Université de Montréal

Spatiotemporal regulation of the Greatwall – PP2A axis is required for mitotic progression

par

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Thèse présentée à la Faculté de Médecine en vue de l'obtention du grade de PhD en Biochimie et Médecine Moléculaire

Septembre, 2014

Résumé

Le cycle cellulaire est hautement régulé par la phosphorylation réversible de plusieurs effecteurs. La kinase dépendante des cyclines Cdk1 déclenche la mitose en induisant le bris de l'enveloppe nucléaire, la condensation des chromosomes et la formation du fuseau mitotique. Chez les animaux métazoaires, ces évènements sont contrés par la protéine phosphatase PP2A-B55, qui déphosphoryle plusieurs substrats de Cdk1. La kinase Greatwall (Gwl) est activée par le complexe cycline B-Cdk1 en début de mitose et induit ensuite l'inhibition de PP2A-B55 via Endos/Arpp19. Toutefois, les mécanismes moléculaires qui régulent Gwl sont encore peu connus.

Nous avons montré que Gwl a une activité s'opposant à PP2A-B55, qui collabore avec la kinase Polo pour assurer l'attachement du centrosome au noyau et la progression du cycle cellulaire dans le syncytium de l'embryon de la drosophile. Ensuite, nous avons trouvé dans des cellules de drosophile que Gwl est localisée au noyau pendant l'interphase, mais qu'elle se relocalise au cytoplasme dès la prophase, avant le bris de l'enveloppe nucléaire. Nous avons montré que cette translocation de Gwl est cruciale pour sa fonction et qu'elle dépend de la phosphorylation de plusieurs résidus de la région centrale de Gwl par les kinases Polo et Cdk1. Cette région centrale contient également deux séquences de localisation nucléaire (respectivement NLS1 et NLS2). De plus, nos résultats suggèrent que la phosphorylation de Gwl par la kinase Polo promeut sa liaison avec la protéine 14-3-3ε, ce qui favorise la rétention cytoplasmique de Gwl. Le rôle de Cdk1 dans cette translocation reste quant à lui inconnu. De plus, nous avons montré que le complexe cycline B-Cdk1 entre dans le noyau avant que Gwl ne soit transportée dans le cytoplasme. Cdk1 pourrait donc activer Gwl et phosphoryler ses substrats nucléaires, à l'abri de PP2A-B55 qui est largement cytoplasmique. Gwl est ensuite exclue du noyau et relocalisée dans le cytoplasme afin d'induire l'inhibition de PP2A-B55. Cela permet de synchroniser les événements de phosphorylation se produisant dans le noyau et dans le cytoplasme. Fait intéressant, un mécanisme de régulation de la localisation de Gwl similaire à cela a été découvert chez l'humain et chez la levure, suggérant que ce mécanisme est conservé entre différentes espèces.

Mots clés: Greatwall (Gwl), Cycline B-Cdk1, PP2A-B55, Cycle cellulaire, Mitose.

Abstract:

Reversible phosphorylation of proteins, triggered by cyclically activated kinases and phosphatases, is a key mechanism to control cell cycle progression. CyclinB-Cdk1 is a crucial kinase phosphorylating a large number of substrates to trigger mitotic entry. However, in metazoans, it is counteracted mainly by a Protein Phosphatase 2A carrying the B55 regulatory subunit (PP2A-B55). On the other hand, the Greatwall (Gwl) kinase is activated by CyclinB-Cdk1 upon mitotic entry and subsequently induces the inhibition of PP2A-B55 by Endos/Arpp19, thus promoting mitotic entry and maintenance. Nonetheless, the regulatory mechanisms of Gwl are less clear.

We demonstrated that in *Drosophila* syncytial embryos, PP2A-B55 is negatively regulated by Gwl, but collaborates with Polo kinase to ensure both nucleus attachment of centrosome and faithful cell cycle progression. Later, we discovered that in *Drosophila*, the subcellular localization of Gwl changes dramatically throughout the cell cycle. Gwl is nuclear in interphase but suddenly becomes mostly cytoplasmic in prophase before nuclear envelope breakdown. Such translocation is important for Gwl's function and requires the phosphorylation of Gwl by both Polo kinase and Cdk1 in the region containing two Nuclear Localization Signals (NLSs). Phosphorylation of Gwl by Polo likely promotes its association with14-3-3ε thereby promoting Gwl cytoplasmic retention, whereas Cdk1's role in this translocation remains elusive. Moreover, I found that most cyclin B is imported into the nucleus before Gwl translocates to the cytoplasm. Therefore, Cdk1 can activate Gwl and phosphorylate its nuclear substrates without the perturbation of PP2A-B55 which is largely cytoplasmic. Subsequently, Gwl translocates into cytoplasm to mediate the inhibition of PP2A-B55 so that the phosphorylation events can be synchronized between the nucleus and the cytoplasm. Interestingly, similar spatial regulation of Gwl was also uncovered in mammal cells and in yeast, implying a conserved regulatory mechanism across species.

