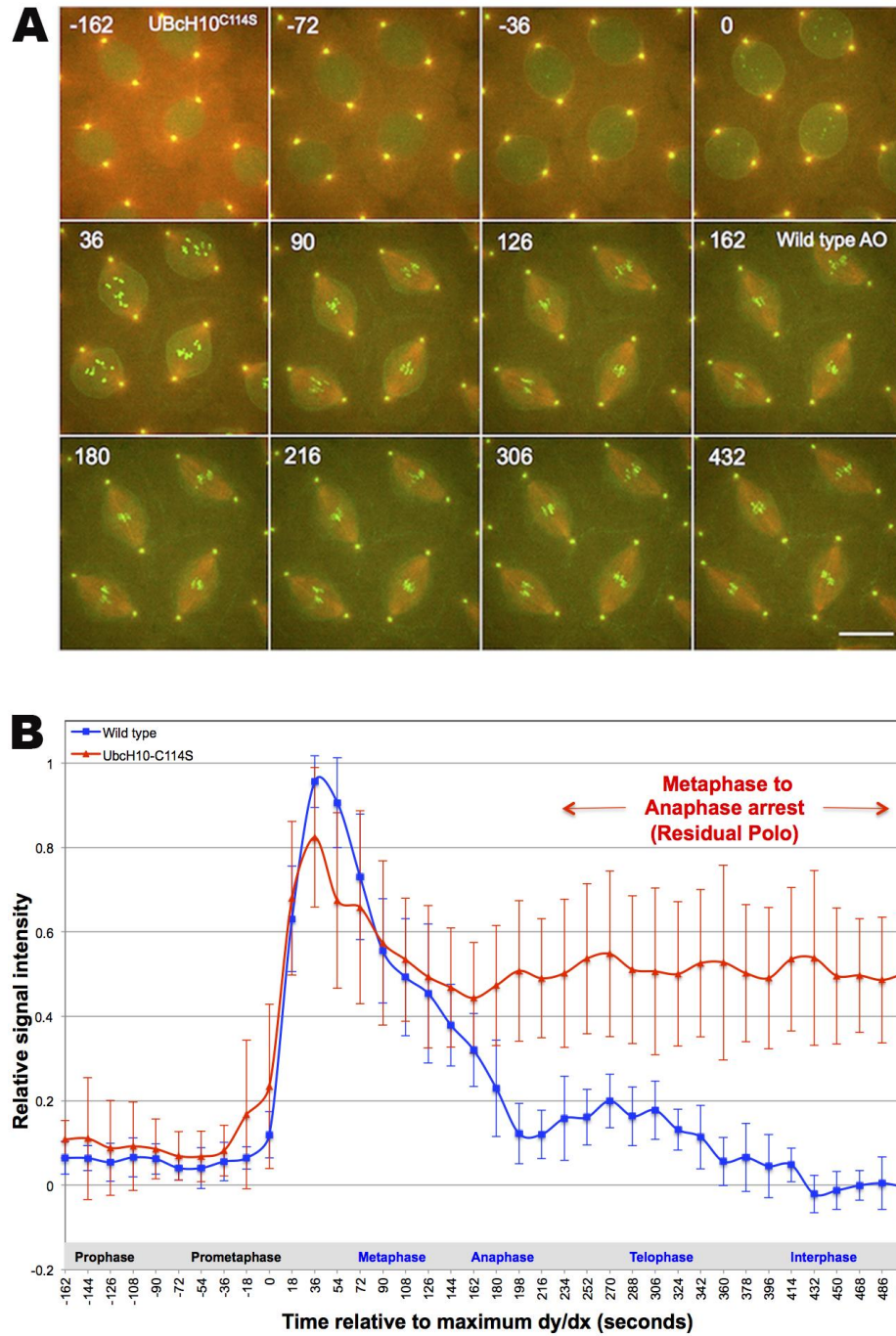


Figure 5.11. Injection of Ubch10C114S did not prevent the first Polo drop

A. Time series of Polo-GFP and mRFP-Tubulin syncytial embryos after injecting 30mg/ml of Ubch10^{C114S}. This led to an arrest in metaphase to anaphase transition state. B. Plot of normalised intensities of Polo-GFP as a function of time. Dynamics of wild type Polo is shown in blue and Polo injected with Ubch10^{C114S} shown in

red. The data is aligned on the X-axis with reference to maximum change in slope between any two-time points i.e. maximum dy/dx . Scale bar $10\mu m$

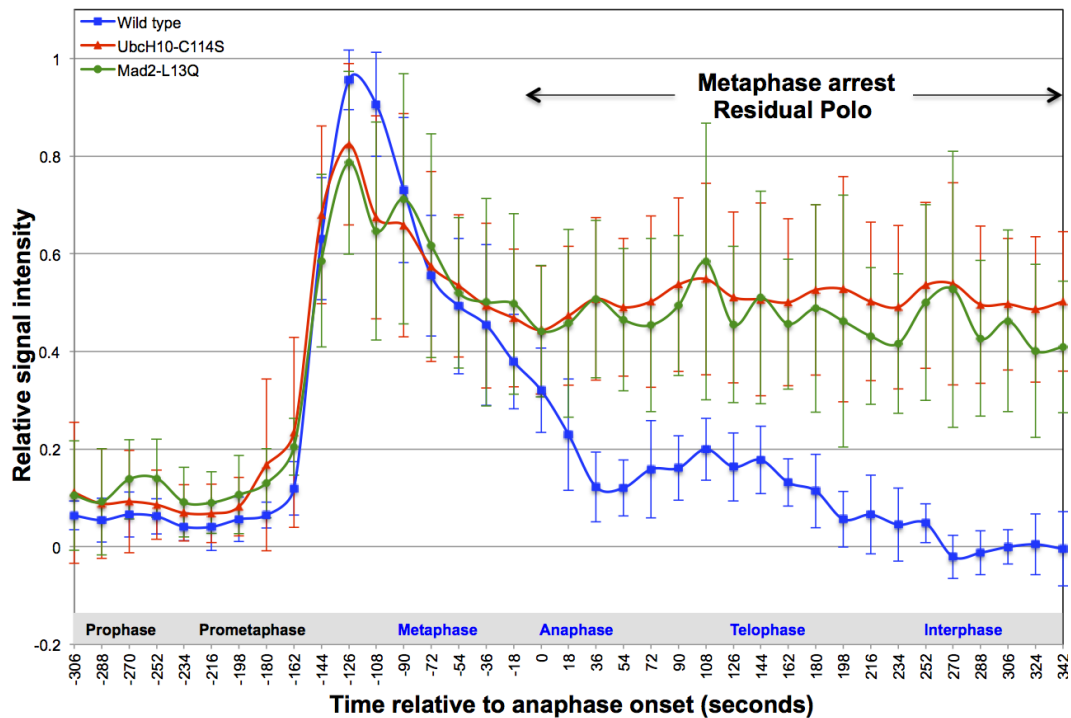


Figure 5.12. Injection of UbcH10C114S or Mad2L13Q did not prevent the first Polo drop
Plot of normalised intensities of Polo-GFP signal as a function of time. Dynamics of wild type Polo, Polo injected with Mad2^{L13Q} and Polo injected with Mad2^{L13Q} shown blue, red and green respectively.

5.5 First Drop of Polo Depends on Process of Silencing Spindle Assembly Checkpoint

Comparison of Polo dynamics with an established SAC protein i.e. BubR1 (fig. 5.17) suggested that the first drop of Polo can be consequence SAC silencing process. SAC silencing requires two conditions to be satisfied a) KT's must be completely occupied by the MT's which can be established by observing complete loss of Mad2 signal⁶⁰⁵ b) Sister KT's must be under maximum tension by the MT's from opposite poles (also referred as amphitelic or bi-oriented configuration) and can be established by observing highly reduced levels of BubR1⁶⁰⁵.

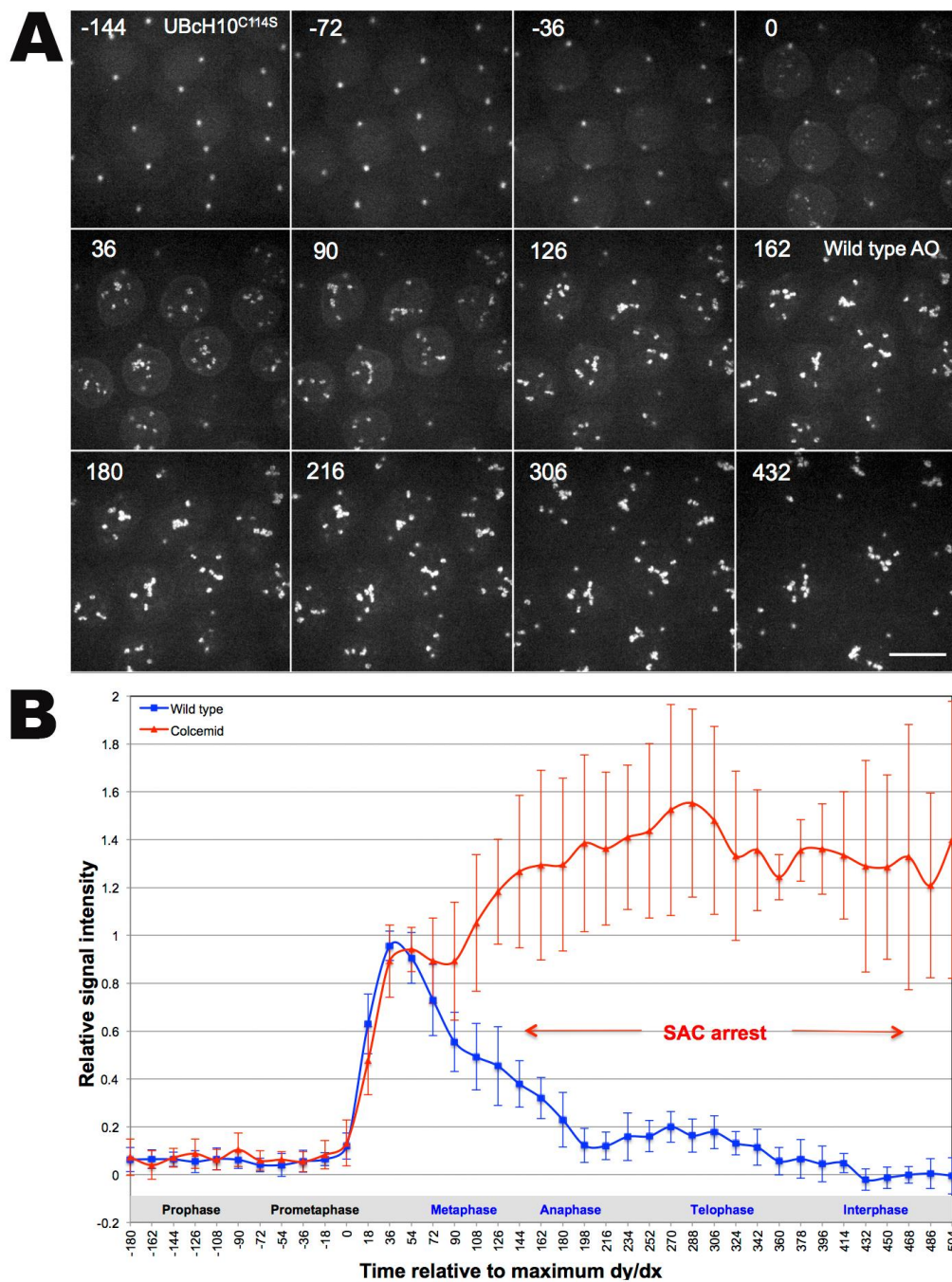


Figure 5.13. Impairing KMT attachments prevents the first Polo drop

A. Time series of Polo-GFP in cycle 12 syncytial embryos after injecting 250 μ M Colcemid. The Colcemid injection leads to a SAC dependent arrest in mitosis. B. Plot of normalised Polo-GFP intensity as a function of time. Dynamics of wild type Polo shown in blue and Polo injected with Colcemid shown in red. The data is

aligned on the X-axis with reference to maximum change in slope between any two-time points i.e. maximum dy/dx . The Colcemid data was normalised to maximum KT intensity of wild type Polo. Scale bar 10 μ m.

If injecting Colcemid (MT poison) prevents the first Polo drop, then it is a consequence of impaired KMT attachments (hence no sister KT tension) that prevents the process of SAC silencing. Injection of 250 μ M Colcemid in Polo-GFP syncytial embryos resulted in SAC dependent arrest in mitosis. On comparison of wild type with Colcemid plot (fig. 5.13) it was clear that the loading dynamics of KT Polo are unchanged even after Colcemid injection. However, the first Polo drop was completely abolished with a surge in the levels of KT Polo.

Additionally, to confirm if the drop was indeed related to process of silencing the SAC, GFP-BubR1 was injected with 250 μ M Colcemid, due to its established role in SAC. Injection of Colcemid in GFP-BubR1 embryos resulted in SAC dependent block in mitosis with depolymerised MTs (fig. 5.14). Comparison of wild type (control) with Colcemid plot of BubR1 showed unchanged loading dynamics of BubR1 after Colcemid injection. However, the drop in BubR1 levels was abolished and there was a surge in its level. This surge in the level of BubR1 was exactly at the same time point as the surge in Polo level (shown in fig. 5.15 at time point -126s) establishing that the impaired KMT attachments prevent the process of satisfaction of SAC. Furthermore, when levels of Colcemid injected Polo and BubR1 surged at time point -126s the levels of wild type (control) Polo, BubR1 and Mad2 were at its peak (and not started their unloading phase) and hence process of SAC silencing had not yet begun at time point -126s (fig. 5.15 and 5.17). Even though this experiment shows that first drop of Polo is due to the process of SAC silencing it is not able to ascertain if completion of two events a) KT occupancy by MTs b) maximum tension on sister KTs happen simultaneously or sequentially.

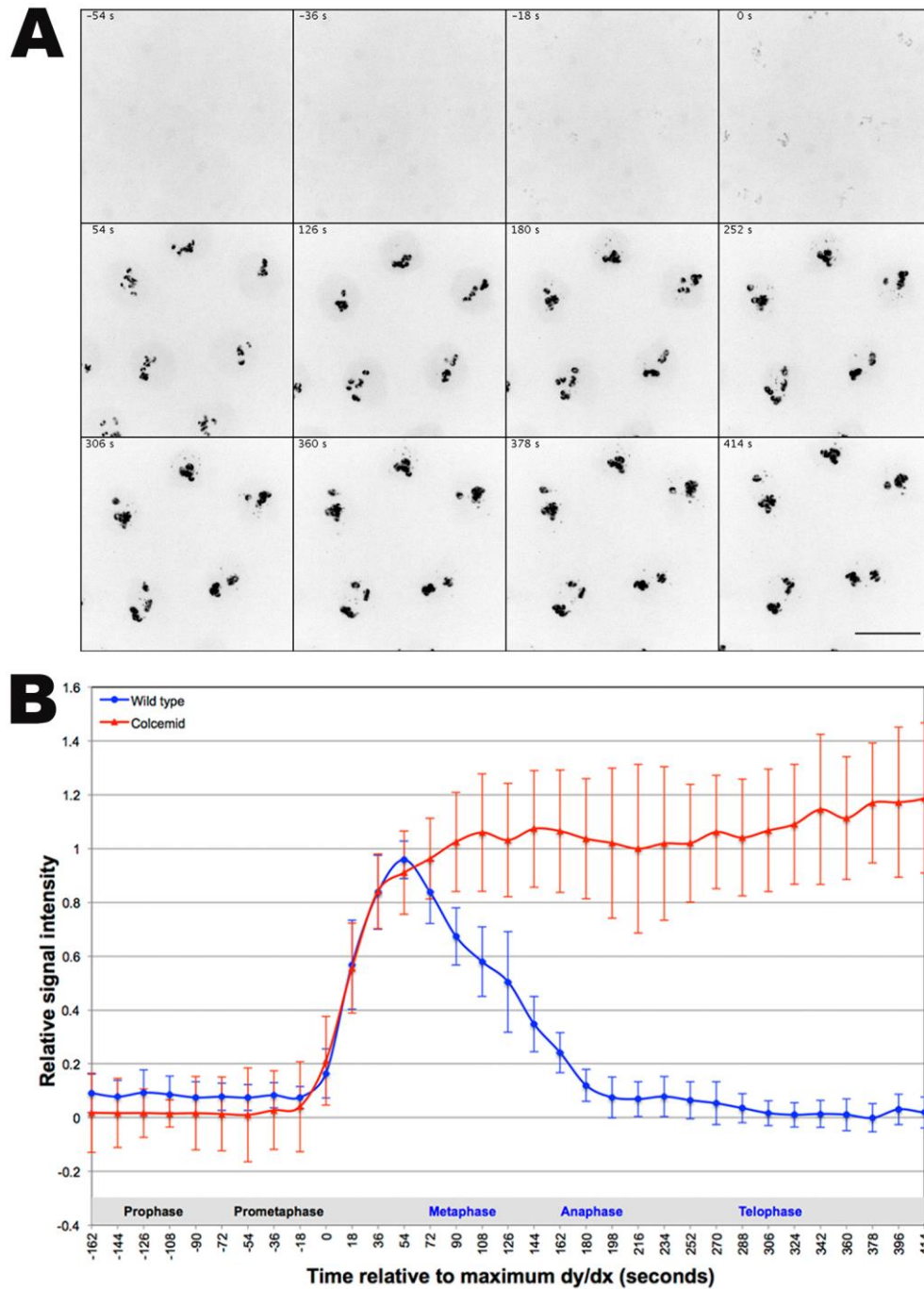


Figure 5.14. Impairing MT-KT attachments keeps the SAC and prevents the first KT-GFP-BubR1 drop
A. Time series of GFP-BubR1 in cycle 12 syncytial embryos after injecting 250 μ M Colcemid. The embryos are in a SAC dependent mitotic arrest. B. Plot of relative GFP-BubR1 signal as a function of time. Dynamics of wild type BubR1 shown in blue and BubR1 injected with Colcemid shown in red. The data is aligned on the X-

axis with reference to maximum change in slope between any two-time points i.e. maximum dy/dx . Scale bar 10 μ m.

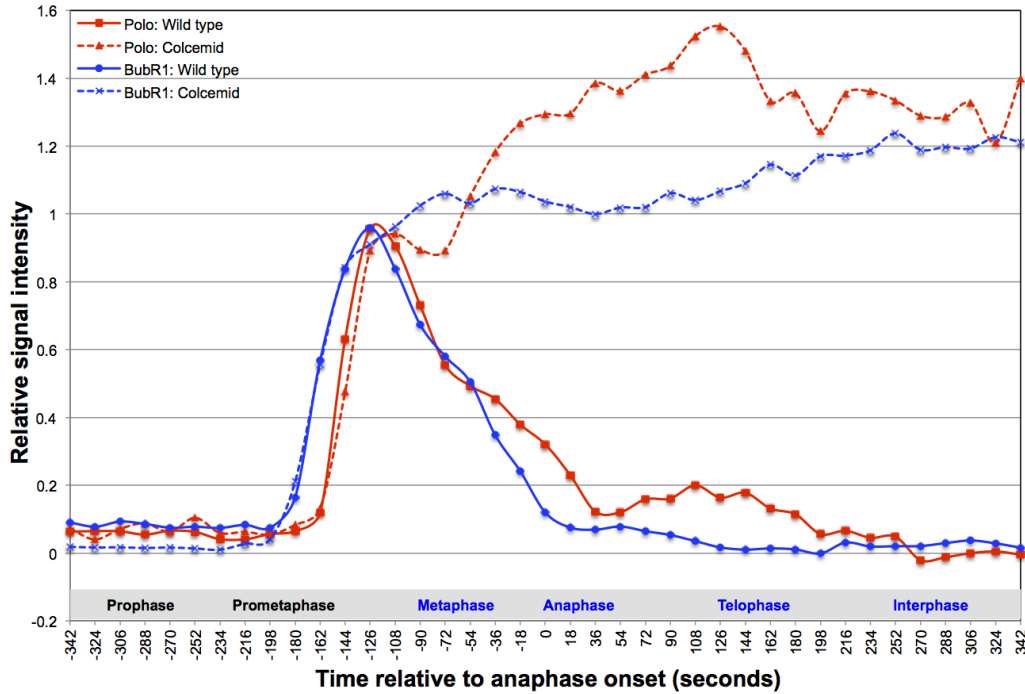


Figure 5.15. Plot of normalised GFP signal intensity of wild type and Colcemid injected Polo and BubR1. Dynamics of wild type Polo (red), Polo injected with Colcemid (dashed red), wild type BubR1 (red) and BubR1 injected with Colcemid (dashed blue).

5.6 Polo Senses Kinetochore-Microtubule Attachments

A reverse experiment was performed in order to confirm the previous finding that Polo senses aberrant KMT attachments via a surge in its KT level. A MAT block with amphitelic attachments and silenced SAC was obtained by injecting 30mg/ml of UbcH10^{C114S} (fig. 5.16 images 1-4). Once the levels of Polo stabilised 250 μ M Colcemid was injected to depolymerise the KMT attachments (fig. 5.16 images 5-12) that reactivated SAC and lead to surge in Polo levels. This and previous observations show that Polo senses KMT attachments in a manner similar to Mad2 (i.e. when KMT attachments are severed level of Mad2 remains increase).

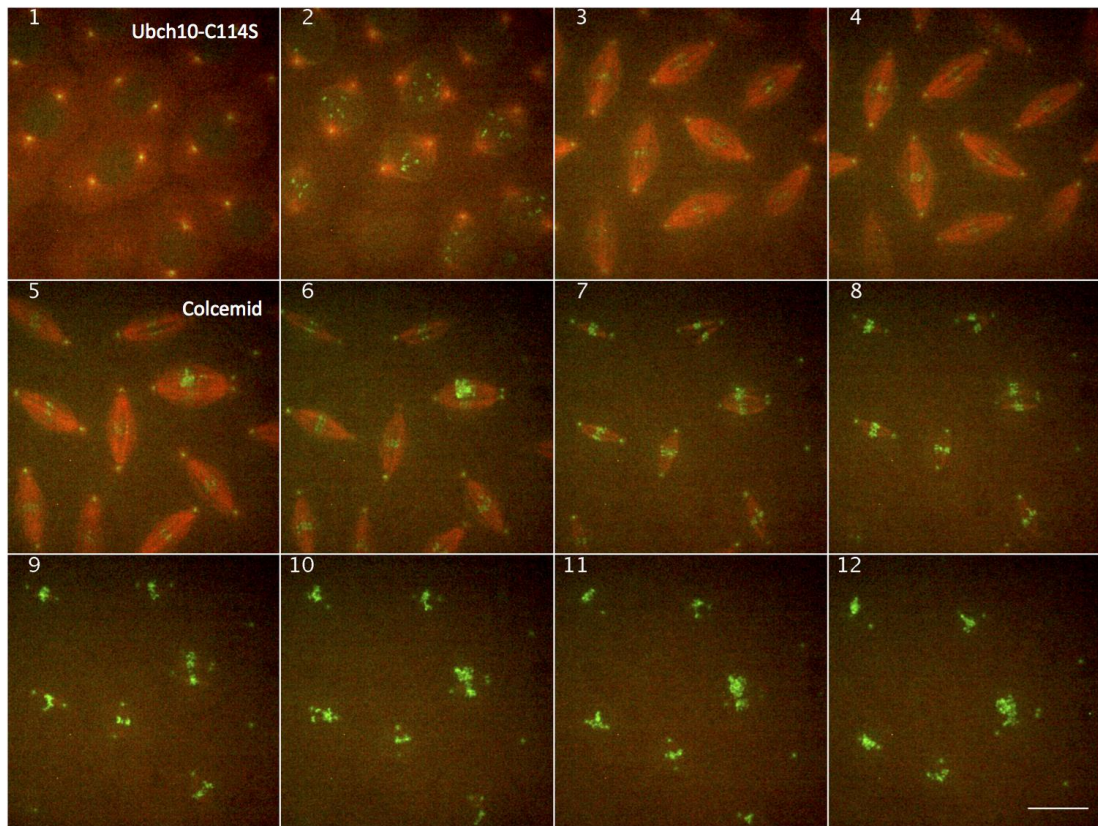


Figure 5.16. Levels of Polo-GFP increase when the SAC is reactivated

Ubch10^{C114S} was injected to cause a stable mitotic arrest. This is followed by injection of Colcemid that lead to MT depolymerisation and reactivation of SAC. Time series of Polo-GFP in cycle 12 syncytial embryos. 3 or more embryos were analysed. Scale bar 10µm.