Key words: Greatwall (Gwl)**,** CyclinB-Cdk1, PP2A-B55**,** Cell cycle**,** Mitosis.

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ABBREVIATIONS LIST

Arpp: cAMP-regulated phosphoprotein

APC/C: Anaphase Promoting Complex/Cyclosome

ATM: Ataxia Telangiectasia Mutated

ATP: Adenosine Triphosphate

ATR: Ataxia Telangiectasia Related

CAK: Cdk Activating Kinase

cAMP: cyclic Adenosine MonoPhosphate

Cdc: Cell division cycle

Cdh1: Cdc20 homolog 1

Cdk: Cyclin dependent kinase

Chk: Checkpoint Protein Kinase

CKI: Cdk Inhibitor

DNA: DeoxyriboNucleic Acid

Endos/Ensa: α-Endosulfine/Endosulfine alpha

FEAR: Cdc Fourteen Early Anaphase Release

GFP: Green fluorescent protein

Gwl: Greatwall

kD: kiloDalton

MEN: Mitotic Exit Network

MPF: Maturation Promoting Factor

MTOC: Micro Tubule Organizing Center

NEBD: Nuclear Envelop BreakDown

NES: Nuclear Export Signal

NLS: Nuclear Localization Signal

OA: Okadaic Acid

PLK: Polo-Like Kinase

PP2A: Protein Phosphatase 2A

PP1: Protein Phosphatase 1

pRb: Retinoblastoma Protein

RNA: RiboNucleic Acid

SAC: Spindle Assembly Checkpoint

SCF: Skp/Cullin/F-Box

TORC1: Tor complex 1

ACKNOWLEDGEMENTS

First, I would like to thank Dr Vincent Archambault, not just because he gave me the opportunity to perform my PhD training in his laboratory, he also helped me so much throughout the course of my PhD projects. He not only taught me almost all the techniques frequently used during my PhD training but also guided me in my fellowship applications, my postdoctoral application, my English, my French, writing techniques…… Without his generous help, I need certainly several years more to complete this thesis.

I am also thankful to all the past and present members of the Archambault's laboratory. Especially, I want to thank to very kind Xavier Pinson and adorable Karine Normandin, they helped me so much and it is always very enjoyable to talk with them. I want to thank to very talented David Kachaner, nice Maxime Cormier, hard-working Haytham Mehsen, and young elites Myreille Larouche, Élizabeth Michaud and Guillaume Lépine. It is very agreeable to work with all these wonderful people and I am grateful for their support and friendship. Thanks Haytham for his very helpful advices for my English writing.

I would like to thank the members of my thesis committee, Dr Gerardo Ferbeyre, Dr Eric Lécuyer, Dr Philip Branton, for taking time to evaluate my work. I would like to thank to Dr Jacques Montagne, Dr Jean-Philippe Parvy, Dr Annie Sainsard-Chanet, Dr Christian Vélot, without their help and support I couldn't get the chance to continue my PhD study. I would like to thank Dr Marc Therrien, Dr Sébastien Carréno, Dr Gregory Emery, Dr Benjamin Kwok, Dr Damien D'Amours and all fly-lab members, for their constructive advices and guidance for my research projects.

Finally, I would like to thank to my loving wife Haijin, she encouraged me to continue my study in biology. I am so sorry to have left her alone for numerous weekends and evenings because of lab work, her support and consideration have been essential in the last five years. I also want to thank my lovely daughter Emma Lujia for her sweet smile what's her special encouragement to me. I thank my father Chuanfa and my mother Guizhi for their selfless love and support.

CHAPTER 1

INTRODUCTION

Background and general organization of chapters

When I began my PhD training in Dr. Archambault's laboratory in 2009, it has been shown that the Greatwall kinase (Gwl) is required for the inhibition of an unknown okadaic acid (OA) ─ sensitive phosphatase to promote Cdc25 activation and M phase entry in *Xenopus* egg extracts. However, the precise molecular mechanisms by which Gwl functions were still unclear. On the other hand, Dr. Archambault has discovered that Gwl genetically antagonizes Polo kinase and PP2A-Tws (Tws is the sole member of the B55 regulatory subunit familly in *Drosophila*), whereas Polo and PP2A-Tws genetically collaborate with each other in *Drosophila* syncytial embryos. However, the nature of these interactions remains uncharacterized. Soon I demonstrated that Polo activity affects Gwl phosphorylation state, whereas Gwl activity has no clear effect on Polo. This result led me to suppose that Gwl appears to be an antagonist of Polo because it strongly antagonizes the potent collaborator of Polo ─ PP2A-Tws, whereas Polo might be a potential regulator of Gwl. Thus, I carefully studied the interplay between Polo and PP2A-Tws and the interaction between Gwl and PP2A-B55, as well as the possible role of Polo in the regulation of Gwl. My work was published in PLoS Genetics (2011) and the Journal of Cell Biology (2013).

Although numerous emerging articles indicated that Gwl kinase is important for mitotic progression, Gwl can't replace cyclin B-Cdk1 to command mitotic entry. Clear evidences support the notion that Gwl is just an assistant of cyclin B-Cdk1 by opposing the activity of PP2A-B55 at mitotic entry. Indeed, Gwl can even be bypassed under certain conditions in *Drosophila* and is known to be absent in *C.elegans*. Therefore, despite the fact that Gwl is the main interest of my PhD, I described in detail the regulation of cyclin-Cdks complexes, particularly the regulation of cyclin B-Cdk1 and the involved reversible phosphorylation control mechanisms in mitosis. Moreover, as my background is clinical medicine, ecology and genetics rather than cell biology, herein I would like to take advantage of my thesis redaction to further increase my knowledge about the cell cycle. That's the reason why I started my thesis with a comprehensive text-book like introduction (chapter 1**)** about the cell cycle (section **1.1**) and its regulatory mechanisms (section **1.2**). This introduction refers to early studies and reviews and will be useful for understanding my thesis work. For example, I described the centrosome cycle and gave some details about microtubule dynamic because PP2A-B55 is involved in the

regulation of these events, etc. In **section 1.3**, I described why *Drosophila* is an excellent model organism for cell cycle study and some approaches that were applied in flies in my PhD work. In **section 1.4**, I described in detail the main reversible phosphorylation events involving Cdk1-Cdc25-Wee1/Myt1-PP2A at mitotic entry, and I also delineated mathematical models which are helpful for understanding the switch-like and irreversible mitotic entry. Although we recently published a review on a similar subject to **section 1.5**, here I presented differently the Greatwall kinase and PP2A-B55 phosphatase axis in mitotic progression control. In this section, I highlighted the role of PP2A-B55 in mitotic progression as well as its other functions and its regulatory mechanisms, and also described in details the functions of Gwl and its regulatory mechanisms.

In chapter 2, I presented my work published in PLoS Genetics in 2011, where we demonstrated that Polo collaborates with PP2A-Tws to ensure correct centrosomes attachment to nuclei and nuclei divisions. We also showed that Gwl antagonizes PP2A-Tws in both meiosis and mitosis.

In Chapter 3, I presented my work published in The Journal of Cell Biology in 2013, where I found that the localization of Gwl is nuclear in interphase and suddenly becomes mostly cytoplasmic in prophase before nuclear envelope breakdown (NEBD). Such translocation is important for Gwl's function and requires the phosphorylation of Gwl by both Polo and Cdk1 in the region containing two Nuclear Localization Signals (NLSs). Phosphorylation by Polo likely promotes the binding of Gwl to 14-3-3ε which subsequently targets Gwl to cytoplasm. I also found that Cdk1 regulates Gwl translocation from the nucleus to the cytoplasm.

In Chapter 4, I discussed some major questions which had not been discussed in our published articles but yet remain to be clarified. I also discussed some recent but still preliminary results obtained during the redaction of my thesis. Finally, in the perspective section, I described experiments which can be performed to further advance our knowledge following the accomplished work.

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1.1 The cell cycle

All living organisms are made of a basic structural and functional unit ─ the cell. Many multi-cellular organisms are developed from one single fertilized cell. The current estimation of total number of cells in a human body is 3.72×10^{13} [1]. Moreover, new cells are constantly generated to replace old ones at a rate of millions per second. Such tremendous proliferation is ensured by a fundamental process, the cell cycle. **The Cell cycle** consists of a series of highly regulated events leading to the division of one mother cell to two genetically identical daughter cells. In eukaryotes, the cell cycle is composed of two main steps: interphase, during which cell grows, doubles its DNA and prepares materials for cell division; and an M phase (mitosis plus cytokinesis), during which the replicated DNA is equally distributed into the two daughter cells. In the paragraphs below, I will describe different phases of the cell cycle

1.1.1 Interphase

As mentioned above, **interphase** is the phase during which DNA replication takes place. The cell cycle spends most of its time in interphase. In a canonical cell cycle, interphase can be divided into two gap phases (G1 and G2) and one DNA synthesis phase (S) (Figure 1.1).