5.7 The End of First Polo Drop Represents Complete Formation of Kinetochore-Microtubule Attachments

Previous experiments show that the first drop in the level of Polo is due to the process of silencing SAC. However, due to the use of Colcemid it was not possible to tease apart if the first Polo drop was due to KT occupancy or tension, both of which are achieved during process of SAC silencing. In order to address this, I investigated if the completion of two events, i.e. KT occupancy by the MTs and maximum tension on sister KTs, happen simultaneously or sequentially. In the former scenario, the KTs should split as soon as Mad2 levels disappear i.e. KT occupancy is complete⁶⁰⁵. In the latter scenario, the BubR1 should reach its minimum level

after complete loss of Mad2 and at this minimum BubR1 level the KT should split (i.e. KT should not split when Mad2 levels are lost). This and the following section establish if the two events are simultaneous or sequential.

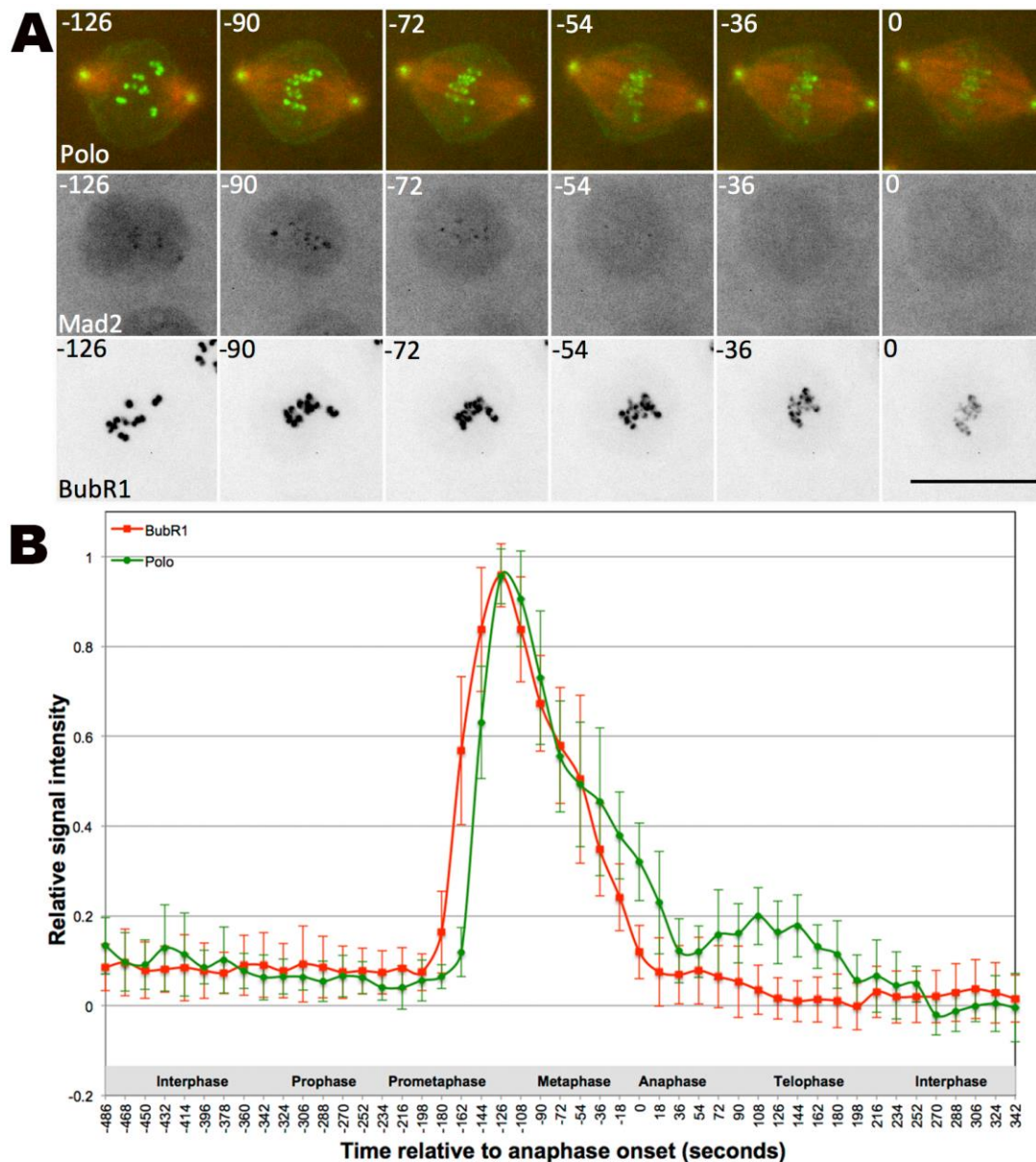


Figure 5.17. Comparing the dynamics of GFP tagged Polo, BubR1 and Mad2

A. Stills from live cell imaging of Polo-GFP; mRFP-Tubulin, GFP-Mad2 and GFP-BubR1. B. Plot of relative Polo-GFP and GFP-BubR1 signal intensity as a function of time. GFP-Mad2 could not be accurately quantified due to very high background. At time point -54s levels of Mad2 almost disappear from the KT as compared to

levels of Polo and BubR1 suggesting that the KT's are completely occupied by the MT's i.e. spindle attachment checkpoint is satisfied. Scale bar 10 μ m.

Fig. 5.17 shows that at time point -54s, KT Mad2 disappeared establishing that KT's are completely occupied by the MT's. Similarly, at -54s level of BubR1 and Polo were reduced compared to their peak levels at -126s. These observations show that both Polo and BubR1 can sense the KT occupancy similar to Mad2 (the difference being Mad2 completely disappears as opposed to a partial decrease in Polo and BubR1). The results are in agreement with outcome of the mathematical analysis done in previous chapter that estimated the first drop of Polo (or the first biological event) to end at -72s. However, there was a minor deviation in the mathematically estimated timepoint (-72s) and the experimentally observed value (-54s). This difference can be improved by higher time resolution imaging. However, higher resolution imaging can cause photobleaching that may impact dynamics and ultimately the mathematical estimations. Thus, the first Polo drop (i.e. from -126s to -54s) is likely due to the process of forming KMT attachments that is completed at -54s.

5.8 The End of Second Polo Drop Likely Represents Silenced Spindle Assembly Checkpoint

For the SAC satisfaction, in addition to complete KT occupancy by the MT's, the sister KT's must also be bi-oriented⁶⁰⁵. AO initiates when both the attachment and tension requirements are satisfied. BubR1 is bonafied marker of satisfied SAC as levels of BubR1 are a minimum when SAC is turned off^{112,605}. As observed KT's did not split at -54s when KT occupancy was complete but at 0s (fig. 5.17A). The levels of Polo and BubR1 further reduce after -54s reaching the minimum levels when the KT's split at 0s (fig. 5.17). These results show that in syncytial embryos there is a time gap of approximately 54s between completion of two

events i.e. KT occupancy and achieving maximum tension on sister KTs (when KTs split). Thus, the two events end sequentially rather simultaneously. These observations suggest that Polo can be used as a marker for sensing silenced SAC just like BubR1, as its level reach minimum levels like BubR1 when maximum tension on sister KTs is achieved and SAC is satisfied. I further wanted to ascertain if the time point at which BubR1 and Polo reaches minimum level (when SAC is satisfied) corresponds to time point at which the sister KTs split. Hence, I designed an experiment to compare levels of MAT arrested and SAC silenced BubR1 and Polo with their levels in wild type embryos at time point when the sister KTs split (AO). I quantified and plotted the intensities of wild type and UbCH10^{C114S} injected GFP-BubR1 and GFP-Polo embryos (fig. 5.18 and 5.11). The GFP-BubR1 intensities in fig 5.18 are aligned to the time point of maximum increase in loading kinetics (maximum change in dy/dx) instead of AO (which in other plots was marked as time point 0s). Images of syncytial embryo expressing GFP-BubR1 in cycle 12 after injecting UbCH10^{C114S} are also shown in fig. 5.18. The intensity plot in fig. 5.18 was remapped and aligned to AO (fig. 5.19), as the time interval between NEBD and AO was highly reproducible. Fig. 5.19 shows that the minimum levels of both BubR1 and Polo correspond to the time point at which the sister KTs split (i.e. completion of obtaining maximum tension on sister KT at 0s) in wild type embryos. The observed lowest intensities of both Polo and BubR1 are at the end of second drop of Polo and BubR1. Thus, the second drop of BubR1 and Polo i.e. -54s till 0s, represents the process of achieving maximum tension on sister KTs. The maximum tension of sister KTs is achieved at the end of second drop of Polo and BubR1 at time point 0s. These results are in agreement with the mathematical estimation done in previous chapter that a second biological event governs the unloading dynamics of Polo i.e. -72s till 0s. As discussed earlier, the deviation in mathematically

estimated time point -72s and observed time point of -54s is due to potential noise. These reduced levels of Polo and BubR1 observed at time point 0s upon injection of UbcH10^{C114S}, are henceforth also referred as “residual”.

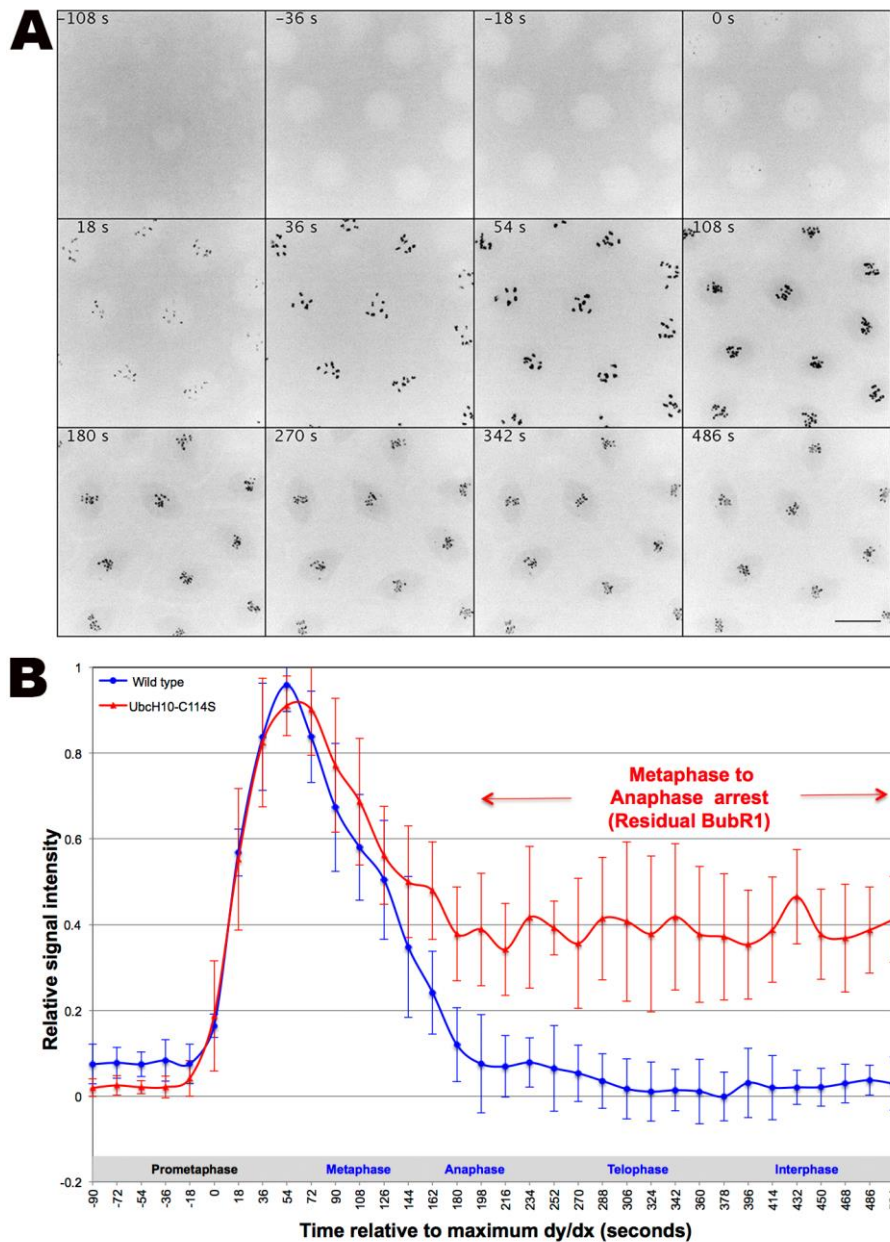


Figure 5.18. Comparison of the dynamics wild type and UbcH10C114S injected GFP-BubR1
A. Images of GFP-BubR1 syncytial embryo cycle 12 after injecting UbcH10^{C114S}. 30mg/ml of UbcH10^{C114S} was injected at time point -108s that leads to a mitotic arrest. B. Plot of normalised GFP-BubR1 intensities as a function of time. The data is aligned on the X-axis with reference to maximum change in slope between any

two-time points i.e. maximum dy/dx . Dynamics of wild type GFP-BubR1 is shown in blue and dynamics of GFP-BubR1 after injecting UbcH10^{C114S} are shown in red. Scale bar 10 μ m.

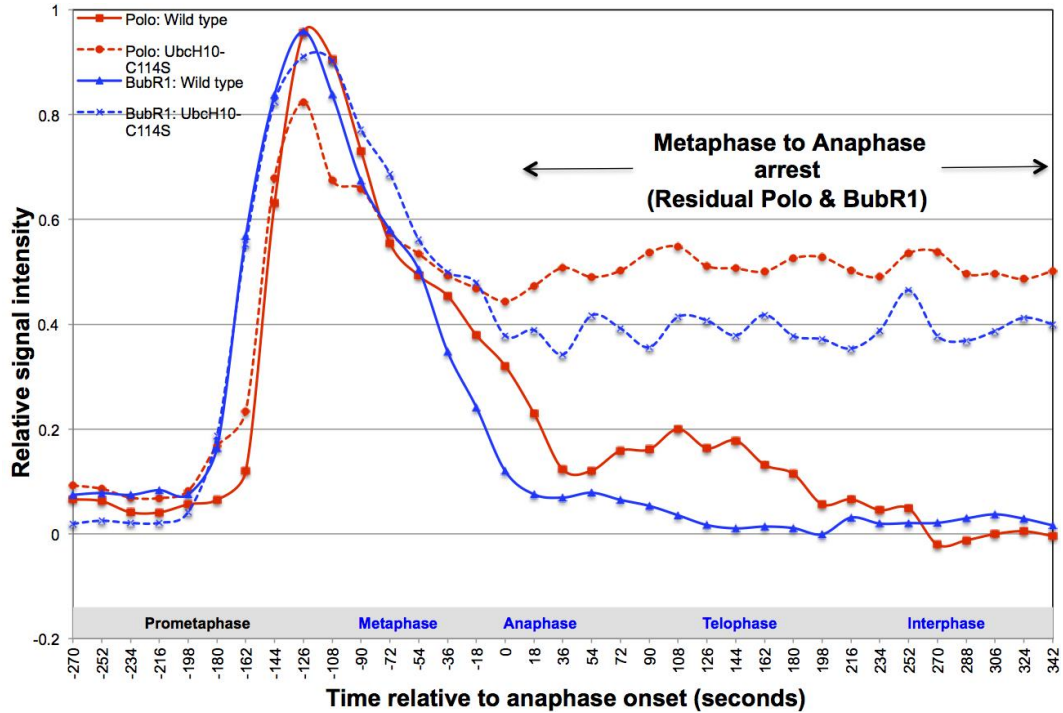


Figure 5.19. Dynamics of wild type and UbcH10C114S injected Polo and BubR1
Dynamics of wild type Polo-GFP, Polo-GFP injected with UbcH10^{C114S}, Wild type GFP-BubR1 and BubR1-GFP injected with UbcH10^{C114S} are shown in red, dashed red, blue and dashed blue respectively.

5.9 Summary

In this chapter, I have experimentally verified and characterised the biological events predicted by mathematical analysis. The monophasic loading dynamics of Polo is dependent on its own activity and activation by AurB. The biphasic Polo drop is shaped by two different events. The first Polo drop i.e. -126s to -54s is a) independent of destruction box dependant Polo degradation b) does not depend on degradation of prometaphase and metaphase APC (i.e. APC^{Cdc20}) substrates like CycB (and hence independent of Cdk1-CycB activity) c) independent of degradation of all APC (i.e. APC^{Cdc20} and APC^{Cdh1}) substrates covered by ubiquitin mediated destruction pathway. The first drop of Polo (-126s to -54s) is due to the process of KT

occupancy by MTs which is completed at -54s marked by absence of Mad2 and reduced levels of BubR1 and Polo. However, the KT's do not split at time point -54s and there is a further drop in level of BubR1 and Polo till the sister KT's split at AO i.e. at 0s. The second drop of Polo from -54s till 0s due to the process of generating tension which is maximum and completed at time point 0s i.e. AO. These observations are aligned to the estimated outcomes from mathematical analysis. An interesting observation is that Polo is not completely removed (like Mad2) when KT occupancy is complete at time point -54s. A partial pool of KT Polo remains. One of the explanations for the partial loss of Polo can be that a certain pool of Polo is removed from the KT's (directly or indirectly) as its occupancy increases. However, there is one more possible explanation. It is known that Mad2 localisation depends on Mad1. Bub1-Mad1 interaction serves as a receptor for Mad2 and the former interaction is enabled by Mps1 phosphorylation of Bub1²³⁹. An alternate hypothesis would be that as the distance between the kinases like AurB and Mps1 and their substrates like Polo and Bub1 increase due to formation of amphitelic attachments, the kinases are no longer able to phosphorylate their substrates which is required for their optimal localisation. This hypothesis also supports the importance of intra-KT stretch in SAC signalling^{157,158}.

In the following chapter, it is investigated if Polo can sense tension when the KMT attachments are not severed, the localisation dependency of residual Polo and its function.

Chapter 6 Investigating the Dynamics of Residual Polo

6.1 Introduction

The second drop of Polo and BubR1 starts from -54s (i.e. when KT occupancy is complete) and ends at 0s when maximum tension on sister KTs is established, SAC is satisfied and KTs split (AO). The second Polo drop is due to the process of generating maximum tension on the sister KTs. The observations in Chapter 4, show that Polo responds to aberrant KMT attachment by showing a surge in its levels and sensing tension by reaching minimum levels when sister KTs are under maximum tension. However, it was not clearly established if Polo can be used as a tension sensor like BubR1. Furthermore, the function and localisation dependency of residual Polo is investigated in this chapter.

The scope of this chapter is to investigate the following questions: 1) What does the localisation of residual Polo depend on? To answer this question, I specifically wanted to investigate if Cdk1-CycB activity is involved in localisation of residual Polo. 2) What is the function of residual Polo? To answer this question, I specifically wanted to investigate the role of residual Polo in maintaining amphitelic KMT attachments after SAC satisfaction. 3) Does Polo act as tension sensor like BubR1? To answer this question, I specifically wanted to investigate if Polo levels would increase when tension on amphitelic attachments is lost after SAC satisfaction without breaking attachments.

6.2 Localisation of Residual Polo Depends on Cdk1-CyclinB Activity.

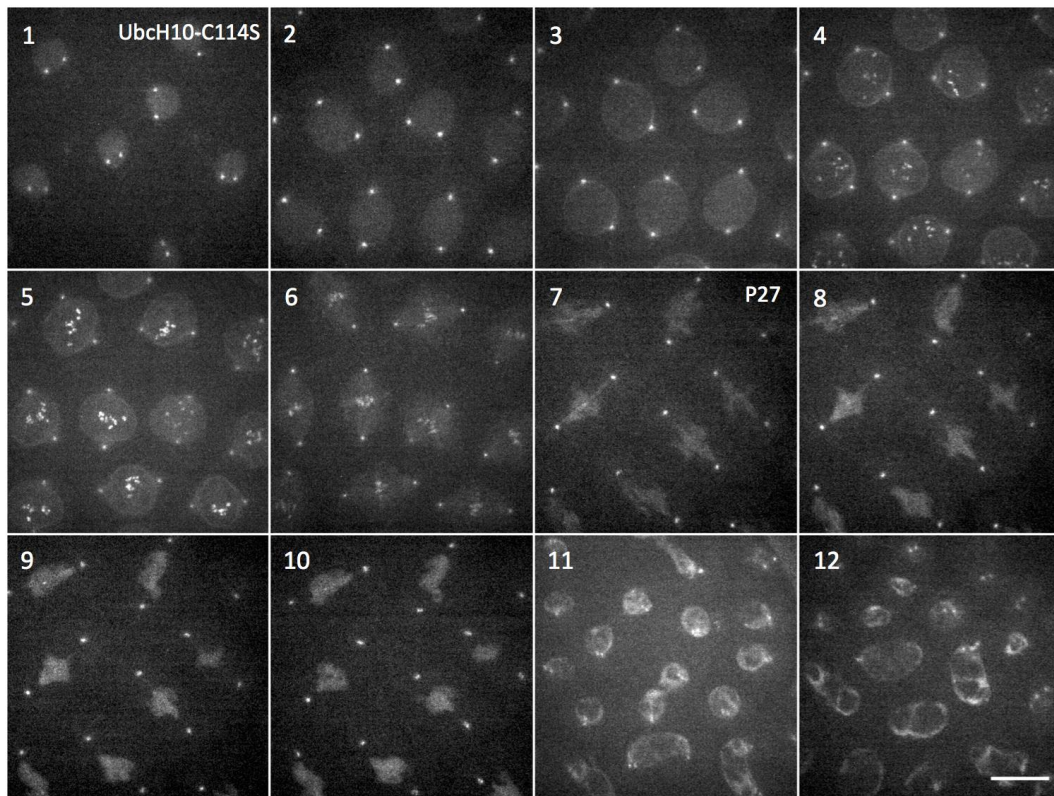


Figure 6.1. Localisation of residual Polo depends upon Cdk1-CyclinB activity

Still images from live imaging of Polo-GFP syncytial embryo in cycle 12s. Mitotic arrest in metaphase to anaphase transition caused by injection of 30mg/ml UbchH10^{C114S} (image 6). Injection of 1.5mg/ml P27 (Cdk1 inhibitor) leads to the loss of residual Polo at the kinetochores (image 7-10). Artificial inhibition of Cdk1 activity by p27 leads to premature exit from mitosis with chromosome segregation errors (image 11-12). Scale bar 10µm

UbchH10^{C114S} prevents the downregulation of Cdk1-CycB activity leading to arrest in MAT¹¹². To investigate if Cdk1-CycB activity is required for the localisation of residual Polo, 30mg/ml UbchH10^{C114S} was first injected into Polo-GFP syncytial Polo causing MAT arrest (as shown in fig. 6.1 image 1-6) and once the stable residual levels of Polo were achieved 1.5mg/ml of p27 (Cdk1 inhibitor) was injected in the same embryo (fig. 6.1 image 7). Purified Human p27 protein was a gift from Dr. Raquel Oliveira and is the only potent inhibitor of Cdk1 in *D. melanogaster* syncytial embryos¹¹². KT associated residual Polo-GFP was completely lost on

p27 injection and the nuclei showed a premature exit from mitosis (fig. 6.1 image 11-12). The premature exit happens because Cdk1-CycB activity is artificially inhibited while the Cohesin rings are not cleaved. This leads to CS errors represented by chromosome aggregates and bridges (fig. 6.1 image 11-12). These results show that the localisation of residual Polo depends on Cdk1-CycB activity.