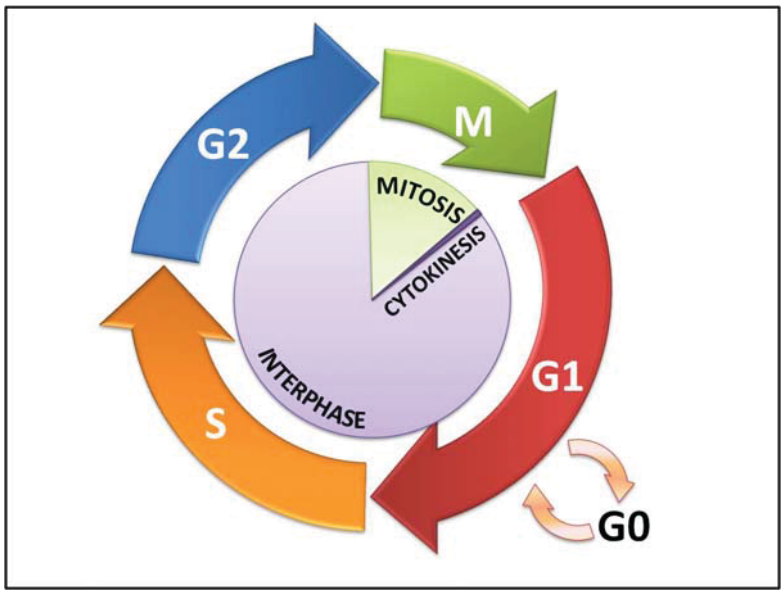


Figure 1.1. The cell cycle phases. The cell cycle has two major phases: interphase, the phase taking place between different mitotic events; and M phase, during which a nuclear division (mitosis) is followed by a cytoplasmic division (cytokinesis). G1: gap phase 1. G0: a specialized resting state in G1. S: the phase during which DNA synthesis occurs. G2: gap phase 2. M phase: mitosis, at the end of which cytokinesis takes place. The G1-S-G2 is collectively called interphase.

The **G1** phase is the first step of the archetypical cell cycle in eukaryotic cells. During this phase, the cell constantly takes in nutrients, increases its size, and manufactures RNAs and proteins. The G1 phase is largely restrained by environmental conditions such as growth factor and temperature. Under unfavourable conditions, G1 could be dramatically prolonged and the cells can even enter a quiescent state called **G0**. Whether cells enter in G0 or progress into S phase is determined by a single point named by Pardee as the restriction point (R) [2]. Once this point is crossed, the cell will progress into S phase independent of external conditions such as growth factors. Actually, the period between the initiation of the G1 phase and the restriction point is the only part of the cell cycle that must be growth factor dependent [3].

The **S** (synthesis) phase is an essential step of the cell cycle, during which DNA replication takes place once and only once exactly. During DNA replication, cells continue to synthesize proteins required for DNA replication, DNA repair and nascent DNA assembly [4]. Parallel to the replication of DNA, the centrosome is also duplicated. The **centrosome**, principal **M**icro**t**ubule **O**rganizing **C**enter (MTOC) of animal cells, consists of a pair of centrioles surrounded by a cloud of amorphous **p**eri**c**entriolar **m**aterial (PCM) which nucleates most microtubules of the cell [5]. In S phase, centrioles are less capable of accumulating PCM consequently leading to reduced microtubule nucleation capacity of the centrosome [6].

The **G2** phase is the last step of interphase, during which cells continue to grow and synthesize proteins required for cell division. During G2 phase, centrioles continue to accumulate more PCM proteins and γ-tubulin ring complexes which consequently nucleate more microtubules to form the radial asters. Parental centrioles begin to disconnect from each other and finally generate two distinct centrosomes. G2 is also the last chance for the cell to determine whether it's ready for mitosis.

However, gap phases can be absent in some specific developmental strategies employed by many organisms. For example, in *Drosophila* and *Xenopus* syncytial embryos, the cell cycle lacks the two gap phases, and is known as S/M cycle. Therefore, there is only a very short interphase consisting exclusively of an S phase. Such S/M cell cycle relies on maternally deposited stockpiles of mRNA and proteins during oogenesis. Thus, the genes that are normally expressed in gap phases are no more necessary. However, nuclei progressively reduce their size

because embryos have no external source of nutrition and completely depend on reserves within the egg [7].

1.1.2 M phase

During **M** phase, the cell stops growing but undergoes the most dramatic architectural rearrangements in the cell cycle, such as chromosome condensation and mitotic spindle establishment etc. The M phase is composed of two major events: nucleur division (mitosis) and cytoplasmic division (cytokinesis).

Mitosis is conventionally subdivided into five sequential phases defined mainly by the chromosomal behavior followed by microscopy [8] (Figure 1.2).