6.3 Residual Polo Prevents Reactivation of Spindle Assembly Checkpoint by Maintaining Kinetochore-Microtubule Attachments

It has been reported that in Plk1 depleted cells, the mitotic spindle cannot generate enough tension on the sister kinetochores to stabilise KMT interactions and hence unable to silence the SAC⁴⁶⁷. Studies have also shown that inhibiting Plk1 activity by BI2536 prevents the formation of KT fibres⁴⁶⁵. However, the function of residual Polo once the amphitelic attachments are established and after SAC satisfaction was not investigated in syncytial embryos.

To address this, 30mg/ml of UbcH10^{C114S} was first injected into the embryo leading to MAT arrest with silenced SAC (fig. 6.2 image 1-4) followed by a second injection of 25µM BI2536 in same embryo (fig. 6.2 image 5) to inhibit the residual KT Polo activity. Injecting BI2536 caused the stable amphitelic KMT attachments to break letting the KTs free (fig. 6.2 image 9-12). This result shows that the active residual Polo is required to: a) maintain the already established amphitelic KMT attachments after SAC satisfaction, thus preventing SAC reactivation b) faithful CS.

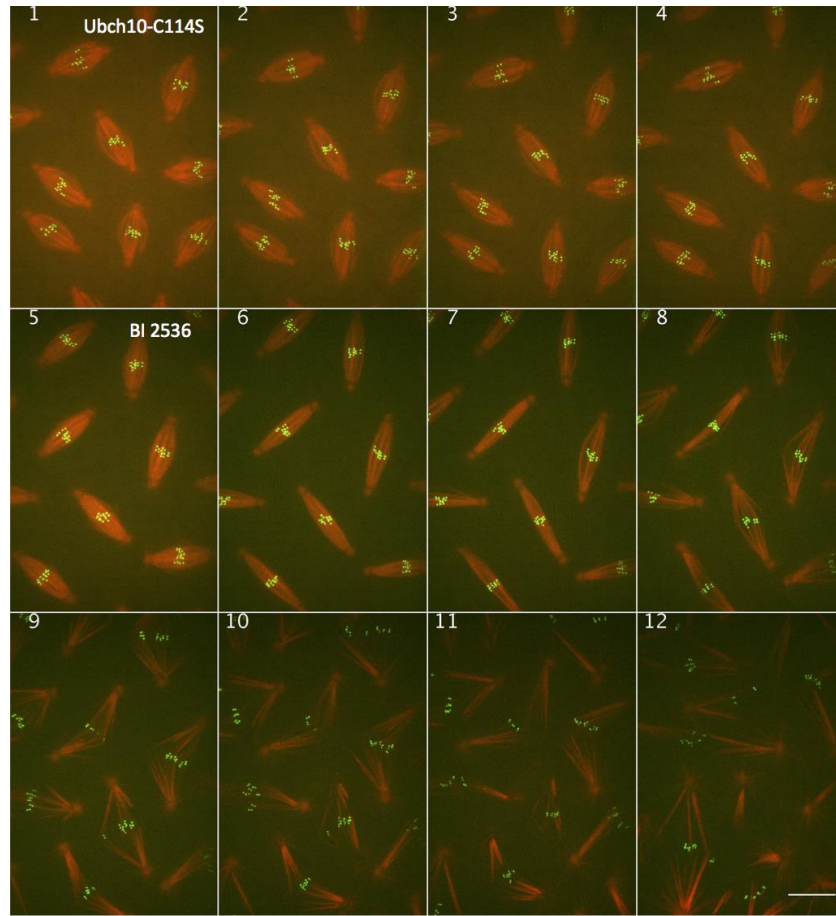


Figure 6.2. Residual Polo maintains amphitelic KMT attachments and prevents reactivation of SAC
 Images from live imaging of syncytial embryo in cycle 12 expressing GFP-CID and Tubulin mRFP. Injection of 30mg/ml Ubch10^{C114S} caused an arrest in metaphase to anaphase transition (image 1-4). MT-KT attachments were broken once 25μM BI2536 was injected. Scale bar 10μm.

6.4 Polo Acts as Tension Sensor

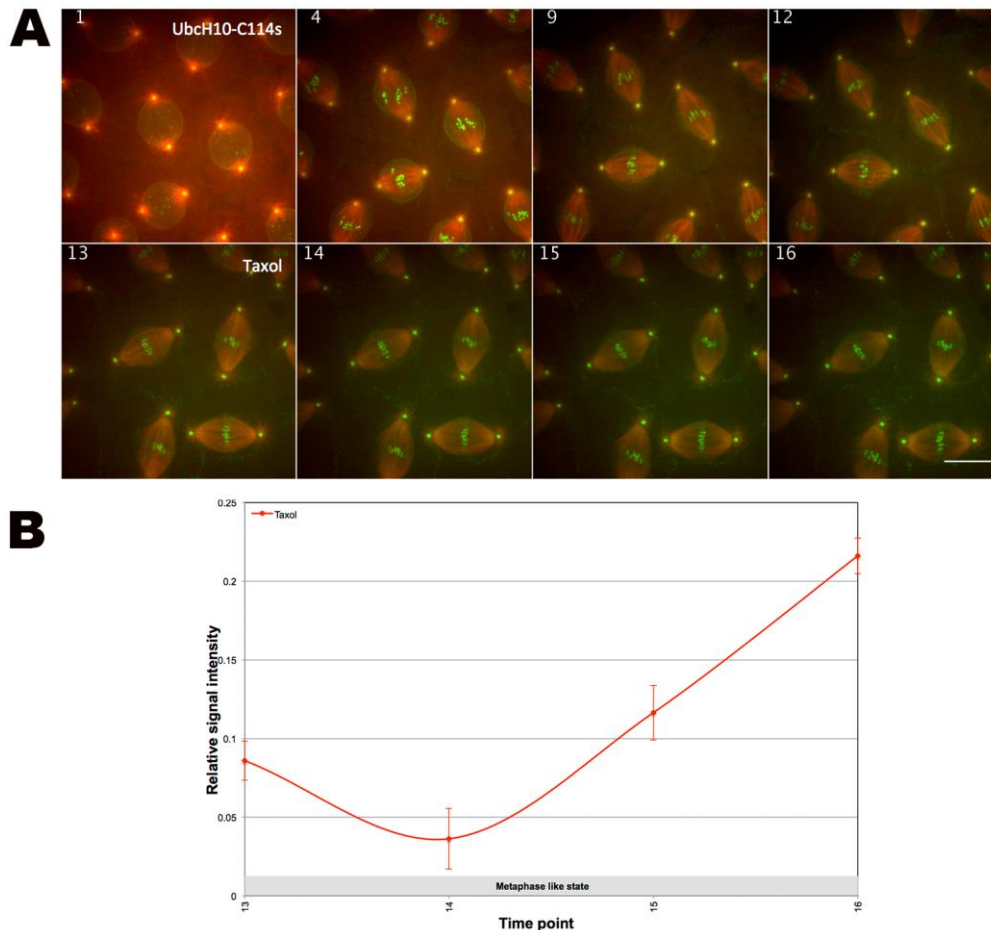


Figure 6.3. Levels of Polo-GFP increase with decrease in KMT tension

A. Images of Polo-GFP and Tubulin mRFP cycle 12 syncytial embryo when injected with 30mg/ml UbCH10^{C114S} (image 1-12) and after injection of 250µM Taxol (image 13-16) B. Plot of Polo-GFP signal intensity as a function of time after Taxol injection (image 13-16). Scale bar 10µm.

Experiments discussed earlier did not conclusively establish if Polo can sense tension like BubR1 i.e. does the level of Polo increase if the tension on amphitelic attachments is lost without breaking the attachments. To address this double tagged Polo-GFP and mRFP-Tubulin syncytial embryos were first injected with 30mg/ml of UbCH10^{C114S} leading to MAT arrest with amphitelic attachments. This was followed by injecting 250µM of Taxol as it stabilises the MTs leading to loss of tension on the KTs^{606–608}. Taxol is not known to break KMT attachments as Mad2 does not localises at KT in Taxol treated PtK1 cells⁶⁰⁶. Injection of UbCH10^{C114S} lead

to a MAT arrest as observed in fig. 6.3A image 1-12. This was followed by a second injection of Taxol to stabilise the MTs (fig. 6.3A image 13-16). In fig. 6.3 and 6.4 the start of frame number 13 represents time point 0s as Taxol was injected at this time point and end of frame 16 represents timepoint 72s as each frame is 18s apart. Polo-GFP signals were quantified and plotted as a function of time (fig. 6.3B). The results show that the levels of Polo-GFP at the KTs more than doubled.

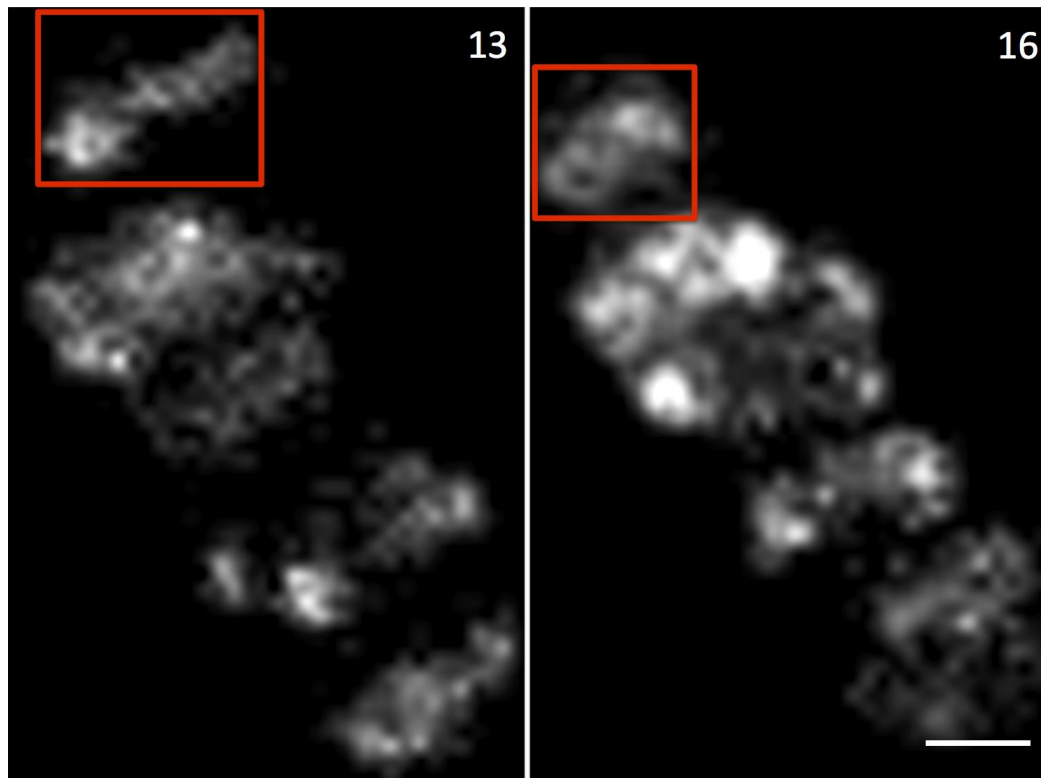


Figure 6.4. Distance between sister kinetochores is reduced when tension is lost after Taxol injection
Image 13 represents the first time point after Taxol injection. Image 16 represents 72s after Taxol injection in Polo-GFP syncytial embryo. Scale bar 100nm

Injection of Taxol leads to stabilised MTs causing a loss of tension on the KTs⁶⁰⁶. If this is the case then the metaphase plates should become more compact, as the MTs are no longer able to pull the KTs in opposite direction. Polo-GFP has a smear of protein that marks the width between two sister kinetochores. On comparison of the compactness of the metaphase

plate immediately after Taxol injection (fig. 5.4 image 13) and after 72s (fig. 5.4 image 16) it was observed that distance between the sister kinetochores was reduced and the metaphase plate was more compact.

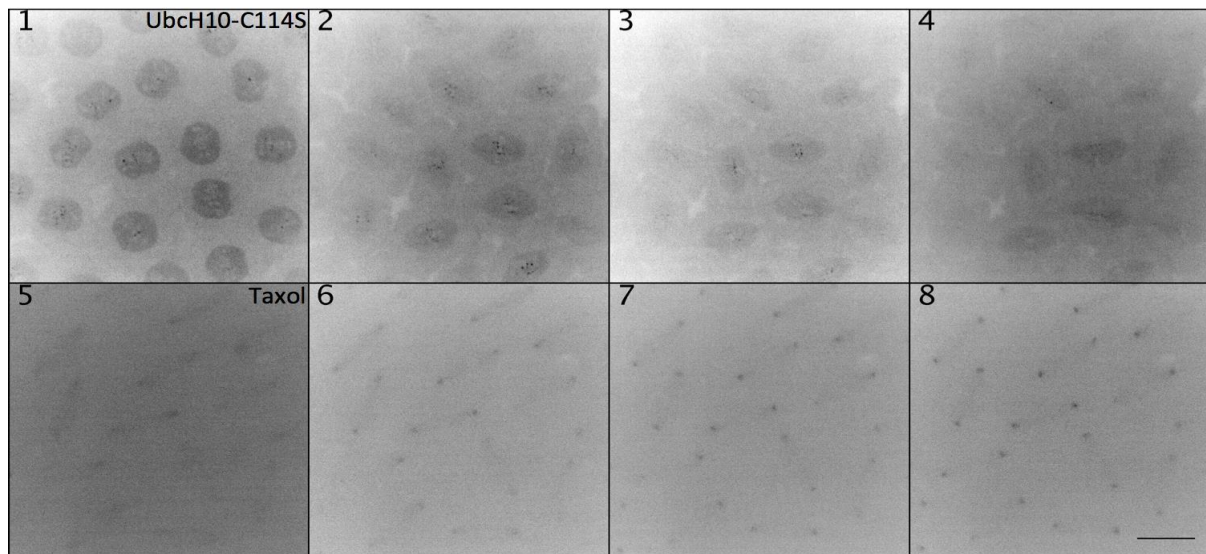


Figure 6.5. Injection of Taxol does not lead to impaired MT-KT attachments

Images of GFP-Mad2 cycle 12 syncytial embryos. 30mg/ml UbcH10^{C114S} was injected at Image 1. There is a complete disappearance of Mad2 levels at the KT's after the mitotic arrest caused by injecting UbcH10^{C114S} (image 4). Injecting 250μM Taxol (image 5) does not cause the levels of KT Mad2 to increase (Images 5-8). Scale bar 10μm.

However, the effect of Taxol in *D. melanogaster* syncytial embryos in double microinjection experiment has not been studied. Hence, it was crucial to observe the behaviour of Mad2, to check if injecting 250μM Taxol breaks KMT attachments. If this concentration of Taxol does not impair the KMT attachments but only causes the loss in tension, then levels of BubR1 should rise and the levels of Mad2 should not increase. These controls would help to establish if Polo indeed senses loss of tension like BubR1. To ascertain that injection of Taxol does not lead to impaired KMT attachments the above experiment was repeated using GFP-Mad2. As expected, injection of UbcH10^{C114S} lead to an arrest in MAT with complete disappearance of GFP-Mad2 at the KT's (fig. 6.6. image 1-4). After stable mitotic arrest 250μM of Taxol was injected in the same embryo (fig. 6.5 image 5). There was no increase in the levels

of Mad2 (fig 6.5 image 5-8) establishing that 250 μ M of Taxol does not impair KMT attachments. The start of frame 5 in fig. 6.5 represents time point 0s as Taxol was injected at this timepoint and end of frame 8 represents 72s as each frame is separated by 18s.

I performed the above described experiment on GFP-BubR1 syncytial embryos. After injecting UbCH10^{C114S} followed by Taxol injection in the same embryo. The intensity of GFP-BubR1 was quantified and plotted as a function of time (fig. 6.6 image 1-4). The start of frame 1 in fig. 6.6 represents time point 0s as Taxol was injected at this timepoint and end of frame 4 represents 72s as each frame is separated by 18s. The levels of BubR1 surged indicating the tension on the KTs was lost even in the presence of stable KMT attachments. These experiments show that Polo acts as a tension sensor like BubR1

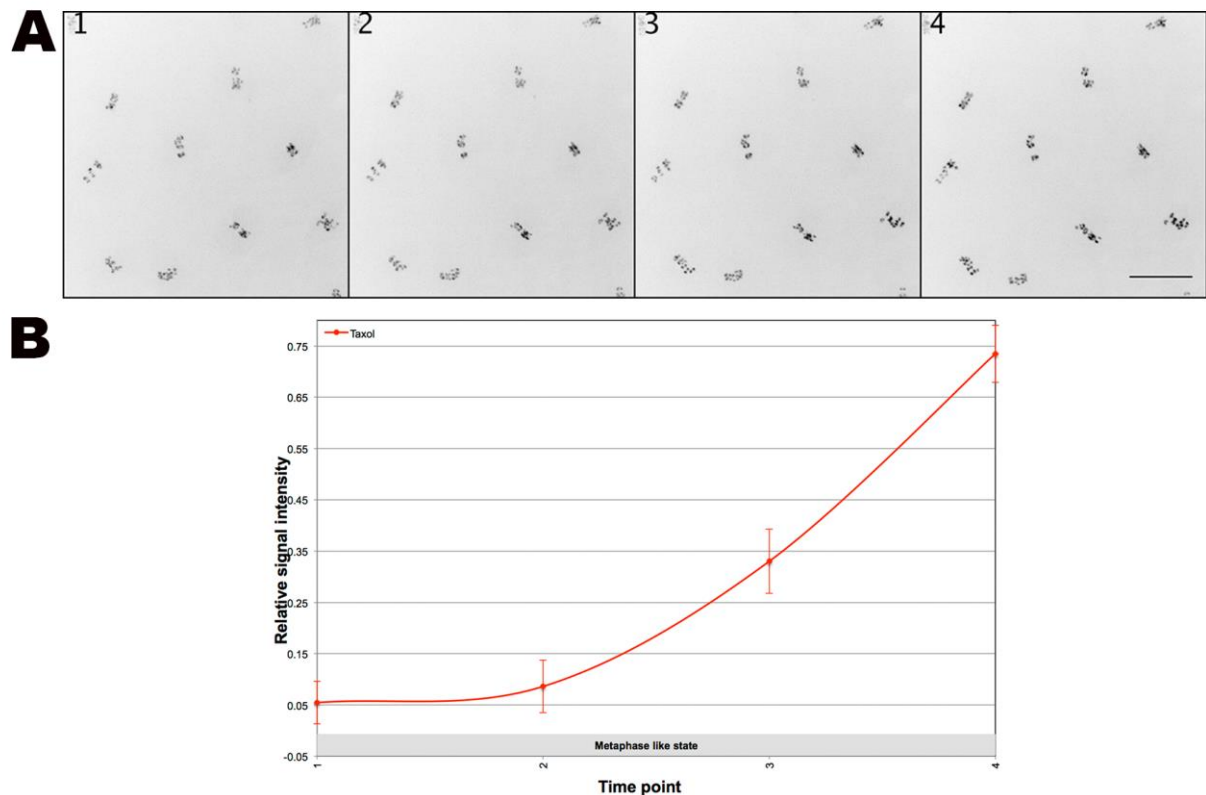


Figure 6.6. Levels of BubR1 increase in response to loss of tension even when the MT-KT attachments are maintained

A. Images of GFP-BubR1 cycle 12 syncytial embryo. 30mg/ml UbCH10^{C114S} was injected prior to time point 1 to cause a stable mitotic arrest with reduced levels of BubR1. Injecting 250μm Taxol at time point 1 polymerises the MTs causing a loss of tension on the KT's. B. Levels of BubR1 rise in response to loss of tension. Scale bar 10μm.

6.5 Summary

It was observed from previous chapters that stable levels of residual Polo were obtained at -54s when injected with UbCH10^{C114S} at MAT arrest with satisfied SAC. In this chapter it was established, using p27, that localisation of residual Polo depends on Cdk1 activity. Using double microinjection experiments (UbCH10^{C114S} and BI2536) it was shown that residual Polo enables maintenance of stable amphitelic attachments after SAC satisfaction to allow for error free CS and preventing reactivation of SAC. Although, it was shown that Polo can sense tension when there KMT attachments were damaged (by showing a surge) and when SAC was satisfied

(by reaching minimum levels), it was not established if Polo levels would increase if the tension on amphitelic KMT attachments is lost without breaking the attachments. Using UbcH10^{C114S} and Taxol double microinjection experiments it was established that Polo levels like BubR1 increase when tension on amphitelic attachments is lost without breaking the attachment. Thus, Polo can act as a tension sensor like BubR1.

Chapter 7 Modelling of Polo Dynamics

7.1 Model

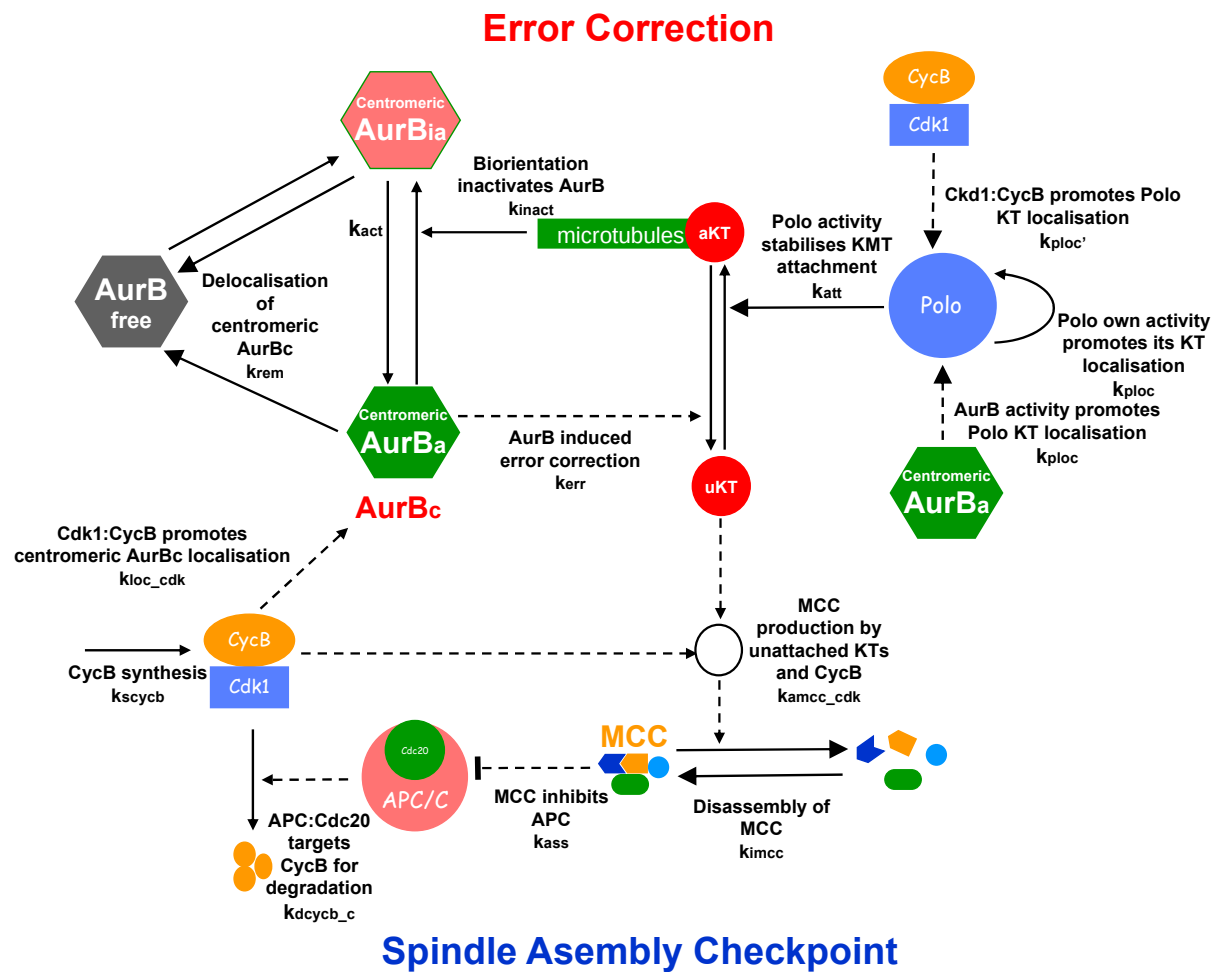


Figure 7.1. Wiring diagram of the model

The experimental results from previous chapters show that KT Polo recruitment is governed by AurB activity in addition to its own activity. Additionally, Cdk1-CycB promotes AurB centromeric localisation^{312,609} thus aiding Polo recruitment. To investigate how modulating Polo dynamics under different conditions affects its a) own KT recruitment b)

KMT attachments c) APC dynamics d) CycB dynamics and e) assembled MCC dynamics an initial version of the mathematical model is developed that quantitatively simulates the experimental data. The model is a modification of Mirkovic et. al⁶⁰⁹ model.

The model (fig. 7.1) consists of two modules namely, EC and SAC. The EC module describes the formation KMT attachments dependent on Polo and CycB activity; while the destabilisation of KMT attachments is dependent on centromeric AurB activity that is attenuated by KT tension with mitotic progression. The SAC module describes APC dependant CycB degradation and its inhibition by MCC that is produced by unattached KTs. In this dual feedback model, it is assumed that EC and SAC module mutually activate each other via Cdk1-CycB dependency in activating AurB and AurB stabilising CycB via SAC.

7.2 Model Construction

Fig. 7.1 shows the qualitative interactions that underly the EC and the SAC modules. The interactions were converted into reaction rate equations that formed a system of coupled ordinary differential equations (ODEs). The ODEs were parametrised to reproduce the normal mitotic timings and Polo dynamics under control condition. I then compared the simulations of mitotic cells under different conditions with the corresponding experimental findings. The following part of this section discusses the detailed assumptions that informed the construction of the model and development of the ODE. The model can be simulated by running the “.ode” file in appendix with the freely available software package XPPAUT (<http://www.math.pitt.edu/~bard/xpp/xpp.html>)⁶¹⁰. The model was implemented using a stiff method for solving ODE. The initial conditions of the model assumed that no MTs were

attached, absence of APC activity as its bound in a complex with MCC, non-significant centromeric AurB and KT Polo localisation with high Cdk1-CycB activity. The start of the stimulations corresponds to NEBD when the mitotic spindle starts to capture the KTs, APC is activated by Cdk1-CycB activity.

7.2.1 Anaphase Promoting Complex or Cyclosome

The total cellular APC level $[APC_T]$ is assumed to be a constant and set to 1. $[APC_T]$ composed of active, free APC i.e. $[APC_{free}] = [APC]$ and inactive, MCC bound APC i.e. $[APCMCC]$ forms (equation 7.1). The level of $[APC]$ decreases when it binds rapidly to $[MCC_{free}]$ to form $[APCMCC]$ and increases when $[APCMCC]$ disassociates and disassembles (equation 7.2 and 7.3)⁶¹¹.

$$[APCMCC] = [APC_T] - [APC_{free}] = 1 - [APC] \text{ Equation 7.1}$$

$$\frac{d[APC]}{dt} = (k_{diss} + k_{imcc}) \cdot [APCMCC] - k_{ass} \cdot [APC] \cdot [MCC_{free}] \text{ Equation 7.2}$$

Substituting for $[APCMCC]$ in equation 7.2 we get the following form:

$$\frac{d[APC]}{dt} = (k_{diss} + k_{imcc}) \cdot (1 - APC) - k_{ass} \cdot [APC] \cdot [MCC_{free}] \text{ Equation 7.3}$$

The association and dissociation rate constants are ($k_{ass} = 5\text{sec}^{-1}$) and ($k_{diss} = 0.001\text{sec}^{-1}$) respectively as MCC binds tightly to APC⁶¹² while the disassembly $[APCMCC]$ by MCC inactivation is $k_{imcc} = 0.035\text{sec}^{-1}$. At the start of mitosis, as all the APC is bound to MCC there is very little free MCC available and hence the initial conditions of $[APC] = 0$ and $[MCC_{free}] = 0.1$.

7.2.2 Mitotic Checkpoint Complex

The total MCC i.e. $[MCC_T]$ is the sum of assembled $[MCC_{asmb}]$ and free MCC subunits not yet assembled i.e. $[preMCC]$ (equation 7.4, 7.6, 7.7 and 7.8) and is assumed to be in excess of $[APC_T]$ i.e. 1.2 as it is a requirement for stoichiometric APC inhibition⁶¹³. $[MCC_{asmb}]$ is present in two forms namely a) $[APCMCC]$ b) $[MCC_{free}]$ (equation 7.5). $[MCC_{free}]$ is produced by unattached KTs i.e. $[uKT]$ from pool of $[preMCC]$ at a rate proportional to the level of limiting component of $[preMCC]$, Cdk1-CycB activity i.e. $[CycB]$ ($k_{amcc,cdk} = 0.25\text{sec}^{-1}$) (equation 7.9 and 7.10) and . The level of $[MCC_{free}]$ increases when $[APCMCC]$ dissociates ($k_{diss} = 0.001\text{sec}^{-1}$) and decreases when it associates with $[APC]$ ($k_{ass} = 5\text{sec}^{-1}$) and when the $[MCC_{free}]$ disassembles ($k_{imcc} = 0.035\text{sec}^{-1}$) (equation 7.9 and 7.10).

$$[MCC_T] = [MCC_{asmb}] + [preMCC] \text{ Equation 7.4}$$

$$[MCC_{asmb}] = [APCMCC] + [MCC_{free}] \text{ Equation 7.5}$$

Rearranging equation 7.4 and substituting for $[MCC_{asmb}]$ we get equations 7.6 and 7.7 respectively.

$$[preMCC] = [MCC_T] - [MCC_{asmb}] \text{ Equation 7.6}$$

$$[preMCC] = [MCC_T] - [MCC_{free}] - ([APC_T] - [APC]) \text{ Equation 7.7}$$

Substituting for $[preMCC]$ and $[APC_T] = 1$ equation 7.6 we get the following form:

$$[preMCC] = [MCC_T] - [MCC_{free}] - (1 - [APC]) \text{ Equation 7.8}$$

$$\begin{aligned} \frac{d[MCC_{free}]}{dt} = & (k_{amc,cdk} \cdot [CycB]) \cdot uKT \cdot [preMCC] + k_{diss}[APCMCC] - k_{ass} \cdot [APC] \\ & \cdot [MCC_{free}] - k_{imcc} \cdot [MCC_{free}] \text{ Equation 7.9} \end{aligned}$$

Substituting for $[preMCC]$ and $[APCMCC]$ in equation 7.9 we get the following form:

$$\begin{aligned} \frac{d[MCC_{free}]}{dt} = & (K_{amc,cdk} \cdot [CycB]) \cdot uKT \cdot ([MCC_T] - [MCC_{free}] - (1 - [APC])) + k_{diss} \\ & \cdot (1 - [APC]) - k_{ass} \cdot [APC] \cdot [MCC_{free}] - k_{imcc} \\ & \cdot [MCC_{free}] \text{ Equation 7.10} \end{aligned}$$

7.2.3 Kinetochore-Microtubule Attachments

$[uKT]$ can be expressed as a sum of total KT's i.e. $[KT_T]$ (set to the value of 1) minus the attached KT's i.e. $[aKT]$ (equation 7.11). At the start of mitosis, all the KT's are unattached i.e. $[uKT] = 1$. As the mitosis progresses $[aKT]$ increases while the number of $[uKT]$ decreases. $[uKT]$ is increased as the EC mechanism of centromeric associated active AurB i.e. $[AurB_a]$ destabilises KMT attachments ($k_{err} = 0.05\text{sec}^{-1}$) (equation 7.12 and 7.13). While the KMT stabilisation by Polo i.e. $[Polo]$ reduces the number of $[uKT]$ with Polo dependant and independent rate constants ($k_{att} = 0.02\text{sec}^{-1}$ and $k_{att'} = 0.05\text{sec}^{-1}$) (equation 7.12 and 7.13). It is assumed that KT associated Polo is in its active form.

$$[uKT] = [KT_T] - [aKT] \text{ Equation 7.11}$$

$$\begin{aligned} \frac{d[uKT]}{dt} = & k_{err} \cdot \frac{AurB_a}{(1 + Inh_aur)} \cdot [aKT] - \left(k_{att'} + k_{att} \cdot \frac{[Polo]}{(1 + Inh_polo)} \right) \\ & \cdot [uKT] \text{ Equation 7.12} \end{aligned}$$

Substituting for $[aKT]$ in equation 7.11 we get the following form:

$$\frac{d[uKT]}{dt} = k_{err} \cdot \frac{AurBa}{(1 + Inh_{aur})} \cdot (KT_T - uKT) - \left(k_{att'} + k_{att} \cdot \frac{[Polo]}{(1 + Inh_{polo})} \right) \cdot uKT \text{ Equation 7.13}$$

In equation 7.13, Inh_{aur} and Inh_{polo} represents the concentration of AurB and Polo inhibitors (i.e. Binucleine2 and BI2536) respectively relative to its IC50 value.

7.2.4 Cyclin Dependant Kinase-1 and Cyclin-B

CycB is synthesised with a constant rate ($k_{scycb} = 0.00015\text{sec}^{-1}$) and its association with Cdk1 (present in excess) is rapid to form Cdk-CycB complex $[Cdk1CycB]$. Hence, $[Cdk1CycB]$ activity depends on levels of CycB i.e. $[Cdk1CycB] = [CycB]$ (equation 7.14). $[CycB]$ is inactivated by CycB degradation that has an APC independent rate constant ($k_{dcycb} = 0.00015\text{sec}^{-1}$) and APC dependant rate constant ($k_{dcycb} = 0.02\text{sec}^{-1}$) (equation 7.15). As the start of mitosis, $[CycB]$ activity is maximum represented by 1.

$$[Cdk1CycB] = [CycB] \text{ Equation 7.14}$$

$$\frac{d[Cdk1CycB]}{dt} = k_{scycb} - (k_{dcycb} + k_{dcycb} \cdot [APC]) \cdot [CycB] \text{ Equation 7.15}$$

Substituting for $[Cdk1CycB]$ in equation 7.15 we get the following:

$$\frac{d[CycB]}{dt} = k_{scycb} - (k_{dcycb} + k_{dcycb} \cdot [APC]) \cdot [CycB] \text{ Equation 7.16}$$

7.2.5 Aurora-B Localisation and Activity

The total AurB pool i.e. $[AurB_T]$ is composed of the centromeric (c) and non-centromeric AurB pool i.e. $[AurB_c]$ and $[Aurb_{nc}]$ respectively (equation 7.17) and assumed to be 1. The localisation of centromeric pool of AurB depends on $[CycB]$ ($k_{loc,cdk} = 0.25\text{sec}^{-1}$) while its dissociation follows first order kinetics ($k_{rem} = 0.04\text{sec}^{-1}$) (equation 7.18 and 7.19). During mitotic entry, there is no significant measurable centromeric AurB localisation i.e. $[AurB_c] = 0$.

$$[AurB_T] = [AurB_c] + [Aurb_{nc}] = 1 \text{ Equation 7.17}$$

$$\frac{d[AurB]_c}{dt} = (k_{loc,cdk} \cdot [CycB]^M) \cdot ([Aurb_{nc}]) - k_{rem} \cdot [AurB_c] \text{ Equation 7.18}$$

Substituting for $[Aurb_{nc}]$ in equation 7.18

$$\frac{d[AurB]_c}{dt} = (k_{loc,cdk} \cdot [CycB]) \cdot ([AurB_T] - [AurB]_c) - k_{rem} \cdot [AurB_c] \text{ Equation 7.19}$$

It was assumed that only a fraction (which is in its active form) i.e. $[AurB_a]$ of $[AurB_c]$ can cause the KMT destabilisation, via its EC activity. Thus $[AurB_c]$ is composed of $[AurB_a]$ and inactive form i.e. $[AurB_{ia}]$ (equation 7.20). It was assumed that recruited $[AurB_c]$ is in its inactive form and hence $[AurB_a] = 0$. As $[AurB_{ia}]$ converts to active form level of $[AurB_a]$ increases ($k_{act} = 0.025\text{sec}^{-1}$) (equation 7.21 and 7.22). While, $[AurB_a]$ level decreases ($k_{inact} = 2.5\text{sec}^{-1}$) with increase in $[aKT]$ and dissociates with first order kinetics ($k_{rem} = 0.04\text{sec}^{-1}$) (equation 7.21 and 7.22)

$$[AurB_c] = [AurB_a] + [AurB_{ia}] \text{ Equation 7.20}$$

$$\frac{d[AurB_a]}{dt} = k_{act} \cdot ([AurB_{ia}]) - k_{inct} \cdot ([AurB_a] \cdot [aKT]^N) - k_{rem} \cdot [AurB_c] \quad \text{Equation 7.21}$$

Substituting for $[AurB_{ia}]$ and $[aKT]$ in equation 7.20 we get the following form:

$$\frac{d[AurB_a]}{dt} = k_{act} \cdot ([AurB_c] - [AurB_a]) - k_{inct} \cdot ([AurB_a] \cdot [1 - uKT]^N) - k_{rem} \cdot [AurB_c] \quad \text{Equation 7.22}$$

7.2.6 Polo Localisation and Activity

The total Polo pool i.e. $[Polo_T]$ is composed of the centromeric Polo i.e. $[Polo_c] = [Polo]$ and non-centromeric Polo pool i.e. $[Polo_{nc}]$ (equation 7.23). The localisation of $[Polo]$ depends on $[CycB]$ ($k_{ploc'} = 0.015\text{sec}^{-1}$), $[AurB_a]$ and its own activity ($k_{ploc} = 1.5\text{sec}^{-1}$) (equation 7.24 and 7.25). While its dissociation follows first order kinetics ($k_{prem} = 0.015\text{sec}^{-1}$) (equation 7.24 and 7.25). At the start of mitosis there is no significant centromeric Polo i.e. $[Polo] = 0$.

$$[Polo_T] = [Polo_c] + [Polo_{nc}] = [Polo] + [Polo_{nc}] \quad \text{Equation 7.23}$$

$$\frac{d[Polo]}{dt} = \left(k_{ploc'} \cdot [CycB] + k_{ploc} \cdot \frac{[AurB_a]}{(1 + Inh_{aur})} \cdot \frac{[Polo]}{(1 + Inh_{polo})} \right) \cdot ([Polo_{nc}]) - k_{prem} \cdot [Polo] \quad \text{Equation 7.24}$$

Substituting for $[Polo_{nc}]$ in equation 7.24 we get the following form:

$$\frac{d[Polo]}{dt} = \left(k_{ploc'} \cdot [CycB] + k_{ploc} \cdot \frac{[AurB_a]}{(1 + Inh_{aur})} \cdot \frac{[Polo]}{(1 + Inh_{polo})} \right) \cdot ([Polo_T] - [Polo]) - k_{prem} \cdot [Polo] \quad \text{Equation 7.25}$$

In equation 7.24 and 7.25, Inh_{aur} and Inh_{polo} represents the concentration of AurB and Polo inhibitors (i.e. Binucleine2 and BI2536) respectively relative to its IC50 value.

7.3 Numerical Simulation of the Model

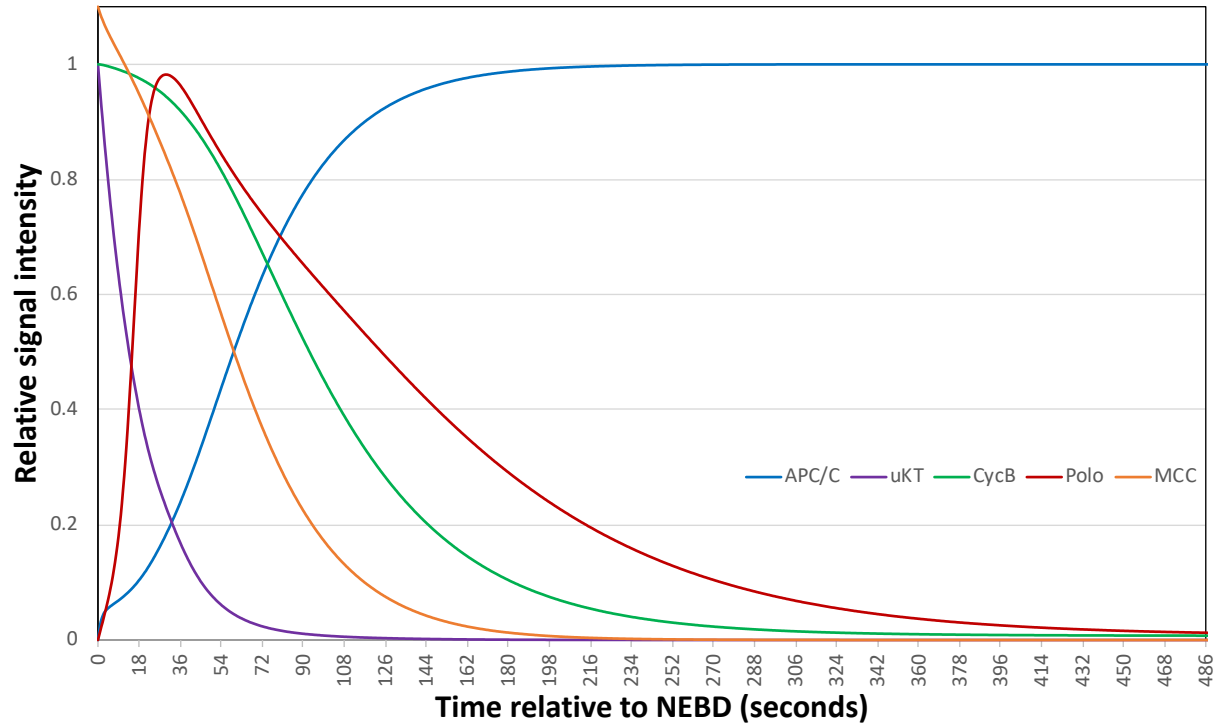


Figure 7.2. Simulation of normal mitosis

Fig. 7.2 shows the model simulation under normal mitotic conditions in syncytial embryos. Centromeric Polo (red) simulation corresponds well to the experimental data and mitotic timing (fig. 4.6). While the simulations of APC (in blue), uKT (in purple), CycB (green) and assembled MCC (in orange) (fig. 7.2) are as expected. As per the initial conditions during mitotic entry: a) uKTs and CycB activity is high (captured by setting $[uKT]$ and $[CycB]$

= 1 b) APC is inactive as it forms a complex with most of the freely available MCC (captured by setting $[APC] = 0$ and $[MCC_{free}] = 0.1$). As the mitosis progresses, the levels of uKT decreases as KMT attachments start to form. APC is activated which in then degrades CycB captured by sigmoidal curves of increasing APC and decreasing CycB that corresponds well to previously published results⁶⁰⁹. The mitotic timing and KMT attachments correspond to the experimental data.

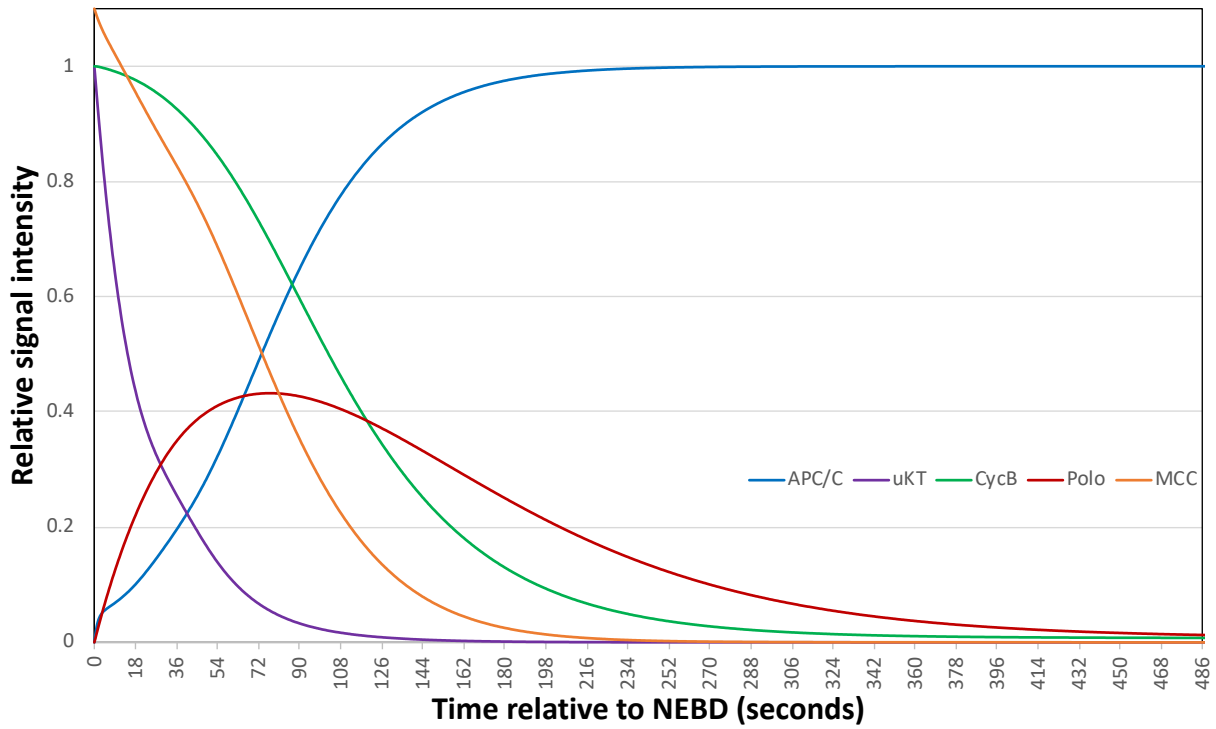


Figure 7.3. Simulation of Polo inhibition

As per the experimental data (fig. 5.1) Polo activity plays a vital role in KT Polo recruitment. Inhibiting Polo activity leads to partial Polo recruitment. This scenario is captured in the model simulation (fig. 7.3) by setting the parameter Inh_polo to 100 i.e. inhibiting the Polo activity. The simulated Polo levels are highly reduced. Furthermore, the uKT levels reduce slower i.e. KMT attachments take longer to form and stabilise due to inhibited Polo activity, as Polo plays a role in formation and maintaining KMT attachments^{600,614}.

The experimental data (fig. 5.2) shows that AurB activity enables KT Polo recruitment. Inhibiting AurB activity reduces optimal Polo recruitment. This scenario is captured in the model simulation (fig. 7.4) by setting the parameter `Inh_aurb` to 100 i.e. inhibiting the AurB activity. The simulated Polo levels are highly reduced. Additionally, the uKT levels reduce slightly faster i.e. KMT attachments happen quicker due to inhibited AurB activity, as AurB plays a role in destabilising erroneous KMT attachments^{600,614}.