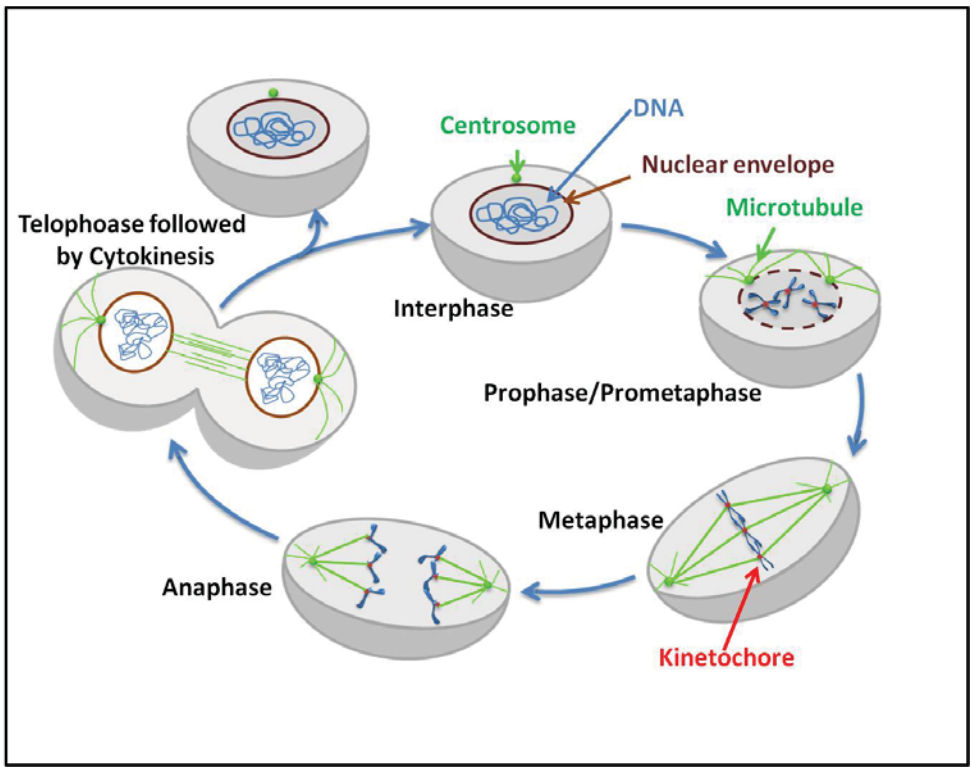


Figure 1.2. Different stages of mitosis followed by cytokinesis. Animal cell mitosis is divided into five stages: prophase, prometaphase, metaphase, anaphase, and telophase.

Prophase is the starting phase of mitosis, during which the chromatin threads condense and twist into visible chromosomes. Each chromosome consists of two sister chromatids connected at the centromere. The asters nucleated by centrosomes start to separate from each other across the nuclear envelope due to the lengthening of microtubules between them. The nuclear envelope provides a direct path for the separation of centrosomes and promotes the formation of the bipolar spindle in cultured human cells [9]. Meanwhile, microtubules organized by the

centrosome exert the pulling force that causes the nuclear envelope invaginations and facilitates the nuclear envelope breakdown (NEBD) [10]. NEBD defines the transition between prophase and prometaphase in higher eukaryotes.

Prometaphase is a transition period between prophase and metaphase. During this phase, the nuclear envelope breaks into small pieces which retract into the membrane system of the endoplasmic reticulum (ER) [11]. Asters further separation becomes dependent on the attachment to an actin- and myosin- based moving cell cortex at the plasma membrane [12]. When asters are aligned on either side of the chromosomes, they stop the migration. Some spindle microtubules emanating from opposite asters can now bind to chromosomes via kinetochores, which are composed of proteins attached to a centromere. Although chromosomes will undergo very active movements that are often described as 'the dance of chromosomes', they will ultimately congress to the spindle equator which represents the transition to the next phase - metaphase.

Metaphase, in most cells, is a very brief stage before anaphase. The chromosomes are aligned on the equatorial plate in the center of the spindle. The spindle consists of three distinct sets of microtubules whose minus ends point toward the centrosome. Astral microtubules radiate outward from the poles and interact with the cortex of the cell to help to position the mitotic spindle at the site of cell division [13]. Kinetochore microtubules emerge from the opposite asters and attach to sister chromatid pairs at kinetochore. Interpolar microtubules, which emanate from the opposite asters, do not link the chromosomes but instead interdigitate in the center of the cell. Shortly after chromosomal alignment, the link between the sister chromatids is broken and the cell enters anaphase.

At **anaphase**, new daughter chromosomes separate and move toward opposite poles of the cell, which is known as chromosome segregation. During early anaphase (anaphase A), the kinetochore microtubules shorten to pull the chromosomes toward the poles. During late anaphase (anaphase B), the daughter chromosomes are pushed farther apart due to the spindle elongation. This pushing force is exerted by both the interpolar microtubules and the astral microtubules.

Telophase is technically the last step of mitosis. During telophase, the two sets of daughter chromosomes reach the opposite poles. All kinetochore microtubules are degraded, but the polar microtubules continue to elongate. The nuclear envelope reforms around the two daughter nuclei. Finally, chromosomes begin to decondense back into chromatin.

1.1.3 Cytokinesis

Cytokinesis is a process during which the cytoplasm of the cell divides to form two daughter cells. In animal cells, cytokinesis usually starts in late anaphase characterized by the sudden appearance of the cleavage furrow at the equatorial cortex. The cleavage furrow contains a contractile ring, which forms beneath the plasma membrane and is mainly composed of actin and myosin II filament. The contraction of actin-myosin filaments tightens the ring by pulling the plasma membrane inward and eventually pinches the cell in two. Although cytokinesis occurs in most cell cycles, it can be omitted in some special developmental stages in some organisms. In *Drosophila* embryo, the first 13 rounds of cell cycle take place in a common cytoplasm without intervening cytokinesis, which greatly speeds up early development. The cell division is completed through a special process called cellularization. Since the tenth cycle, the nuclei migrate toward the cortex of the embryo. Finally, at the 14 cell cycle, a simultaneous ingression of membrane around each nucleus occurs in order to form the individual cells.