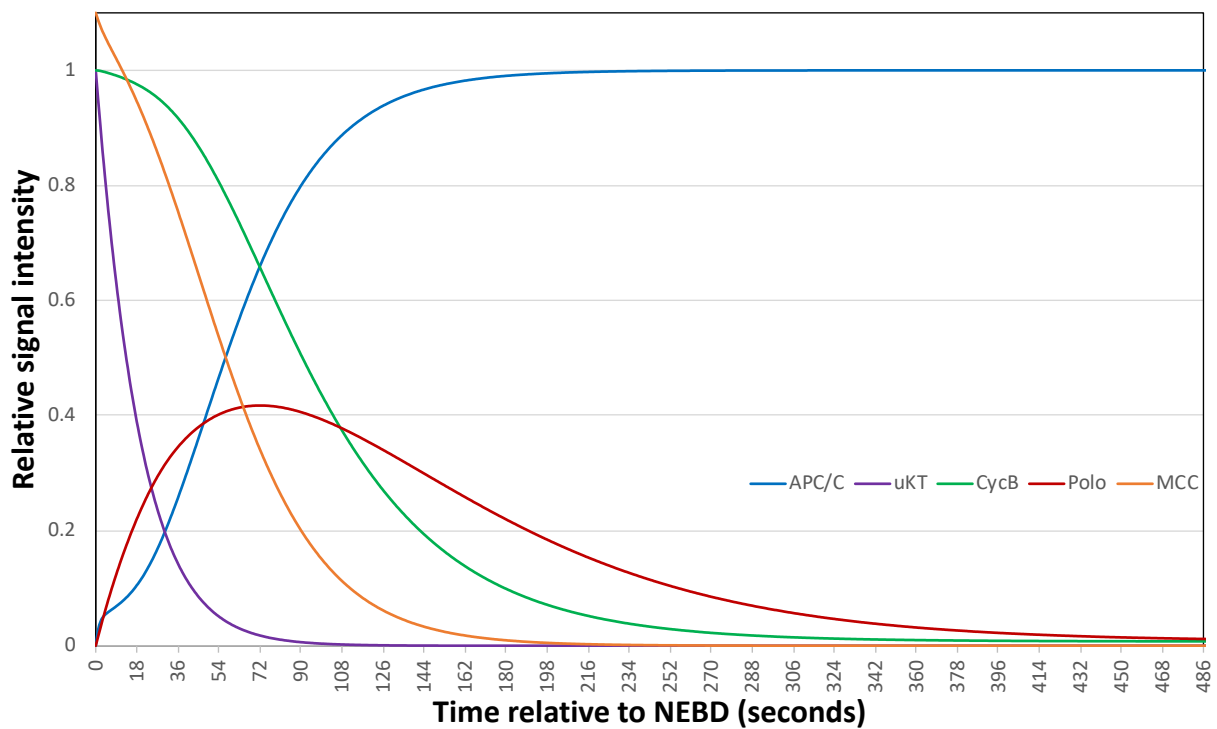


Figure 7.4. Simulation of Aurora-B inhibition

The experimental data (fig. 5.13) shows that injecting Colcemid in syncytial embryos prevents the first drop of Polo as it depolymerises the MTs preventing KMT attachments. The simulation data in fig.7.6 captures this effect. Although the Polo levels remain high, they do not rise as experimentally observed. It can be seen that simulated uKT levels remain high while the CycB levels reduce over time due to mitotic slippage. In the absence of any KMT

attachments, most of the free MCC is in complex with APC thus inhibiting its activity. However, due to mitotic slippage APC activity increases decreasing CycB levels in presence of engaged SAC⁴²⁸. Recent evidence suggests that in order to sustain SAC arrest the cellular pool of CycB needs to be replenished via de-novo synthesis⁶¹⁵. Also, exogenous expression of CycB can delay mitotic slippage. The decrease CycB due to mitotic slippage has some effect on production of free MCC from individual subunits ultimately causing slow decrease in total assembled MCC.

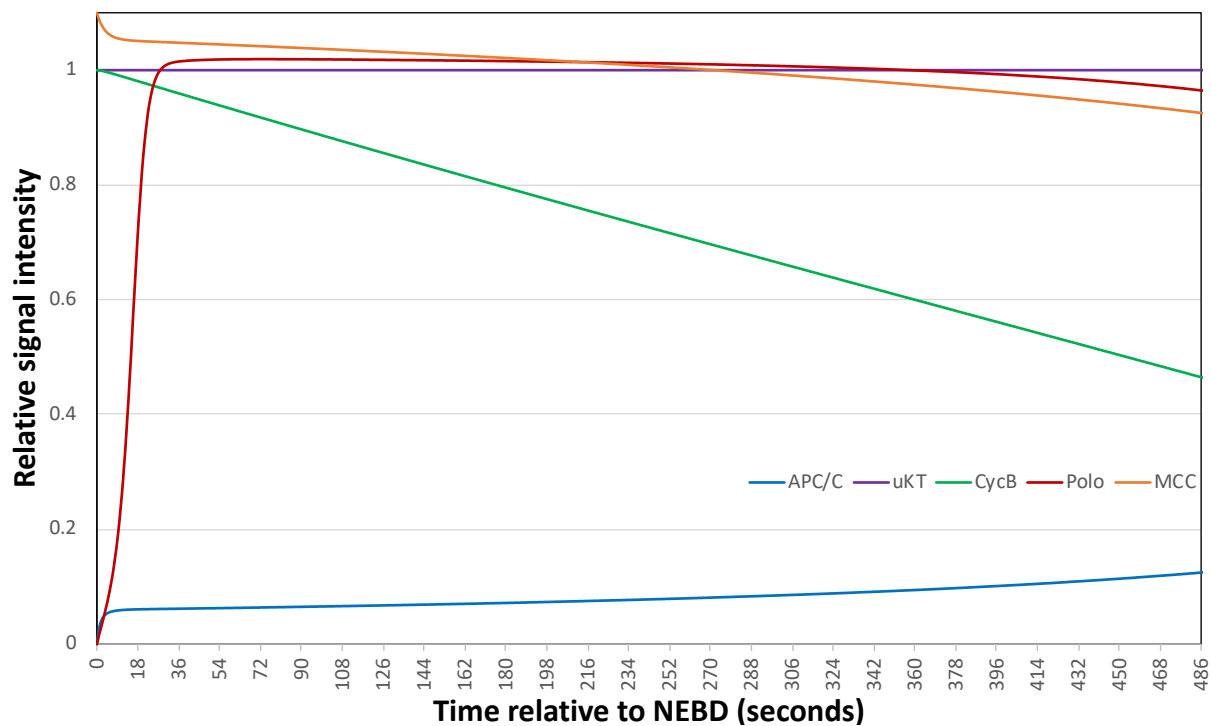


Figure 7.5. Simulation of Colcemid treatment

The experimental data (fig. 5.11) shows that injecting UbcH10 in syncytial embryos leads to a MAT arrest with silenced SAC and reduced levels of Polo. The simulation (fig.7.7) captures this effect. Although Polo levels decrease but to a lesser extent as experimentally observed. It can be seen that simulated uKT levels match that of control mitosis (fig.7.5), CycB

levels stabilise as there is no proteasomal degradation and there is no significant change in APC dynamics compared to control simulation. The satisfaction of SAC is accompanied by KT's being captured and the assembled MCC show similar dynamics as in control simulation.

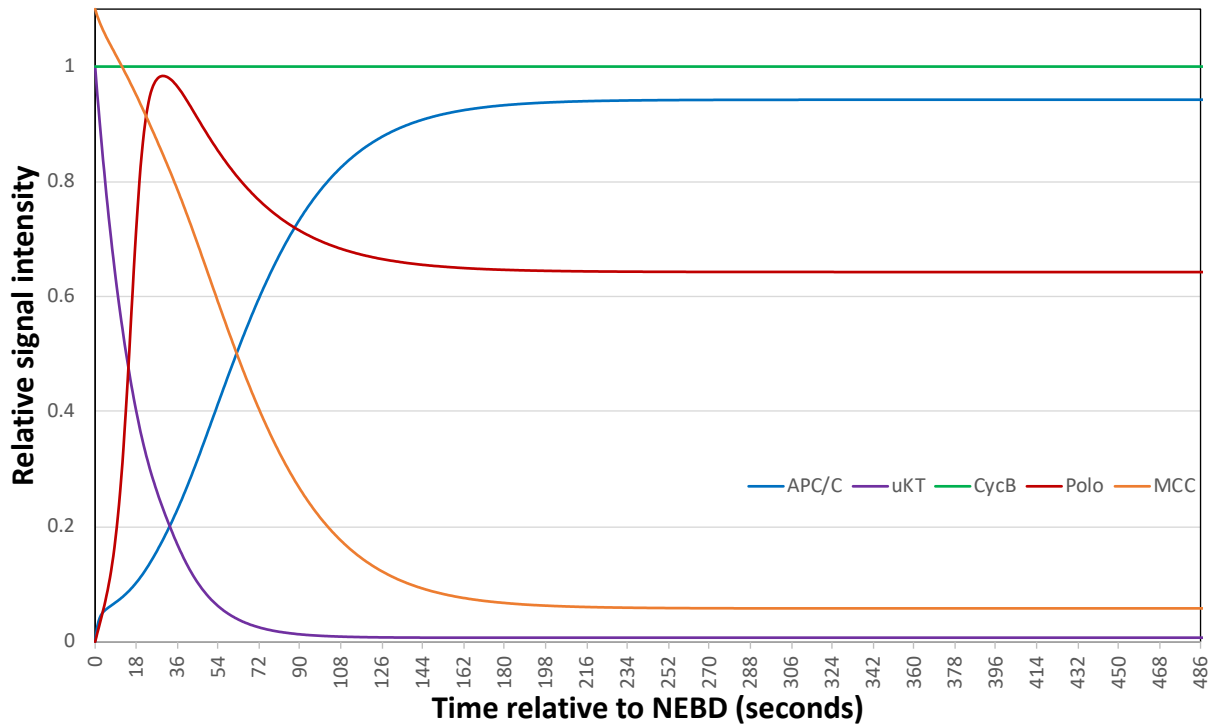


Figure 7.6. Simulation of UbcH10 treatment

7.4 Limitations

This is initial version of the model built by primarily focusing on Polo dynamics under normal and perturbed mitotic conditions to observe its effects on unattached KT's, CycB, APC and assembled MCC. Indeed, the premise of representing the biological system that is spatially heterogenous and the localisation effects by a compartmentalisation, but integrated mathematical system may be subject to inherent limitations and approximations. The model would need further evolution to incorporate the dynamics of individual MCC and CPC subunits. However, the simplicity of the model, gives it more visibility to scrutiny and a

significant agreement with the experimental data, giving the confidence in its validity and future work.

7.5 Summary

The developed dual feedback model highlights the importance of considering the EC and the SAC modules together instead of treating them separately. In this chapter, I have quantitatively explained the experimental results under normal mitotic conditions considering Polo dynamics. The parametrised model is then used to capture the effects of Polo under different conditions (i.e. Polo and AurB inhibition, Colcemid and UbcH10 treatment) on unattached KTs, CycB, APC and assembled APC that agrees with the experimental and other published data.

The following chapter focuses on building and showcasing the functionality of software called DAPPER that that in future can be used for analysing PPI data of a) Polo and BubR1 b) and in combination with other KT and mitotic proteins. The aim would be to understand how PPI interactions govern and regulate Polo and BubR1 dynamics. However, there was no unified platform for such a custom focused analysis. Hence, I collaborated with and Dr. Abbas Haider Sayed to build the software platform and with Dr. Zoltan Lipinszki to find the binding partners of Polo and BubR1 to demonstrate the functionality of the platform. These are discussed in the following chapter.

Chapter 8 Software Development of Database for Protein-Protein Interactions (DAPPER)

8.1 Introduction

Experiments described in earlier chapters focused on a) validating the outcome from mathematical analysis b) functionally characterising the biological events that govern the dynamics of Polo c) developing a quantitative model to investigate Polo dynamics. The biochemical mechanisms that govern the dynamics these events depend on the proteins interaction network and post translational modifications. The branch of “omics” that deals with the identification of protein-protein interactions (PPI) and post translational modifications is called “Proteomics”. PPI interactions are the basis on which the cellular structure and function are built and interacting partners are an immediate lead into biological function that can be exploited for therapeutic purposes⁶¹⁶. Affinity purification (AP) combined with mass spectrometry (MS) is a technique used in proteomics to identify the PPI of a protein. In this chapter the PPI of Polo and BubR1 are identified using AP-MS with a goal to showcase the functionality of software called “Database for protein-protein interaction (DAPPER)”. To analyse the PPI data must be annotated, stored, shared, processed and investigated individually and collectively with other KTs proteins using a unified platform. AP-MS generates large volume of data and there was no ideal versatile in-house platform for this. Hence, there was a clear need for DAPPER ⁶¹⁷.

The scope of this chapter is 1) To build DAPPER that can be used in future to analyse PPI data 2) To identify the binding partners of Polo and BubR1 to showcase functionality of the DAPPER. However, a separate follow-on project had been initiated in Glover Lab to analyse Polo and BubR1 PPI data individually and in combination other KT and mitotic proteins. The aim of this follow-on future work was to experimentally validate and functional analyse the interactions that play a role in shaping the dynamics of Polo and BubR1 and more broadly other KT proteins.

For developing DAPPER I collaborated with Dr. Abbas Haider Sayed from Department of Computing, University of Cambridge. I developed one of the most crucial algorithm and code of DAPPER required for parsing the XML mascot data file that was obtained from the mass spec facility after AP-MS. The code for the XML parser is present in the appendix E Dr. Abbas Haider Sayed integrated this parser code with the front end of DAPPER that he developed. The freely available Biomart warehouse system 0.7 with which DAPPER was integrated was installed and configured. I defined the concept and build algorithms for DAPPER functions like intersection, intersection prime, distinct and sort. The algorithm for these functions were then converted into web enabled codes by Dr. Sayed as they had to be derived from the Biomart class. DAPPER resides on server at Department of Genetics, University of Cambridge which is maintained by the departmental computing officer. I collaborated with Dr. Zoltan Lipinszki to identify the binding partners of Polo and BubR1. My role was to multiply the GFP-BubR1 and GFP-Polo flies, collect and treat the embryos and finally store them for AP-MS experiment. The procedure of embryo collection and storage is described in the methods chapter. GFP tagged Polo and BubR1 were affinity purified using GFP-Trap experiment as described in the protocol published by Dr. Zoltan Lipinszki and sent

to mass spectrometry facility to identify PPI⁶¹⁸. The result of AP-MS experiments was provided by the facility in mascot XML file format. Once the binding partners were obtained, DAPPER was used to process the mascot XML to extract the PPI data, store and annotate it. Several functions of DAPPER are also illustrated in this thesis.

8.2 Considerations for AP-MS

AP-MS is one of the most widely accepted methods for understanding PPI network^{619,620}. AP-MS in collaboration with complex algorithms helps in the identification of peptides which are obtained by digesting purified proteins. PPI helps to uncover the molecular mechanism by which a protein functions in a cell. In AP-MS the protein for which the binding partners need to be found is called as the “bait” and it may be bound with a tag protein. Upon expression of the bait protein if the tag is present then the tag is affinity purified (AP). However, when the tag is not present the bait protein is affinity purified. Thus, there are two approaches to do the affinity purification one using an antibody against the bait protein and other by using an antibody against the tag protein which is bound to the bait protein. Antibody against the bait protein can be used if it has a high sensitivity and specificity as this may reduce false positives compared to using a tag. This is because sticky proteins may bind to the tag rather than the bait protein and be detected as interactors after AP-MS. However, if the antibody against the bait protein is of poor sensitivity it may not bind very effectively or if the antibody against the bait protein is of poor specificity it may give false positives. If a highly sensitive and specific antibody against the bait protein cannot be used, then an alternative approach is taken. A tag protein against which a commercially available highly sensitive and specific antibody is available is used to purify the bait protein. This technique assumes that when the tag protein is

affinity purified it pulls the bait protein which in turn pulls its interacting partners. This approach of using the tags may result in detection of false positive interactors. The false positives interactors are those proteins (other than the bait) that are bound to the tag instead of the bait and were pulled down (purified). These false positive interactors can be found by AP-MS of tag protein without being bound to the bait protein in the same type of cell or tissue. If a fluorescent tagged protein like GFP is bound to the bait protein, then the same tissue or cell line used for AP-MS can be used for imaging that produces the dynamics. This can be very useful as it saves resources and especially useful to link the protein dynamics to its interactions. Another aspect to be considered during AP-MS is if the bait protein needs to be expressed endogenously or its expression has to be induced. Each approach has its own advantages and disadvantages based on the expression level of the protein, lethality of overexpression and sensitivity and specificity of the antibodies against the protein. The optimal approach to take for a large-scale AP-MS can often be decided by doing small scale AP-MS using various combination of methods. In case of Polo and BubR1, endogenous GFP tagged syncytial embryos were used for live imaging and AP-MS.

8.3 Data Analysis Pipeline

The data analysis after the AP-MS experiment is complex and cumbersome process. The MS data is submitted to one of the most widely used commercial software package called Mascot (<http://www.matrixscience.com>). This package is used to identify proteins from primary sequence databases. The XML files from Mascot are uploaded to DAPPER for further processing, analysis, annotation, storage and sharing. During the DAPPER upload process, a) the XML mascot files are parsed to extract data to be stored in the database b) automatically

annotated with FlyBase gene name and identifiers using the FlyBase dumps c) any gel images and extra experimental information that are added by the user in addition to the XML files are also parsed and stored in the database. For further annotation or any additional information DAPPER is linked to a local copy of Ensembl *D. melanogaster* database version 75. DAPPER has additional functions like sorting, intersection, intersection prime and distinct for analysing the data interactively with other proteins once it has been uploaded. DAPPER outputs the results as HTML tables. These results can also be downloaded as a tab separated value (TSV) in text format. DAPPER is a very valuable resource to the *D. melanogaster* community because of its versatile features of parsing data from XML files, annotating, storing, sharing, adding any extra experimental information, storing gel images and its functionality for integrative analysis with other proteins using its various functions sorting, intersection, intersection prime and distinct. DAPPER is a web-based software and available for use at: <http://dapper.gen.cam.ac.uk>. The overview of AP-MS and DAPPER pipeline is explained below and represented in fig. 7.1

1. Affinity purified protein is run on the gel to check if AP has been successful. After which it is sent to the mass spec facility.
2. The interaction data from the mass spec facility is obtained in an in an XML format.
3. These XML files were uploaded into DAPPER with a special user interface that has the option to add experimental details and gel images. A programme called the “parser” processes the XML file uploaded from the user interface, combines the additional experimental information and gel images added by the user during the upload process and stores it in the database.
4. DAPPER automatically annotates the data with the corresponding FlyBase gene

identifiers and names from the FlyBase dumps. DAPPER is also linked to a local copy of Ensembl *Drosophila melanogaster* database version 75 to extract any additional information.

5. DAPPER tools like sorting, intersection, intersection prime and distinct can further be used for individual and integrative analysis with other proteins.
6. The results from DAPPER are obtained as HTML tables with hyperlinks for a detailed view as well as in tab delimited text format for download.

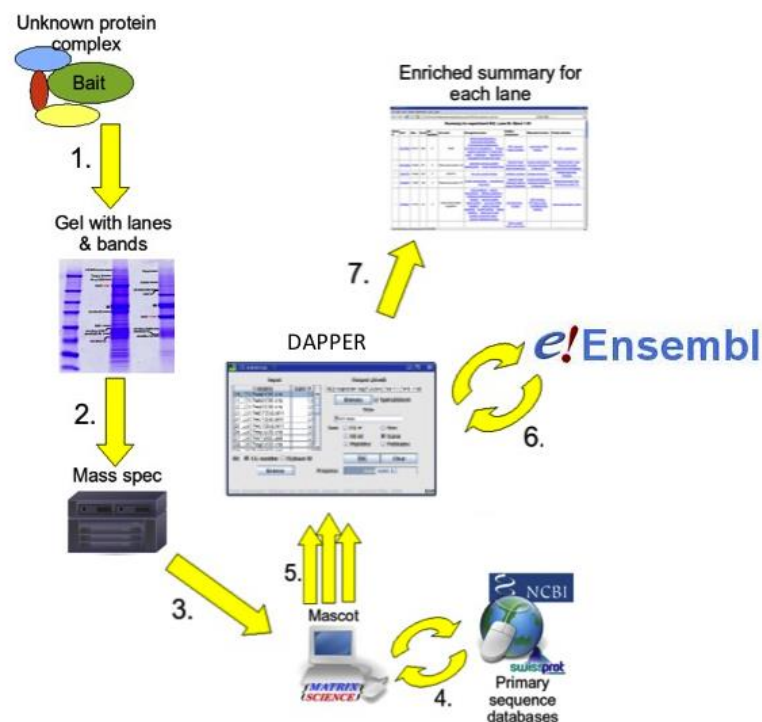


Figure 8.1. Flow chart of how DAPPER usage

1. The bait protein is purified in a gel free manner from cells/tissues. 2. The purified bait protein is run on the gel to check if AP was successful. 3. The purified proteins is sent for mass spec. 4. Mascot software is used to identify the interacting proteins and stored in XML format. 5. Output XML files obtained from mass spec facility is uploaded to DAPPER along with any additional experimental information and gel images. DAPPER automatically annotates with FlyBase gene name and identifies during the upload process. 6. DAPPER is connected to local Ensembl *Drosophila melanogaster* dump for additional information. 7. DAPPER output is in

HTML format which can be downloaded as a tab separated text file. Figure and description guidelines are adapted from initial version by Dr. Marcin Przewolka.

8.4 Parsing of Polo and BubR1 Binding Partners

Binding partners of both Polo and BubR1 were identified with a view to showcase the functionality of DAPPER. The results obtained from the MS facility in XML format were processed using DAPPER parser (fig. 7.2) that generated the following tables (tab. 1 and 2) with annotated binding partners that were as per the protein scores.

Home | Upload | Refresh | MartView | Sign out

Update Existing Experiment

Enter Experiment ID

Retrieve

Delete Existing Experiment

Enter Experiment ID

Delete

Upload XML/Image Files

Add Files

0 Files Uploaded

Experiment Properties

First name*

Last name*

Experiment type* Public

Date of experiment* Nov 07, 2014

Experiment name*

Mass spec facility

Organism* Drosophila melanogaster

Gel* Gel Free

Bait details

Name*

Accession number*

Full length* Yes

Expressed domain start position

Expressed domain end position

Localisation

Function

Terminus* C

Tag* HA

Source* Cells

Promoter for cells* NoPromoter

Expression in files

Tissue & developmental stage

Promoter

Submit Data

Remarks

Drug used before harvesting

Drug 1 name

Drug 2 name

Drug 3 name

Drug 1 concentration (micro molar)

Drug 2 concentration (micro molar)

Drug 3 concentration (micro molar)

Drug 1 treatment time (hours)

Drug 2 treatment time (hours)

Drug 3 treatment time (hours)

Extraction details

Drug 1 name

Drug 2 name

Drug 3 name

Drug 1 concentration (micro molar)

Drug 2 concentration (micro molar)

Drug 3 concentration (micro molar)

Drug 1 treatment time (hours)

Drug 2 treatment time (hours)

Drug 3 treatment time (hours)

Centrifuge details

Units rpm

Speed

Time (hours)

Figure 8.2. Uploading data in DAPPER

The parser that runs behind the upload and edit page process the data from the input XML file along with any extra experimental information. If any gel images are present, the parser stores it in the relevant folder on the server from where it can be retrieved. The parser also plays a role in annotating the data with FlyBase gene identifiers and names. The upload page can also be used to edit any experimental information added during the initial upload and also allows the experiment owner to delete experiments.

Table 1. Top 50s binding partners of Polo

Experiment id	FlyBase Gene Name	Protein score
150	polo	8103
150	sle	3465
150	Map205	3319
150	FK50s6-bp1	2904
150	CG12592	1298
150	Hsc70s-4	999
150	toc	912
150	Bruce	622
150	Nurf-38	618
150	CkIIalpha	558
150	His2A:CG31618	524
150	Ef1alpha48D	462
150	Hsc70s-1	401
150	Hsp60s	401
150	pic	379
150	Hsc70s-3	376

150	alphaTub84B	362
150	dre4	360
150	RpL12	357
150	Grip71	342
150	Yp2	340
150	CG3342	332
150	milt	314
150	RpL7	305
150	RpL7A	301
150	PP2A-29B	285
150	RpLP0s	279
150	Yp3	277
150	sta	271
150	RpLP1	266
150	RpL4	265
150	CklIbeta	265
150	RpL10Ab	259
150	RpS7	256

150	RpLP2	249
150	l(1)1Bi	238
150	Ref1	235
150	CG5261	223
150	spd-2	222
150	nonA	220
150	His3.3A	208
150	CG12272	204
150	CG170s18s	193
150	betaTub56D	191
150	mtrm	190
150	RpL3	181
150	Hsp68	181
150	CG32365	178
150	RpL18s	173
150	l(1)G0s156	167

Table 2. Top 50s binding partners of BubR1

Experiment id	FlyBase Gene Name	Protein score
186	Bub3	21780
186	BubR1	19073
186	Hsc70s-4	2180
186	Hsp60s	856
186	Hsc70s-3	802
186	Yp3	642
186	Ef1alpha48D	60s6
186	Nurf-38	565
186	alphaTub84B	533
186	rod	493
186	Yp2	488
186	Hsc70s-1	464
186	CG8798	392
186	fzy	362
186	Hsp68	345
186	PP2A-29B	304

186	RpS7	303
186	Yp1	286
186	pxt	270
186	betaTub56D	242
186	26-29-p	230
186	me31B	227
186	CG5261	213
186	Jafrac1	197
186	Tcp-1eta	195
186	SdhA	194
186	RnrL	188
186	CG6439	188
186	Aldh	178
186	l(1)G0s156	175
186	Uba1	158
186	sta	158
186	CG17754s	154
186	CG5366	149

186	RpLP0	149
186	dj-1beta	145
186	RpL4	137
186	Hsc70s-5	137
186	Rpd3	135
186	polo	134
186	blw	130
186	RpLP1	128
186	twin	128
186	Pen	124
186	cdc16	122
186	twc	120
186	Trx-2	119
186	eIF3-S10	118
186	DsimGD17470s	118
186	RpS4	117

8.5 System Architecture

A large amount of data had been accumulated from AP-MS experiments in Glover lab with a broader goal to identify and investigate the PPI network of proteins involved in cell cycle regulation of *D. melanogaster*⁶¹⁷. A platform was required to store the data in a unified way so that it can be shared, annotate, processed and analysed integratively with other data sets and ultimately mine information which would otherwise not be obvious⁶¹⁷. Hence, the platform named DAPPER was developed on open source Biomart data warehouse platform (fig. 7.3). Biomart is an open source software available for download and installation. Once configured it can be used to store, view, query and share the data. DAPPER is integrated and configured to run with Biomart. Once the data is uploaded it can be queried and analysed. The queries about the data is passed to the Biomart data warehouse system. The results from the processed queries are shown as HTML tables which can be downloaded as tab separated text file. A query is split into DAPPER based and Ensembl based attributes⁶¹⁷. Both attributes have preselected default settings. DAPPER specific attributes are used to selectively choose from the experimental properties of the data that the user would like to visualise in the final output. These experimental properties are those which were added by the user during initial upload of XML results file but were not present in the XML file. Further, filters are used to place a limit on the attributes. For example, from the long list of experimental properties if the user wants to see only two attributes namely protein name and experiment id in the final output it can be achieved by selecting the check boxes for protein name and experiment id. The user can then filter the final output based on applying conditions to the attributes. These conditions are called as filters. For example, suppose the user wants to see proteins with a score over 100s0s in the final output then the filter for protein score must be activated by checking it and writing the

condition of ≥ 100 s in the protein score text box. Additionally, DAPPER also has Ensembl specific attributes as it connects to a local copy of *D. melanogaster* Ensembl database version 75 to retrieve additional information about the protein. Filters can be applied to Ensembl based attributes in the same way as described for experimental based attributes. Fig. 7.3 shows the schematic system level diagram of DAPPER built on Biomart data warehouse system 0.7. This diagram has been taken from the DAPPER paper published in BioData Mining⁶¹⁷

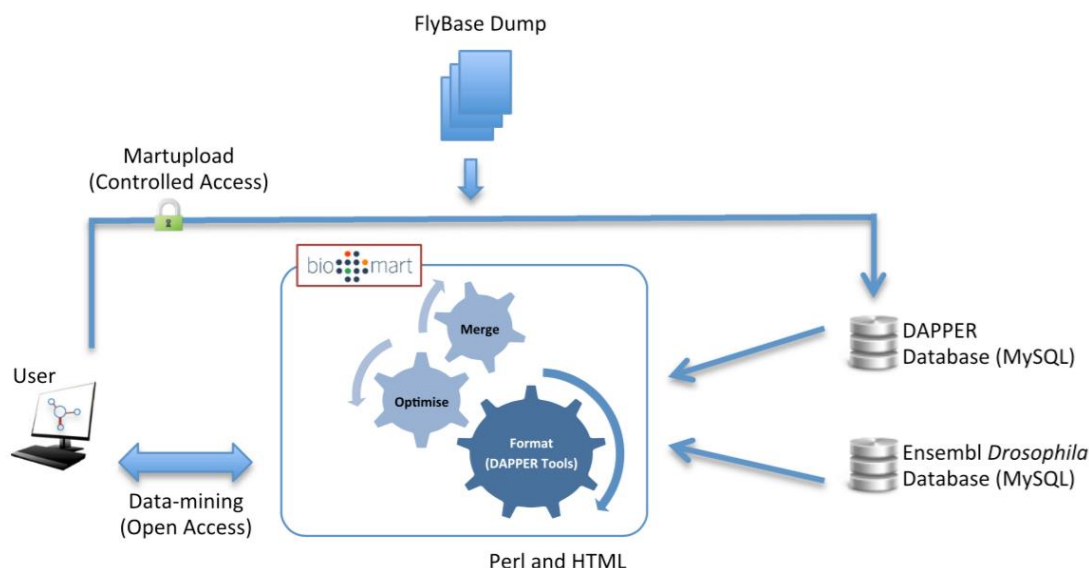


Figure 8.3. Schematic diagram of DAPPER

The user can upload the XML files obtained from Mascot by adding experimental details and/or can query DAPPER for integrative data analysis and/or for any additional information from Ensembl. During the data upload of XML files, the mascot id is automatically annotated by FlyBase gene identifiers and names. This figure has been taken from the DAPPER paper published in BioData Mining⁶¹⁷.

8.6 Tools in DAPPER

In addition to the basic functionalities that DAPPER inherits from Biomart framework it also has customised tools for additional data analysis. The four main customised functions in DAPPER are “Sort”, “Intersection”, “Intersection prime” and “Distinct”. In order to understand these functions, consider three experiments each having a list of interacting proteins identified

by their CG numbers. Experiment 1 = {CG1111, CG1112, CG1113, CG1114, CG1115} and Experiment 2 = {CG1112, CG1114, CG1116, CG1117, CG1118}. Experiment 3 = {CG1112, CG1114, CG1117, CG1115, CG1119}.

Sort: The sort function can be used with a single experiment. It is active by default and when the data is retrieved from the backend as it sorts it based on the protein score in the descending order.

Intersection: This function has to be used with minimum two experiments. Using this function gives the list of common interacting proteins found in selected experiments. For example, if experiments 1 and 2 were selected and the intersection function was applied the result would be the list of proteins with following CG numbers: {CG1112, CG1114}, as these two CG numbers are common between both experiments. This function can be extended to more than two experiments.

Intersection Prime: This function needs at least two experiments to be selected. The intersection prime function gives list of CG numbers that are not present in both the selected experiments i.e. common interacting proteins between the two experiments are not present in the output. For example, if experiments 1 and 2 were selected and the intersection prime function was applied the result would be the list of proteins with following CG numbers: {CG1111, CG1113, CG1115, CG1116, CG1117, CG1118}. CG1112 and CG1114 are not included in the output as they are common elements in both experiment 1 and 2.

Distinct: This function needs at least three experiments to be selected. The distinct function gives list of CG numbers that are not present as common elements between any experiments i.e. gives unique CG numbers found exclusively in one experiment. For example, if

experiments 1, 2 and 3 were selected and the distinct function was applied the result would be the list of proteins with following CG numbers: {CG1111, CG1113 CG1116, CG1118, CG1119}.

8.7 Example of Analysing the Binding Partners using DAPPER

A strong interaction between Polo and Map205 was identified in AP-MS experiment. This interaction does not require any priming phosphorylation of Map205. Cdk1 phosphorylation of Map205 releases interaction between Polo and Map205. The in vivo interaction of Map205 with Polo can stabilise and inhibit its activity. The Polo hypomorph defects of the centrosome are enhanced by overexpression Map205. It has been suggested that Map205 enabled targeting of Polo to the MTs works as a Polo reservoir that can be rapidly released during mitotic entry by Cdk1 phosphorylation⁶²¹.

A strong interaction between BubR1 and Bub3 was identified in AP-MS experiment. Both, BubR1 and Bub3 form a constitutive cell cycle complex. Bub3 is required for stable bipolar end on attachments⁶²². Additionally, the presence of PP2A in BubR1 purification can be due to its interaction via B56, a regulatory subunit of PP2A. This interaction is known for promoting chromosome congression and antagonising AurB activity at the KTs leading to formation of stable KMT attachments⁵⁷⁹. The interaction of PP2A with BubR1 is also required for silencing SAC by dephosphorylating the MELT motifs of KNL1 as discussed earlier.

8.8 DAPPER is a Unique Tool

DAPPER is very unique in comparison to other tools (e.g. DroID⁶²³ etc.) for the following reasons:

1. DAPPER contains PPI data that has been experimentally generated from over 100 AP-MS experiments with some of the interactions validated and functionally tested.
2. DAPPER is unique to *D. melanogaster* but has the functionality to be extended for human data set.
3. DAPPER is built on the widely known and established open source Biomart data warehouse platform which gives the flexibility to view the data in many ways.
4. DAPPER has customized functions like sort, intersection, intersection prime and distinct.
5. DAPPER can automatically annotate the data with FlyBase gene names and identifiers
6. DAPPER has the functionality to connect locally installed database dumps of Ensembl and FlyBase to annotate the proteins.
7. DAPPER allows one to upload user's own data sets to be sliced and diced along with data from pre-existing but experimentally obtained data.

8.9 Summary

This chapter focuses on development of DAPPER platform for processing, sharing, storing and analysing experimentally obtained PPI data from *D. melanogaster*. This chapter also showcases the functionality of DAPPER that includes a) parsing and data sorting b) slicing

and dicing the data using functions like sorting, intersection, intersection prime, distinct c) exporting the data in various formats like tab delimited, HTML etc formats d) analysing PPI data (like in case of Polo, Map205 was identified as one of the binding partners and in case of BubR1, Bub3 was shown as a binding partner). As part of future work, it would be ideal to analyse a) Polo and BubR1 PPI individually and with other proteins to experimentally characterise how its interactions play a role in shaping and governing the dynamics.

Chapter 9

9.1 Image Analysis

One of the goals of this chapter was to compare image analysis approaches for measuring KT signals using live imaging data. Using mean of a constant size ROI to represent KT intensity was often not possible as KTs can be spatially clustered or distributed during the time course. Furthermore, a variable size ROI to measure the KT intensity was also not a viable method since changing the ROI size had an impact on the mean measurement. However, it was observed that the maximum intensity of the ROI was not affected irrespective of the change size of ROI. To represent the KT signal by a single maximum intensity pixel would not be a robust method given that it can be influenced by imaging artefacts. Hence, an Image J plugin was developed to calculate the mean from top N (variable number) maximum intensity pixels to represent KT signal. N was empirically determined to be 10 as the top 10 maximum intensity pixels were always on the KT in the imaging set up used. It was shown that if higher number of N pixels were chosen (as far as the N pixels are on the KTs) then similar Polo dynamics were obtained but with decreased sharpness that could impact the further mathematical analysis. Given that KTs are inherently embedded in the nucleoplasm, three background correction methods were compared. The method that removed the maximum background between NEB and AO was used. However, irrespective of the method used for background correction the trend in the dynamics of Polo was not significantly impacted. Furthermore, Polo

KT dynamics were verified using a more traditional alternate approach. It was also established that there was no significant photobleaching affect in both channels, i.e. green and red, in the imaging set up used. As a part of future work, it would be ideal to develop an automated method to measure intensity of individual KTs at each time point using segmentation and tracking.

9.2 Investigating the Dynamics of Kinetochores Associated Proteins

One of the aims of chapter 4 was to build a protein kinetic map of some of the key KT associated proteins in *D. melanogaster* syncytial embryos. Such a map would be helpful to better understand the formation and regulation of the KTs. This study was initiated by mapping of the dynamics of KMN network proteins²³² and later expanded to include the dynamics of regulatory proteins like CPC, MCC and Polo. Based on the kinetic mapping, the loading of the CPC starts prior to BubR1, Polo and Fzy potentially as AurB is at the apex of both SAC and EC mechanisms and localised closest to the heterochromatin. CPC reached its peak levels just after start of metaphase but before the KMN network proteins. It was observed that AurB KT recruitment happens earlier compared to other CPC subunits and this may be due to several positive feedback loops enhancing its recruitment. The KMN network proteins are recruited to the KTs much before the regulatory proteins enabling them to form the core KT structure to load regulator proteins. The MCC shows tightly regulated and sharp dynamics as anticipated due to its regulatory nature. The dynamics of Mad2 could not be accurately mapped due to background. As a part of the future work, it would be ideal to have all the centromeric and KT proteins on this global map. Another goal chapter 4, was to develop computational approaches to investigate KT protein dynamics. The first method analysed the protein dynamics to predict interdependencies in KMN network proteins that were experimentally validated. The second

method analysed Polo and BubR1 kinetics to estimate the time and number of biological events that shape it. Later chapters verified and characterised these biological events. As a part of the future work it would also be ideal to mathematically establish co-dependency and biological events that shape the dynamics of all proteins in *D. melanogaster*.

9.3 Investigating the Loading and Unloading Dynamics of Polo

Chapter 5 discussed the loading and unloading dynamics of Polo. The loading phase of Polo depends on its own activity and its activation by AurB. AurB phosphorylates Polo at the centromeric region³⁸⁶. The cross talk between the two kinases enables fine tuning of AurB enabled destabilisation and Polo driven stabilisation of KMT attachments. Inhibiting Polo or AurB activity by BI2536 or Binucleine2 respectively prevents optimal Polo recruitment at the KT region. This observation is aligned to the mathematical analysis that suggested monophasic Polo loading phase i.e. shaped by one biological event. As a part of future work, another approach to investigate this would be to make a kinase dead version of AurB transgenic fly and study the dynamics of GFP-Polo in this mutant. However, it is likely that syncytial embryos from kinase dead AurB flies die in early syncytial cycles to make any observations. In this case the expression of AurB kinase dead protein can be put under Gal4 control and expressed in the zygotic embryos. However, one would need to repeat all the previous experiments to validate the observations in a zygotic embryo.

It was experimentally established that the first Polo drop is a) independent of degradation via its destruction-box mechanism b) does not depend on degradation of prometaphase and metaphase APC (i.e. APC^{Cdc20}) substrates including CycB (and hence independent of Cdk1-CycB activity c) independent of any APC (i.e. APC^{Cdc20} and APC^{Cdh1}) degradation substrate covered by ubiquitin mediated destruction pathway. However, as the

dynamics of Polo closely matched BubR1, it was speculated that the first Polo drop was dependant on SAC silencing process⁶⁰⁵ that requires completion of KT occupancy by MTs and establishment of maximum tension on sister KTs. Injection of Colcemid in Polo and BubR1 established that first Polo drop was due to the process of silencing SAC. However, due to the use of Colcemid it was not possible to tease apart if the first Polo drop was due to KT occupancy or tension, both of which are achieved during process of SAC silencing. In order to address this, I investigated and established that the completion of two events happen in a sequential manner. The KTs are completely occupied by the MTs at -54s and establishment of maximum tension happens at AO i.e. 0s. These observations are in alignment with the mathematical analysis that two biological events shape the unloading phase of Polo. The first event is the completion of KT occupancy at -54s and the second event is establishment of maximum sister KT tension at 0s. As a part of future work, these results could also be verified by using super resolution live microscopy on dual tagged transgenic flies expressing mCherry-Polo-GFP-BubR1, mCherry-Polo-GFP-Mad2, GFP-Polo-mRFP-Tubulin. The dynamics obtained from these double labelled transgenic flies could show the drop in the levels of Polo and Mad2 in the same fly, a drop-in level of Polo when captured by MTs and a drop in the level of Polo and BubR1 in the same fly at time point 0s. However, using mCherry for certain proteins may not give similar level of brightness to see the details and can photobleach quicker causing fluorophore bias. Asking a reverse question of what would happen if the Polo drop is prevented would be interesting. Such studies have not yet been done in vivo system of *D. melanogaster* syncytial embryos. To address this, the levels of Polo can be kept constitutively high by using different kinds of chimeric fusions for KT Polo. Ideally, three kinds of transgenic flies be helpful to investigate: a) Wild type Polo chimeric fly b) Constitutively active Polo chimeric fly c) Kinase dead version of Polo chimeric fly. This would enable to answer some of the questions like what happens if Polo or constitutively active version of Polo is artificially kept high at the

KTs. One can observe these chimeras in different backgrounds of Histone, Tubulin, CID, Mad2 and BubR1. Histone backgrounds can be used to see if there are any effects on chromosome condensation or any other defects. CID and Tubulin background can be used to find out if there are any defects during process of KMT attachments. While Mad2 and BubR1 can be used to study any impact on KT occupancy and tension. The kinase dead version of Polo would act as a suitable control to establish no aberrant observations are due to the use of chimeric fusion proteins.

9.4 Investigating the Dynamics of Residual Polo

Chapter 6 focused on characterising residual Polo. Using double microinjection experiments (i.e. UbcH10^{C114S} and p27) it was shown that localisation of residual Polo depends on Cdk1-CycB activity. Furthermore, it was established using double microinjection experiments (i.e. UbcH10^{C114S} and BI2536) that activity of residual Polo is required to maintain stable amphitelic attachments after SAC satisfaction enabling correct CS and preventing reactivation of SAC. Using double microinjection experiments (i.e. UbcH10^{C114S} and BI2536) and with appropriate control experiments using Mad2 and BubR1 it was established that Polo acts as a tension sensor like BubR1 i.e. its levels increase when tension on amphitelic attachments are lost without breaking the attachments.

9.5 Modelling Polo Dynamics

Chapter 7 discusses the quantitative model characterising Polo dynamics. The developed dual feedback model build using Polo dynamics captures the experimental data quantitatively under normal and perturbed mitotic conditions (i.e. Polo and AurB inhibition, Colcemid and UbcH10 treatment). This quantitative approach allows to characterise the effects

of Polo on unattached KTs, CycB, APC and assembled APC that agrees with the experimental and published data. However, as the current model primarily accounts for Polo dynamics it can be evolved to encompass kinetics of all MCC and CPC subunits as the second step, with the ultimate goal to have a comprehensive model considering kinetics of all KT proteins that play a role in SAC and EC.

9.6 Software Development of Database for Protein-Protein Interactions (DAPPER)

Chapter 8 discusses software development of DAPPER that in future can be used for analysing PPI data of Polo and BubR1, to understand how it biochemically shapes their dynamics. DAPPER can store, share, annotate interaction data using various functionalities and collectively analyse it with other databases like Ensembl. The functionalities of DAPPER was showcased by using Polo and BubR1 interacting partners obtained via AP-MS. It was a part of another project in Glover Lab to use DAPPER for: a) analysing Polo and BubR1 PPI data b) further extend the analysis considering other KT and mitotic proteins to experimentally characterise and validate them. The ultimate future goal is to investigate how PPI interaction data shapes the dynamics of *D. melanogaster* KT proteins starting with Polo and BubR1.

Chapter 10 Overall Conclusions

The overall goal of the thesis was to investigate the KT protein dynamics in *D. melanogaster* syncytial embryos using experimental and computational approaches. Both the approaches compliment and validate each other completing the systems approach as discussed below.

The first results chapter i.e. Chapter 3, compares various image analysis methods. Based on this analysis a novel approach to analyse the KT dynamics, for proteins with nucleoplasmic and cytoplasmic background, was developed. This method takes into consideration the photobleaching and background correction to find the dynamics. Chapter 4 applies this image analysis method to build a global loading and unloading kinetic map of key KT proteins, giving insight into their order. Chapter 4 also discusses two predictive computational approaches based on the kinetics to a) identify the recruitment dependencies KT complexes using KMN network proteins as an example that is experimentally verified in this chapter and b) identify the number and the timing of the events that govern the dynamics of KT proteins using Polo and BubR1 as examples. It was predicted that Polo and BubR1 are monophasic and biphasic in their loading and unloading kinetics respectively. Polo and BubR1 kinetic phases are experimentally verified and functionally characterised in chapter 5 and 6. Chapter 7, focuses on supporting and validating the experimental findings from previous chapters by comparing and combining it with known literature findings using a quantitative mathematical model. This chapter also discusses the limitations and the further evolution of this mathematical model. In order to understand what governs the KT dynamics of proteins it is vital to identify its binding partners using AP-MS and to investigate this in relation to other KT proteins. Hence there is need for a computational platform to store, annotate and analyse the AP-MS data. Chapter 8 discusses the development of such a software called DAPPER using experimentally obtained Polo and BubR1 PPI data. Chapter 9 focuses on discussion and future work in more detail. Chapter 10 highlights how the systems approach was used in this thesis by combining experimental and computational approaches.

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Appendix A Protocols and Images

Gateway Cloning (taken from Invitrogen Clonase II manual)

The Gateway Technology uses lambda recombination system to transfer heterogenous DNA sequences between vectors. This is enabled by two recombination reactions that use proprietary enzymes called BP clonase and LR clonase respectively and rely on specific Gateway “att” sites.

BP Reaction: This facilitates recombination of attB substrate (attB-PCR product) with donor vector having attP site. This creates entry clone with attL site and an attR by-product. See figure below taken from Invitrogen clonase II manual.



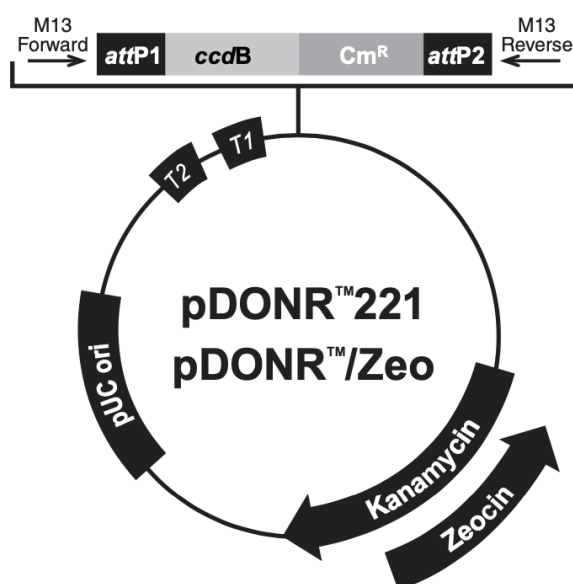
LR Reaction: This facilitates recombination of attL substrate (i.e. entry clone) with a destination vector having attR site. This creates expression clone with attB site and an attP by-product. See figure below taken from Invitrogen clonase II manual.



ccdB and *Cm^R* gene: This gene allows for negative selection of donor and destination vectors in *E. coli*, following recombination and transformation, by interfering with DNA gyrase that inhibits most *E. Coli* strain (e.g. DH5 α , TOP10). The *ccdB* gene is replaced by the gene of interest following a recombination reaction. By-product molecules having *ccdB* gene and cells that take up unreacted vectors with *ccdB* gene fail to grow, allowing for high efficiency transformation. The *Cm^R* gene is the Chloramphenicol resistance gene that plays a role in counter selection and is present in donor and destination vectors.

Vector: For donor vector in BP reaction pDONOR221 or pDONOR Zeo was used.

The following is the vector map.