1.2 Cell cycle regulation

As described above, the cell cycle is a complex and orderly series of events leading to cell division. The whole process must be tightly regulated, and unchecked cell cycle may eventually lead to cancer. The essential events of the cell cycle, such as DNA replication and chromosomes segregation, are monitored by a cell cycle control system. This system consists of a network of regulatory proteins that govern the orderly progression of a eukaryote cell through the sequential stages of cell cycle. In the paragraphs below, I will describe the major regulatory mechanisms which ensure the faithful progression of the cell cycle.

1.2.1 The cyclins and cyclin-dependent kinases (Cdks) are the central components of the cell cycle control system.

1.2.1.1 Cdks and cyclins

Cyclin-dependent kinases (Cdks) play an essential role in driving cell cycle progression. Cdks are a family of serine/theronine protein kinases that add negatively charged phosphate groups (PO_4^{2-}) to a protein or other organic molecule in a process called phosphorylation. The cyclically activated and inactivated Cdks can trigger abrupt change in the phosphorylation state of the key proteins that initiate or regulate the essential processes of the cell cycle, such as DNA replication, mitosis, and cytokinesis. For example, in mammal cells, Cdk2 becomes active at the end of G1 phase, and it is responsible for driving the cell into S phase. Cdk1 activity dramatically increases at the beginning of mitosis, which is needed to trigger the phosphorylation of different mitotic substrates that control chromosome condensation, spindle assembly, nuclear envelope breakdown, etc. Thereafter, these kinases are inactivated, which allows the completion of mitosis and cytokinesis.

Cdks alone are inactive, unless they bind tightly to their regulatory proteins - cyclins. Cyclins binding activates Cdks by causing their structural rearrangements [14] (Figure 1.3). Unlike Cdks, whose concentrations are constant throughout the cell cycle, cyclins undergo a periodic synthesis and ubiquitin-mediated degradation [15]. Therefore, the cyclical change of Cdks activity largely depends on the oscillation in levels of cyclins during the cell cycle. There are four classes of cyclins (Table 1.1). Different cyclins are produced at different cell cycle stages to induce the assembly and the activation of a set of cyclin-Cdk complexes. Different cyclin-Cdk complexes subsequently trigger distinct steps in the cell cycle (Figure 1.4). Cyclins which help drive the cell through the restriction point R toward S phase are called G1 cyclins. Correspondingly, the active complexes that G1 cyclins form with their Cdks partners are called G1-Cdks. Respectively, G1/S-Cdks and S-Cdks promote S phase entry and initiate DNA replication while M-Cdks launch the phosphorylation events to trigger mitotic entry. Among the four classes of cyclins, G1 cyclins are the only ones whose concentrations depend on the extracellular signals rather than on specific phases of the cell cycle.

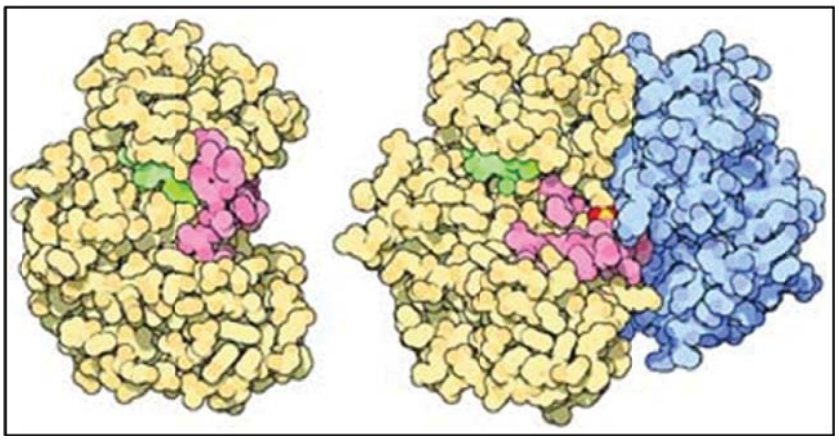
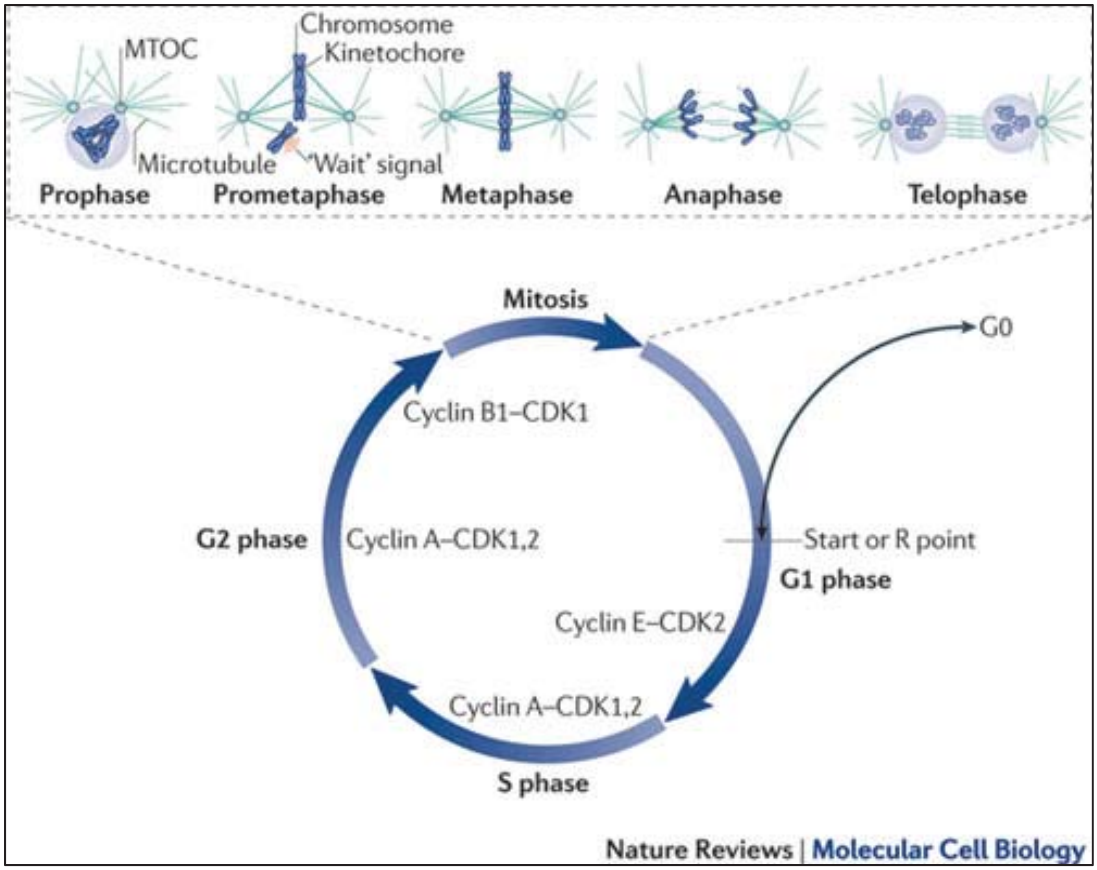


Figure 1.3. Cyclin and cyclin-dependent kinase. The kinase shown on the left has a deep groove that binds ATP, shown in green. ATP provides the phosphate that is transferred during the phosphorylation reaction. A loop of the kinase, shown in pink, folds up and blocks the active site when the enzyme is free. When cyclin (shown in blue on the right) binds, this loop is pried away, opening the active site and allowing the complex of cyclin and kinase to add phosphate groups to proteins in the cell-division machinery. The kinase enzyme itself is also activated by phosphorylation: a phosphate group (shown here in bright red and yellow) is added to the kinase loop to bring it into its fully active form. Coordinates were taken from entries 1hck and 1jst at the Protein Data Bank (http://www.pdb.org). This figure was adapted from David S. Goodsell, (2004) [16], with permission from the publisher John Willey & Sons.

Tableau 1.1. The major cyclins and Cdks in vertebrates. Note that there are also the subtypes of these cyclins.

Cyclin classe	Cyclin	Cdk partner
G1	Cyclin D	Cdk4, Cdk6
G1/S	Cyclin E	Cdk2
S	Cyclin A	Cdk2
M	Cyclin B	Cdk1

Figure 1.4. Each of the main phases of the cell cycle — G1, S (when DNA synthesis occurs), G2 and mitosis — is controlled by a cyclin–cyclin-dependent kinase (CDK) complex. In animal cells, the main cyclin–CDK complexes are formed by cyclin A, cyclin B and cyclin E. Cyclin B1–CDK1 triggers entry to mitosis, an expanded view of which is shown at the top of the figure. In prophase, the chromosomes condense, the centrosomes separate and the nuclear envelope breaks down. In prometaphase, the chromosomes attach to the mitotic spindle, and any unattached chromosomes generate a 'wait anaphase' signal. In metaphase, all the chromosomes attach, and the wait anaphase signal is turned off. In anaphase, the sister chromatids separate, and in



telophase the DNA decondenses, the nuclear envelope reforms and the two daughter cells form by cytokinesis. MTOC, microtubule-organizing centre; R, restriction. From Johnathon Pines (2011) [17], with permission from publisher Nature Publishing Group.

1.2.1.2 Cyclins regulate the substrate specificity of cyclin-Cdk complexes

The major differences among cyclins are thought to confer substrate specificity to cyclin-Cdk complexes [18]. For example, in fission yeast, M cyclin promotes mitosis whereas G1 cyclins do not [19], suggesting that different cyclin-Cdks complexes may have intrinsically distinct substrate preferences. One way that cyclins may contribute to substrate specificity of cyclin-Cdk complexes is by directly interacting with substrates. Cyclin D (G1-cyclin), but not cyclin A (S-cyclin), was shown to contain the binding motif (Leu-x-Cys-x-Glu) of pRb which is the only essential substrate of cyclin D-Cdks [20, 21].