Comments for:

	pDONOR™221 4762 nucleotides	pDONOR™/Zeo 4291 nucleotides
<i>rrnB</i> T2 transcription termination sequence (c):	268-295	268-295
<i>rrnB</i> T1 transcription termination sequence (c):	427-470	427-470
M13 Forward (-20) priming site:	537-552	537-552
<i>attP1</i> :	570-801	570-801
<i>ccdB</i> gene (c):	1197-1502	1197-1502
Chloramphenicol resistance gene (c):	1847-2506	1847-2506
<i>attP2</i> (c):	2754-2985	2754-2985
M13 Reverse priming site:	3027-3043	3027-3043
Kanamycin resistance gene:	3156-3965	---
EM7 promoter (c):	---	3486-3552
Zeocin resistance gene (c):	---	3111-3485
pUC origin:	4086-4759	3615-4288

(c) = complementary strand

The destination vectors used during LR reaction to create a PrA and EGFP expression clone were custom made in Glover lab under USAp promoter i.e. PUASp-PrA-C-Term and PUSAp-C-Term EGFP tag

The working concentration used during transformation were 100 µg/ml for Ampicillin, 50µg/ml for kanamycin and Zeocin.

C-terminal fusion PCR primer including shine-dalgarno and kozak for Aurora B, Incenp, Borealin, Survivin and Polo as per Gateway Cloning Invitrogen Manual:

Aurora B forward PCR primer:

GGGGACAAGTTTGTACAAAAAAGCAGGCTTCGAAGGAGATAGAACCATGACGCT
TTCCCGCGCGAAGCACG

Aurora B reverse PCR Primer:

GGGGACCACTTTGTACAAGAAAGCTGGGTCATTTCTGGCCGTGTTCTCCTTGCCG

Incenp forward PCR Primer:

GGGGACAAGTTTGTACAAAAAAGCAGGCTTCGAAGGAGATAGAACCATGGAGG
ACATCTTGGGCGTGCTGG

Incenp reverse PCR Primer:

GGGGACCACTTTGTACAAGAAAGCTGGGTCGTATTTTCGGCAGCTCGGAATAGCG
C

Borealin forward PCR Primer

GGGGACAAGTTTGTACAAAAAAGCAGGCTTCGAAGGAGATAGAACCATGCCGCG
CACCAAGATACCAAAGA

Borealin reverse PCR Primer:

GGGGACCACTTTGTACAAGAAAGCTGGGTCCTTGAACACCGCCTGGCTGGCCTTA

Survivin forward PCR Primer:

GGGGACAAGTTTGTACAAAAAAGCAGGCTTCGAAGGAGATAGAACCATGGAATC
GCCAGTGGTAAACGAAG

Survivin reverse PCR Primer:

GGGGACCACTTTGTACAAGAAAGCTGGGTCTTTTTGATTACGCGTAAACTCATCT

Polo forward: PCR Primer

GGGGACAAGTTTGTACAAAAAAGCAGGCTTCGAAGGAGATAGAACCATGGCCGC
GAAGCCCGAGGATAAGA

Polo reverse PCR Primer:

GGGGACCACTTTGTACAAGAAAGCTGGGTCTGTGAACATCTTCTCCAGCATTTTC

PCR Mix and Reaction (taken from Invitrogen Accuprime Pfx manual):

To do 50µl PCR reactions add the following to microcentrifuge tube (MT) to prepare for PCR mix: 1) 5 µl of 10X AccuPrime Pfx Reaction mix 2) Primer mix (10µm each) 3) Template DNA (10pg-200mg) ≥ 1 µl 4) AccuPrime Pfx DNA Polymerase 0.75µl 5) ddH₂O (double distilled water) to make the total volume up to 50µl.

Perform the PCR amplification using a three-step cycling protocol. Initially denaturation for 2min at 95°C. Then perform 30 cycles of PCR. In each cycle the denaturing temperature is 95°C for 15s, annealing temperature is between 55-64°C for 30s and the extension is 1 minute per kb at 68°C. Maintain the reaction at 4°C or can be stored at -20°C until use. Analyse the PCR product by agarose gel electrophoresis and visualise by ethidium bromide.

PCR purification using Qiagen QIAquick PCR purification kit protocol using microcentrifuge (taken from Qiagen QIAquick Spin Handbook):

To Perform PCR purification: 1) Add 250µl of PB buffer to 50µl of PCR sample and mix. 2) Check that the colour of the mixture is yellow 3) Put the spin column in the 2ml collection tube. 4) To bind the DNA put the sample in the spin column and centrifuge for 30-60s. 5) Discard the flow through and put the spin column in the collection tube. 6) Add 0.75ml of PE buffer for wash and centrifuge for 30-60s. 7) Discard the flow through and spin again for 60s. 8) Put the spin column in a new 1.5ml MT 9) Add 30µl of EB buffer to the MT and wait for 60s and then centrifuge. 10) Analyse the purified DNA on agarose gel by adding 1 volume of loading dye to 5 volumes of purified DNA.

Gel extraction using Qiagen QIAquick kit protocol using microcentrifuge (taken from Qiagen QIAquick Spin Handbook):

For gel extraction: 1) Excise the DNA fragment from agarose gel using a sharp clean scalpel. 2) Weight the cut gel piece and add 3 volumes of QG buffer to 1 volume of gel i.e. (i.e. 300µl to each 100mg of gel). 3) Incubate at 50°C for 10min or till the slice has completely dissolved. Vortex the tube every 2-3 min during the incubation 4) After the gel dissolves the mixture should be yellow in colour 5) Add 1 volume of isopropanol to the dissolved sample in mix i.e. for 100mg add 100µl. 6) Put the spin column in the 2ml collection tube 7) Put the sample mix (not more than 800µl in one go) in this spin column in the collection tube and centrifuge for 1min. 8) Discard the flow through and put the spin column in the collection tube 9) Add 0.5ml of QG buffer to spin column and centrifuge for 1min. 10) Wash the spin column with 0.75ml of PE by centrifuging. 11) Discard the flow through and centrifuge again for 1min at 13000rpm. 12) Place the spin column in new 1.5ml MT tube. 13) Add 30µl of EB buffer to the spin column centre, wait for 1 min and then centrifuge for 1min. 14) Purified DNA can be analysed on gel by adding 1 volume of loading dye to 5 volume of purified DNA

Transforming using competent cell Dh5α cells (taken from Thermofisher manual):

1) Thaw on ice competent cells and transfer 50µl to 1.5ml MT. 2) Add about 1 to 5µl of DNA/ligation to the MT and gently tap the vial. 3) Incubate on ice for 15 mins. 4) Incubate in water bath at 42°C for 45sec without shaking or mixing. 5) Put the vial after heat shock on ice for 2min. 6) Add 950µl of SOC at RT to the vial. 7) Shake the MT at 37°C and 300rpm for 1 hour 8) Spread the SOC on two LB plates (one with 25µl and one with 75µl of SOC) with 100µg/ml ampicillin 9) Incubate overnight at 37°C.

BP and LR reaction (taken from Invitrogen Gateway Cloning Manual):

1) For BP reaction add the following in 1.5ml MT tube at RT: attB-PCR product (approx. 15-150ng) 1-7µl. + Donor vector (150ng/µl) 1µl + TE buffer up such that total volume is 10µl 2) Thaw the BP clonase enzyme on ice for 2min and vortex briefly 2 times for 2sec each. 3) Add

2µl of BP clonase to the MT tube, vortex twice briefly. 4) Incubate the mix at 25°C for 1 hour. 5) Add 1µl of Proteinase K solution to the MT to terminate the reaction, vortex briefly and then incubate at 37°C for 10 min.

1) For LR reaction add the following in 1.5ml MT tube at RT: Entry clone (501-150ng) 1-7µl + Destination vector (150nl/µl) 1µl + TE buffer upto 8 µl such that the total volume is 10µl. 2) Thaw the LT clonase on ice for 2 minutes and vortex twice for 2sec each. 3) Add 2µl of LR clonase to the MT and vortex twice briefly. 4) Incubate the mix at 25°C for 1 hour. 5) Add 1µl of Proteinase K solution to the MT to terminate the reaction, vortex briefly and then incubate at 37°C for 10 mins.

HiSpeed Plasmid Maxi Kit protocol from Qiagen (taken from Qiagen handbook):

For Maxi prep: 1) Take a single colony from the streaked plate and inoculate with 5ml of LB medium with appropriate antibiotics (called as the starter culture). Incubate for 8h at 37°C with on a shaker at approx. 300rpm. 2) Dilute the starter culture in 250ml of LB media with appropriate antibiotics. Incubate on shaker for 12-6h at approx. 300rpm. 3) Harvest the cell by centrifuging at 13000 rpm for 15min at 4°C. 4) Discard the supernatant and resuspend the pellet in 10ml of P1 buffer. 5) Add 10ml of P2 buffer and mix well by inverting the tube 4-6 times and incubate at room temperature (RT) for 5min. 6) Add 10ml of prechilled P3 buffer to lysate and mix by inverting the tube 4-6 times 7) Put the lysate in the barrel of the filter cartridge and incubate at RT for 10 mins. 8) Equilibrate the Maxi tip by adding 10ml of QBT buffer and allow the column to empty via gravity flow. 9) Insert the plunger into the Maxi cartridge and filter the lysate into the equilibrated Maxi tip. 10) Allow the lysate to enter the resin by gravity flow. 11) Wash the Maxi tip by 60ml of QC buffer 12) Elute the DNA with 15 ml of QF buffer. 13) Precipitate the DNA by 10.5ml of isopropanol at RT. Mix and incubate at RT for 5min 14) During the incubation remove the plunger from the 30ml syringe and attach the Maxi Module

precipitator on the outlet nozzle. 15) Put the precipitator over a waste bottle and transfer the isopropanol/elute into the 30 ml syringe and insert the plunger. Filter the elute/isopropanol mixture through the precipitator using constant pressure. 16) Remove the precipitator and the plunger from the 30ml syringe. Re-attach the precipitator and add 2ml of 70%ethanol through the precipitator under constant pressure. 17) Remove the precipitator and the plunger from the 30ml syringe. Re-attach the precipitator and the plunger to the 30ml syringe and dry the membrane by passing air through the precipitator quickly and forcefully. Repeat this step. 18) Dry out the outlet nozzle of the precipitator with tissue to prevent the ethanol carry over. 19) Remove the plunger from the new 5ml syringe and attach the precipitator to the outlet nozzle. Hold the outlet of the precipitator over 1.5ml microcentrifuge tube (MT) and add 1ml of TE buffer to the 5ml syringe. Insert the plunger and elute the DNA under constant pressure. 20) Remove the precipitator and the plunger from the 5ml syringe and then re-attach the precipitator. 21) Transfer the elute from step 19 to the 5ml syringe and then elute again in the same 1.5ml MT tube.

Calculations, company names and catalogue number for making stock reagents during His tag protein purification:

1. Trizma-Base-HCl pH 7.5; Company: Sigma-Aldrich; Catalogue number: T1503; M.W: 121.14; To make 1M stock solution add 60.57 g Tris-Base in 0.5 L of ddH₂O to make 1M stock solutions and adjust pH to 7.5 by adding HCL.
2. NaCl; Company: Sigma-Aldrich; Catalogue Number: 31434; M.W: 58.44; To make 2.5 M stock solution add 73.05 g of NaCl to 0.5 L of ddH₂O.
3. EGTA; To make 0.5 M stock solution add 0.5L of ddH₂O to 1 g of EGTA and adjust pH to 8 by adding NaOH
4. Triton X-100; Company: Sigma-Aldrich; Catalogue number: T9284-100 ML

5. PMSF; To make 100 mM stock solution take 100% EtOH pre-chilled on ice and add 0.0871g of PMSF powder in 5 ml of EtOH and vortex till it mixes (always keep on ice between vortexing).
6. Ni-NTA Agarose (25 ml); Company: Qiagen; Catalogue Number: 30210

Gel Images from syncytial CPC and Polo embryos expressing GFP or Pra:

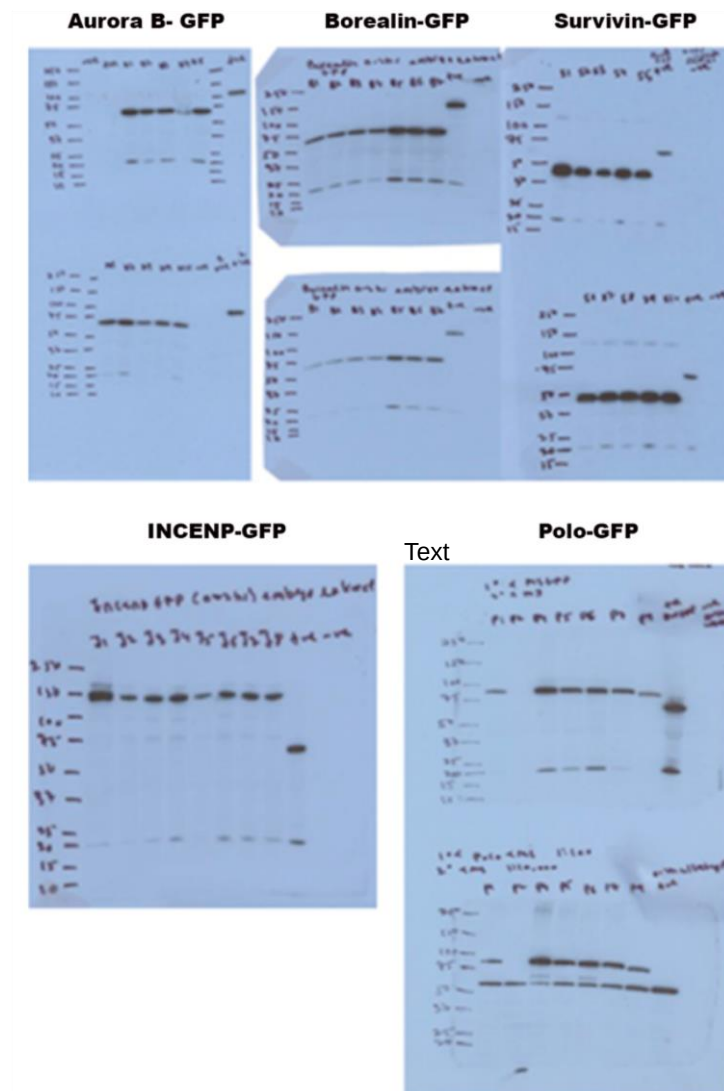


Figure 10.1. Syncytial embryos extract from GFP tagged CPC & Polo flies

The antibodies used is against GFP. The ladder sizes in kDa from bottom up are: 15, 25, 37, 50, 75, 100, 150, 250

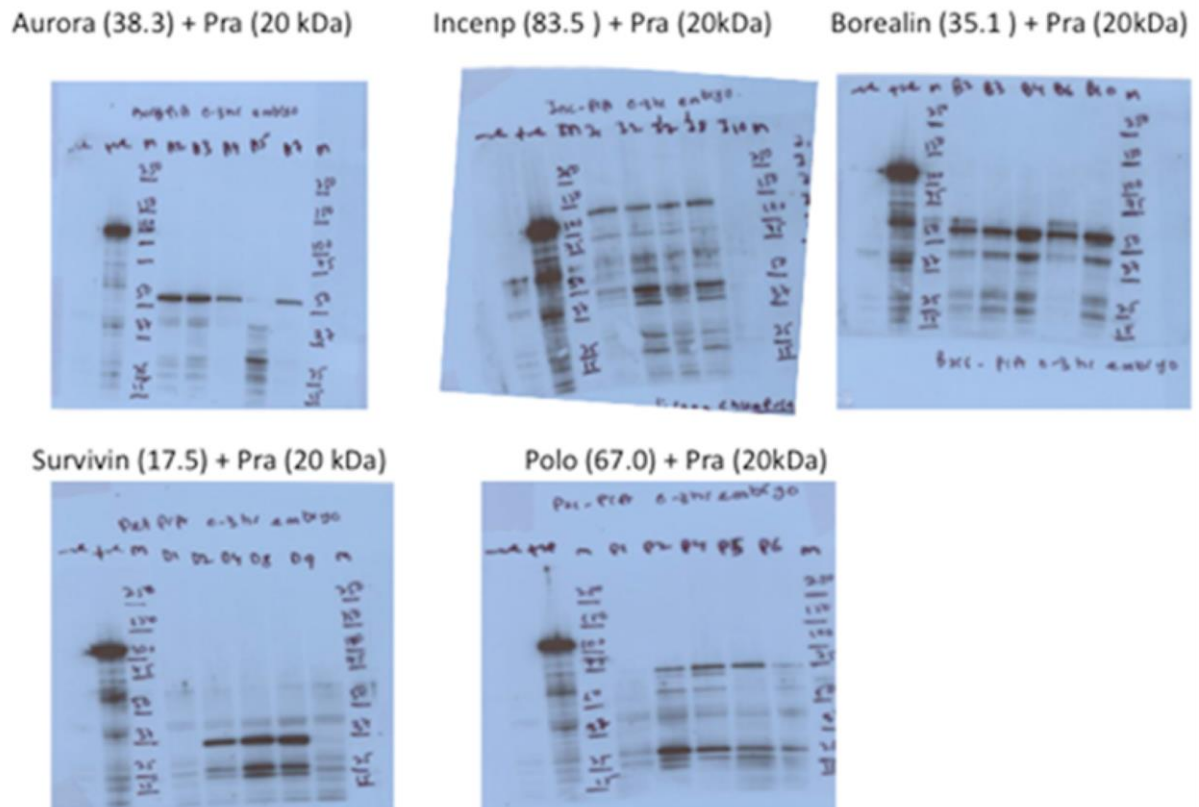
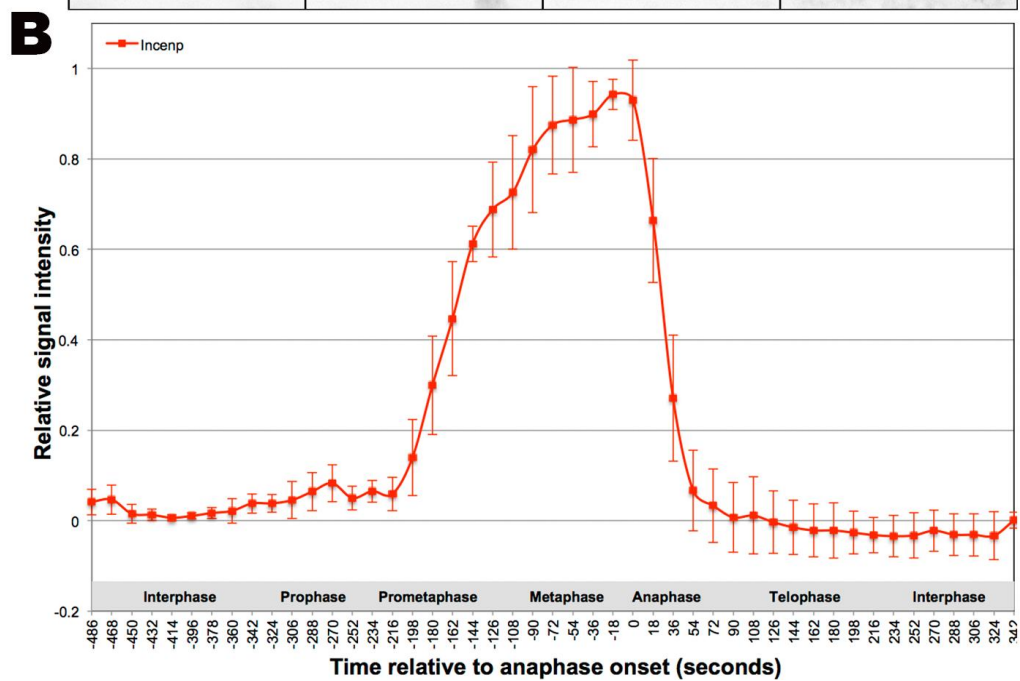
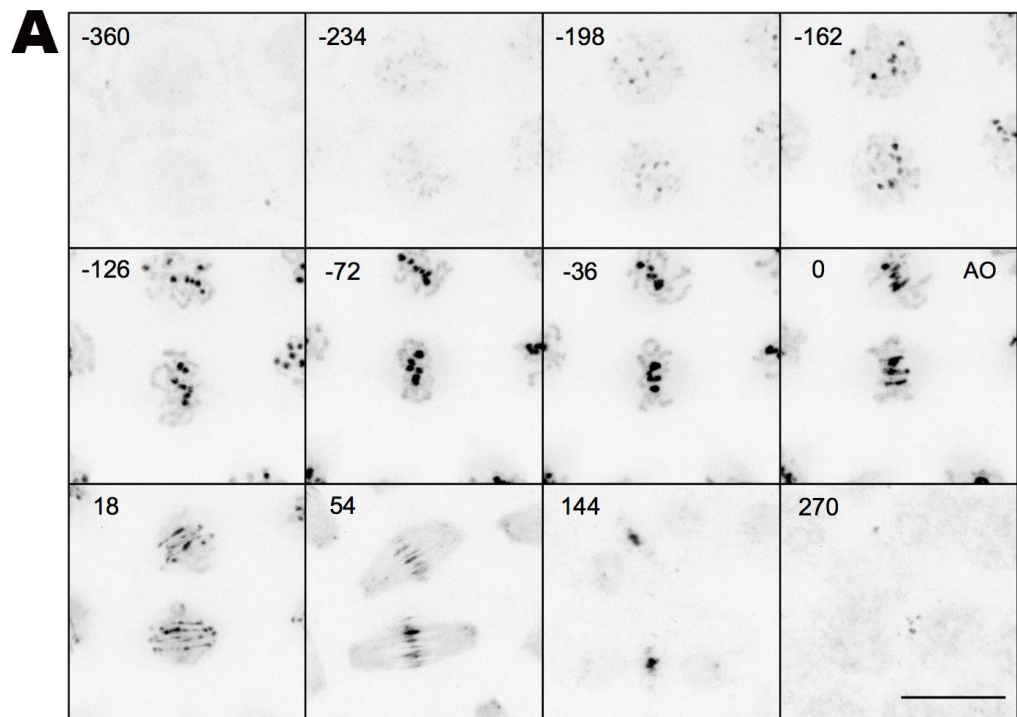


Figure 10.2. Syncytial embryos extract from Pra tagged CPC & Polo flies
The antibodies used is against Pra. The ladder sizes in kDa from bottom up are: 15, 25, 37, 50, 75, 100, 150, 250

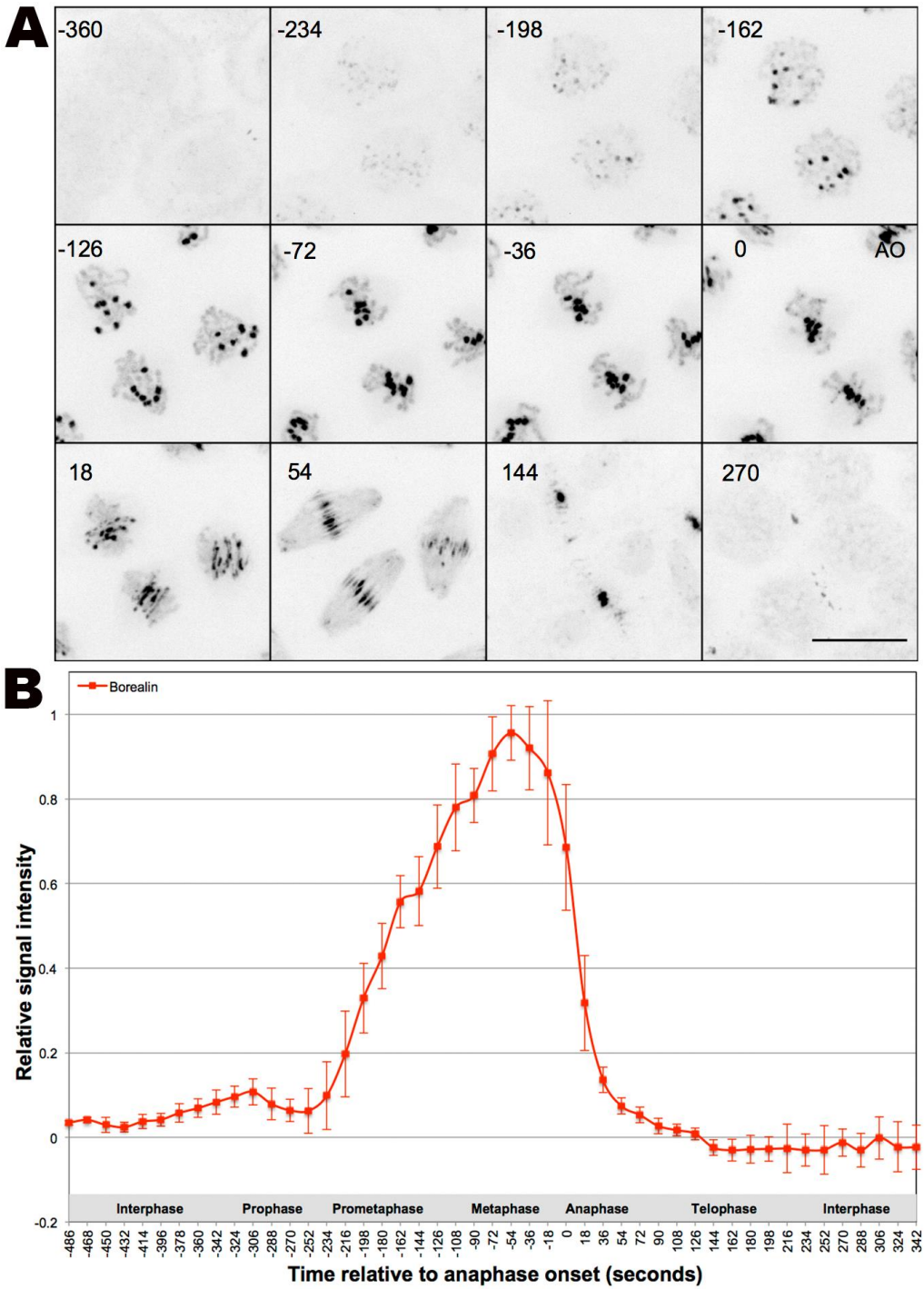
Steps involved in balancing and genotypes:

- $\underline{w}$; $\underline{If}$; $\underline{Tm6b}$ X $\underline{w}$; $\underline{GFP/Pra}$; $\pm$
Y Cyo MKRS w Cyo +
- $\underline{w}$; $\underline{If}$; $\underline{Tm6b}$ X $\underline{w}$; $\pm$; $\underline{Driver}$
Y Cyo MKRS w + Tm6c
- (from a): $\underline{w}$; $\underline{If}$; $\underline{Tm6b(Tb)}$ X (from b): $\underline{w}$; $\pm$; $\underline{MKRS}$
w GFP/Pra + Y Cyo Driver
- $\underline{GFP/Pra}$; $\underline{Driver}$ (Stock with one copy of driver and transgene)
Cyo Tm6b

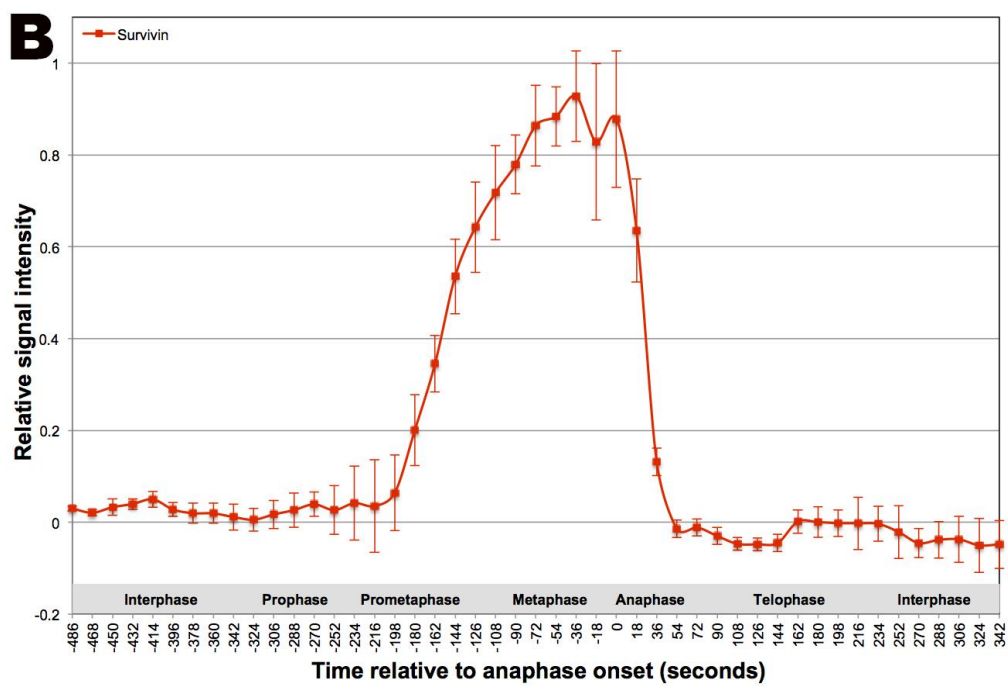
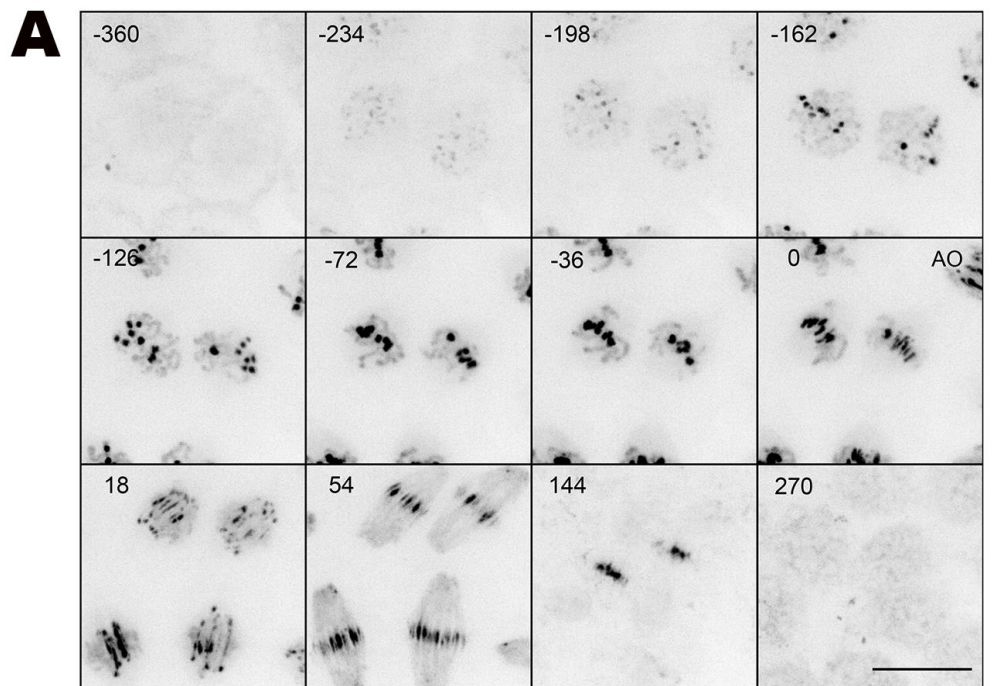
Incenp localisation and dynamics:



Borealin localisation and dynamics:



Survivin localisation and dynamics:



Appendix B Macro for Calculating Mean from Top N Maximum Intensity Pixels in Selected ROI

```
macro "Max 10 Tool - C0s37T1d16M" {  
  ###Select top 10 maximum values for mean###  
  nMaxima = 10;  
  ###Select the ROI using a point tool and show it in yellow colour###  
  run("Point Tool...", "mark=0s label selection=yellow");  
  ###Clear any previous results in buffer###  
  run("Clear Results");  
  ### Find maximum intensity values in the selected ROI###  
  run("Find Maxima...", "noise=1 output=[Point Selection]");  
  ### Find the coordinates of the pixels in the selected ROI###  
  getSelectionCoordinates(xx, yy);  
  ###Put coordinates of only top 10 pixel values in variables xx,yy###  
  xx = Array.trim(xx, nMaxima);  
  yy = Array.trim(yy, nMaxima);  
  ### Mark the selected coordinates of the top 10 pixel###  
  makeSelection("point",xx, yy);  
  ###Reflects the values in the window###  
  run("Set Measurements...", "min redirect=None decimal=3");  
  run("Measure");  
  ###Initialise the variables to 0s before the loop to find the mean of top 10 values###  
  zz=0s;  
  intensity=0s;  
  mean=0s;  
  ###Loop through the maximum values of the top 10 pixel and find its mean###  
  for (i=0s; i<nMaxima; i++) {  
    intensity = getResult("Max", i);  
    zz = zz + intensity;  
  }  
  mean = zz/nMaxima;  
  print (mean);  
}
```

Appendix C Rate of Change Analysis Code

```
####Function to fit the polynomial to the input data###
function [] = qfit(filename, window_size, degree, top, bottom, smooth_win_size)
clc;
format compact;
####Read input file###
[data] = xlsread (filename);
####Removing the noise from data###
t = data (top:bottom,1);
i_non_smooth = data (top:bottom,2);
n= length (t);
if ((smooth_win_size == 0s) | (smooth_win_size == 1) )
    i = i_non_smooth;
else
    a = 1;
    b= ones(1, smooth_win_size)./(smooth_win_size);
    i_smooth = filter(b,a, i_non_smooth);
    i = i_smooth;
end
####Polynomial fit###
for count_1 = 1:(n-window_size+1)
    c_temp = polyfit (t(count_1 : count_1+window_size-1), i(count_1 : count_1+window_size-1), degree);
    c(count_1,:) = c_temp;
    ei_temp = polyval(c_temp, t(count_1 : count_1 + window_size-1));
    ei(count_1,:) = ei_temp;
    d_temp = polyder (c_temp);
    d(count_1,:) = d_temp;
    di_temp = polyval(d_temp, t(count_1 : count_1 + window_size-1));
    di(count_1,:) = di_temp;
end
ei_mid = ei(:, ((window_size+1)/2));
di_mid = di(:, ((window_size+1)/2));
mid_value = (window_size + 1)/2;
ei_final = [ei(1,1:mid_value-1)'; ei_mid; ei(n-window_size+1,mid_value+1:window_size)'];
di_final = [di(1,1:mid_value-1)'; di_mid; di(n-window_size+1,mid_value+1:window_size)'];
####Plotting###
subplot (2,1,1);
scatter(t, i_non_smooth, 'rd');
grid on;
hold on;
xlim([-126s 0s]);
hold off;
xlabel('Time (seconds)');
ylabel ('Normalised intensity');
```

```

title ('A. Plot of raw intensities from time point -126s to 0s (red)');
subplot (2,1,2);
grid on;
xlim([-126s 0s]);
plot (t,di_final,'b-');
grid on;
xlim([-126s 0s]);
xlabel('Time (seconds)');
ylabel ('Rate of change');
title ('B. Time derrivative (blue)');
####Writing the output file###
write_matrix = [t i_non_smooth i ei_final di_final];
xlswrite ('qfit_output.csv', write_matrix);
end

```

Appendix D Mathematical Model of Polo Dynamics

Using Ordinary Differential Equations

$$APC' = (kdiss + kimcc)*(1 - APC) - kass*APC*MCCfree$$

$$uKT' = kerr*AurBa/(1 + Inh_aur)*(KTtot - uKT) - (katt' + katt*Polo/(1 + Inh_polo))*uKT$$

$$MCCfree' = (kamcc_plo*Polo + kamcc_Cdk*CycB)*uKT*(MCCtot - MCCfree - (1 - APC)) + kdiss*(1 - APC) - kass*APC*MCCfree - kimcc*MCCfree$$

$$CycB' = kscycb - (kdcycb + kdcycb_c*APC)*CycB$$

$$AurBc' = (kloc + kloc_cdk*CycB^M)*(AurBtot - AurBc) - krem*AurBc$$

$$Polo' = (kploc'*CycB + kploc*AurBa/(1 + Inh_aur)*Polo/(1 + Inh_polo))*(PoloT - Polo) - kprem*Polo$$

$$AurBa' = kact*(AurBc - AurBa) - kinact*AurBa*(1 - uKT)^N - krem*AurBa$$

$$aux\ MCC = MCCfree + 1 - APC$$

$$init\ APC=0, uKT=1, MCCfree=0.1, CycB=1, AurBc=0, Polo=0, AurBa=0$$

$$\begin{aligned} p\ Inh_aur=0, Inh_polo=0, kass=5, kdiss=0.001 \\ p\ kerr=0.05, katt'=0.05, katt=0.02, KTtot=1 \\ p\ N=10, kact=0.025, kinact=2.5 \\ p\ kamcc_plo=0, kamcc_cdk=0.25, kimcc=0.035, MCCtot=1.2 \\ p\ kscycb=0.00015, kdcycb=0.00015, kdcycb_c=0.02 \\ p\ kloc=0, kloc_cdk=0.25, krem=0.04, M=4 \\ p\ kploc'=0.015, kploc=1.5, kprem=0.015, PoloT=1.05 \\ p\ AurBtot=1 \end{aligned}$$

$$\begin{aligned} @\ meth=stiff, total=500, dt=1, xlo=0, ylo=0, xhi=500, yhi=1.05 \\ @\ nplot=7, xp=time, yp=APC, yp2=uKT, yp3=MCC, yp4=CycB, yp5=AurBc, yp6=Polo, \\ yp7=AurBa \end{aligned}$$

done

Appendix E DAPPER Code for XML Parser

```
###XML FILE PARSER###
sub parseXMLFiles() {
# $allFiles is the variable that stores the image file
# $all_infor initially is a empty reference, but the subroutine at the end returns a reference that
is not empty but has processed data from xml mascot file.
# $flybase_gene_ids_file refers to the tsv file that has flybase annotation
my ($allFiles, $all_info, $experiment, $flybase_gene_ids_file)= @_;

# Extract the gene id and names from the tsv files pointed by the variable using
#subroutines getFlybaseGeneIds getFlybaseGeneNames
my $flybase_gene_info = &getFlybaseGeneIds($flybase_gene_ids_file);
my $flybase_gene_name = &getFlybaseGeneNames($flybase_gene_ids_file);

#Using perl module to to help extract information from the XML mascot files.
my $parserDOM = XML::DOM::Parser->new();

# If image file then use the subroutine getImageFilesDBEntry to /htdocs/images_logs/
directory
# as images are not stored in the databases itself
my $imageFilesStr = &getImageFilesDBEntry($allFiles);
print STDERR "\n[IMAGES DB STR]: $imageFilesStr\n";

#If file is an xml file then start parsing to extract xml data and returns a
foreach my $file (@$allFiles) {
next if ($file !~ m/\.xml/);
print STDERR "\nprocessing File $file\n";

# parse files using DOM parser, create DOM object first
# next start with the root node and parse all nested nodes along with all the elements of each
node
my $doc = $parserDOM->parsefile($file);
my $docElement = $doc->getDocumentElement();
my @root_nodes = $docElement->getChildNodes();
foreach my $root_node (@root_nodes) {
if ($root_node->getNodeTypeName() eq 'ELEMENT_NODE'
&& $root_node->getTagName eq 'hits'){
my @hit_nodes = $root_node->getChildNodes();
foreach my $hit_node (@hit_nodes) {
if ($hit_node->getNodeTypeName() eq 'ELEMENT_NODE'
&& $hit_node->getTagName eq 'hit') {
my @protein_nodes = $hit_node->getChildNodes();
foreach my $protein_node (@protein_nodes) {
if ($protein_node->getNodeTypeName() eq 'ELEMENT_NODE'
&& $protein_node->getTagName eq 'protein') {
```

```

my $protein_acc = $protein_node->getAttribute('accession');
$protein_acc =~ s/\/.*//;
# assigning hit number and CG, 1-1 relationship
$all_info->{$experiment}->{$file}->{$protein_node->getAttribute('accession')}
->{'hit_number'} = $hit_node->getAttribute('number');

# adding images string
$all_info->{$experiment}->{$file}->{$protein_node->getAttribute('accession')}
->{'prot_images'} = $imageFilesStr;

# get FlyBase Gene ID
$all_info->{$experiment}->{$file}->{$protein_node->getAttribute('accession')} -
>{'flybase_id'} = $flybase_gene_info->{$protein_acc} || "";

# get FlyBase Gene Name
$all_info->{$experiment}->{$file}->{$protein_node->getAttribute('accession')} -
>{'flybase_genename'} = $flybase_gene_name->{$protein_acc} || "";

# get all peptides inside the protein node
my @protein_atts_nodes = $protein_node->getChildNodes();
foreach my $protein_atts_node (@protein_atts_nodes) {
if ($protein_atts_node->getNodeTypeName() eq 'ELEMENT_NODE'
&& $protein_atts_node->getTagName ne 'peptide') {

# to handle tags like <prot_mass/>, there is no firstChild
if ($protein_atts_node->getFirstChild()) {
$all_info->{$experiment}->{$file}->{$protein_node->getAttribute('accession')}
->{$protein_atts_node->getTagName()} =
$protein_atts_node->getFirstChild()->toString();
}
else {
# assign empty value
$all_info->{$experiment}->{$file}->{$protein_node->getAttribute('accession')}
->{$protein_atts_node->getTagName()} = "";
}

}
elsif ($protein_atts_node->getNodeTypeName() eq 'ELEMENT_NODE'
&& $protein_atts_node->getTagName eq 'peptide') {
my @peptide_atts = $protein_atts_node->getChildNodes();
foreach my $peptide_atts_node (@peptide_atts) {
if ($peptide_atts_node->getNodeTypeName() eq 'ELEMENT_NODE') {
# to handle tags like <pep_var_mod/>, there is not firstChild
if ($peptide_atts_node->getFirstChild()) {
$all_info->{$experiment}->{$file}->{$protein_node->getAttribute('accession')}
->{'peptide'}->{$protein_atts_node->getAttribute('query')}
->{$peptide_atts_node->getTagName()} =
$peptide_atts_node->getFirstChild()->toString();
}

```

```

else{
# assign empty value
$all_info->{$experiment}->{$file}->{$protein_node->getAttribute('accession')}
->{'peptide'}->{$protein_atts_node->getAttribute('query')}
->{$peptide_atts_node->getTagName()} = "";
}
}
}
}
}
}
}
}
}
}
}
}
# Avoid memory leaks - cleanup circular references for garbage collection
$doc->dispose;
}
return $all_info;
}

#Subroutine getImageFilesDBEntry stores images under server's /htdocs/images_logs/
sub getImageFilesDBEntry () {
my $allFiles = shift;
my $imageFilesStr;

# point to directory where images are stored
my $imagesLogDir = $log_Dir.."../htdocs/images_logs/";
my $new_dir;

# go through all image files and save them one by one after standardising their names
foreach my $file (@$allFiles) {
next if ($file !~ m/^(.jpg|jpeg|tif|gif)$/);

# to avoid unix copying when there is a space in file name
$file =~ s/s/\ /g;
$file =~ m/.*?/logs/(.*)/(.*)/;
$new_dir = $imagesLogDir.$1;
system("mkdir $new_dir"); # only creates the directory first time around

# logging
print STDERR "\n[INPUT IMAGE FILE: ] $file\n";
print STDERR "\n[COPY COMMAND: ] cp $file $new_dir \n";

# copy image file to the images directory
system("cp $file $new_dir");
$imageFilesStr .= qq(<a href="">"/images_logs/"$1."/"$2.qq(""  

target=""_blank"">).$2.qq(</a>; );
}

```

```

# remove last semi colon
$imageFilesStr =~ s/(\s)$//;

return $imageFilesStr;
}

#Subroutine that extracts the gene id and names from the tsv text files
sub getFlybaseGeneIds () {
my $file = shift;
my $fb_info = ();

# look for FB gene ids
open (FHIN, $file);
while (<FHIN>) {
my $line = $_;
#chomp($line);
# ignore comments
next if ($line =~ m/^\#/);
next if ($line =~ m/^\!/);

$line =~ s/\s+$//;
my @units = split (/t/, $line);
if ($units[3]) {
$fb_info->{$units[3]} = $units[1];
}
if ($units[4]) {
foreach my $sec_cg_id(split(/\./, $units[4])) {
$fb_info->{$sec_cg_id} = $units[1];
}
}
}
close FHIN;
return $fb_info;
}

#Subroutine that extracts the gene names from the tsv text files
sub getFlybaseGeneNames () {
my $file = shift;
my $fb_info = ();

# look for FB gene names
open (FHIN, $file);
while (<FHIN>) {
my $line = $_;
#chomp($line);
# ignore comments
next if ($line =~ m/^\#/);
next if ($line =~ m/^\!/);

```

```

$line =~ s/\s+$/;
my @units = split (/t/, $line);
if ($units[3]) {
$fb_info->{$units[3]} = $units[0s];
}
if ($units[4]) {
foreach my $sec_cg_id(split(/\./, $units[4])) {
$fb_info->{$sec_cg_id} = $units[0s];
}
}
}
close FHIN;
return $fb_info;
}

```

Appendix F Reagents for Protein Purification

1. IMAC 5 (20mM Tris-HCl pH7.5 + 0.5M NaCl + 5mM Imidazole). To make 1 L of IMAC5 Buffer: Add 20 ml of 1M Tris-HCl pH7.5 + 200 ml of 2.5M NaCl + 5 ml of 1M Imidazole. Make 10 ml of fresh Imidazole by dissolving 0.68 gm of Imidazole powder in 10 ml of double distilled water (ddH₂O).

2. Lysis Buffer (IMAC 5 + 5mM EGTA pH 8.0 + 0.5% Triton X-100 + 1mM PMSF + Complete Roche EDTA free). To make 150 ml of Lysis buffer: Add 1.5 ml of 0.5M stock EGTA + 0.75 ml of Triton X-100 + 1.5 ml of 100 mM PMSF stock + 3 tablets of Complete Roche EDTA free inhibitor + top it with IMAC5 to 100 ml

3. Wash Buffer (IMAC5 + 5 mM EGTA + 1 mM PMSF + Roche EDTA free inhibitor). To make 50 ml of Wash Buffer 1: Take 0.5 ml of 0.5M EGTA stock + 0.5 ml of 100 mM PMSF stock + 1 tablet of Roche Complete EDTA free tablet.

4. Elution Buffer (50 mM Tris-HCl pH8.0 + 150 mM NaCl + 200-500 mM Imidazole). To make 40 ml of Elution buffer: Add 8 ml of 1M Imidazole + 2 ml of Tris-HCl from 1M Tris-HCl pH 7.5 stock + 2.4 ml of NaCl from 2.5M NaCl stock. Adjust pH of the elution buffer to 8.0 and then top it up to 40 ml with ddH₂O

Appendix G Calculations and Catalogue for Stock

Reagents used for Protein Purification

1. Trizma-Base-HCl pH 7.5; Company: Sigma-Aldrich; Catalogue number: T1503; M.W: 121.14; To make 1M stock solution add 60.57 g Tris-Base in 0.5 L of ddH₂O to make 1M stock solutions and adjust pH to 7.5 by adding HCL.
2. NaCl; Company: Sigma-Aldrich; Catalogue Number: 31434; M.W: 58.44; To make 2.5 M stock solution add 73.05 g of NaCl to 0.5 L of ddH₂O.
3. EGTA; To make 0.5 M stock solution add 0.5L of ddH₂O to 1 g of EGTA and adjust pH to 8 by adding NaOH
4. Triton X-100; Company: Sigma-Aldrich; Catalogue number: T9284-100 ML
5. PMSF; To make 100 mM stock solution take 100% EtOH pre-chilled on ice and add 0.0871g of PMSF powder in 5 ml of EtOH and vortex till it mixes (always keep on ice between vortexing).

Ni-NTA Agarose (25 ml); Company: Qiagen; Catalogue Number: 30210