Another mechanism that cyclins confer substrate specificity to their Cdks partners is by targeting them to specific subcellular locations. An excellent example for such regulation is the

case of cyclin E (G1/S-cyclin) and cyclin B (M-cyclin). Cyclin E promotes DNA replication, whereas cyclin B induces mitosis [22]. Cyclin B is unable to trigger DNA replication probably due to the fact that it cannot reach and phosphorylate proteins which are required for DNA replication, since cyclin E is nuclear whereas cyclin B is cytoplasmic in interphase cells [23, 24]. Indeed, in the frog egg extract where cyclin E was depleted, the addition of wild type cyclin B to the extract can never induce DNA replication. However, in such extract, when cyclin B tagged with the NLS (nuclear localisation signal) of cyclin E was added back, it entered the nucleus and induced substantial DNA replication in absence of the endogenous cyclin E [25]. Therefore, the failure of wild type cyclin B to induce DNA replication is due to fact that the nuclear envelope separates it from the nuclear substrates which are required for DNA replication.

In addition to the mechanisms described above, it was suggested that direct biochemical differences between cyclins may confer their Cdks partners the substrate specificity [26]. Indeed, a collection of cyclin─Cdks structures reveal that both the cyclin orientation and position relative to its respective Cdk can vary significantly, which may have an impact on the substrate specificity [27]. However, their functional significance relative to substrate specificity is yet to be uncovered.

1.2.1.3 Compensatory mechanisms among cyclins and Cdks.

There are 11 Cdks binding to different cyclins generate about 20 cyclin─Cdk complexes in mammals [28, 29]. Among them, only five Cdks and four classes of cyclins are involved in mammal cell cycle control [30]. Each of these cyclin─Cdk complexes was speculated to execute unique functions in a particular phase of the cell cycle. However, several unanticipated compensatory mechanisms among cyclins and Cdks have been discovered both in yeast and in mammals.

B type cyclins are essential components for mitotic initiation. At mitotic entry, cyclin B1-Cdk1 promotes DNA condensation, mitotic spindle assembly and nuclear envelope breakdown, while cyclin B2-Cdk1 induces the reorganisation of Golgi apparatus [31]. Cyclin B1 knockout mice died in early embryonic phase suggesting that cyclin B1 is a nonredundant cyclin and none of the other cyclins is able to compensate its function [32]. In contrast to cyclin B1, cyclin B2

knockout mice were viable and showed no abnormalities indicating that the absence of cyclin B2 was fully compensated by cyclin B1 [32].

The most surprising discovery is that Cdk2 is dispensable for cell cycle progression. Cdk2 was believed to be essential for entry into and progression through S phase, since *in vitro* inhibition of Cdk2 arrest the cell cycle [33]. However, Cdk2 knockout mice were viable and developed normally except for reduced size and sterility [34, 35]. In addition, Cdk2 deficient MEFs released from quiescence proliferate normally with only a slight delay in S phase entry [35]. Thus, it appears that Cdk2 is not essential for cell cycle progression. Two years after those discoveries, Cdk1 was found to bind to cyclin E and promotes G1/S transition in absence of Cdk2 [36]. However, this compensatory mechanism is not as simple as it appears to be. In interphase, cyclin E-Cdk2 localizes in nucleus, whereas Cdk1 localizes in cytoplasm [37, 38]. Thus, a discrepancy emerges: if Cdk1 has to replace Cdk2 to trigger G1/S transition, Cdk1 should enter nucleus; however, if Cdk1 prematurely enters nucleus, it could prematurely initiate mitosis which will be catastrophic. Nonetheless, it was demonstrated that Cdk1 enter the nucleus earlier in absence of Cdk2 and non premature mitotic entry was observed [38]. Therefore, it was speculated that Cdk2 may be necessary to maintain Cdk1 in cytoplasm until mitosis, and that in the absence of Cdk2, Cdk1 can freely enter nucleus. Although Cdk1 enters the nucleus, cyclin E-Cdk1 is probably unable to phosphorylate the substrates whose phosphorylations are required for mitotic entry, or simply these substrates may not yet be available to be phosphorylated by Cdk1in G1 and S phase [39].

In conclusion, widespread compensatory mechanisms among cyclins and Cdks have been uncovered. These findings challenge the dogma that a particular cyclin─Cdk complex performs a unique function. On the other hand, this mechanism might be useful to compensate for the loss of individual cyclins or Cdks from genetic deletion [40].

1.2.1.4 Reversible phosphorylation is an important mechanism which regulates Cdks activity.

Apart from the regulation of periodic synthesis and degradation of cyclins, the activity of cyclin─Cdk complexes is also tightly regulated by phosphorylation and dephosphorylaiton. Indeed, cyclin binding can only partially activate Cdks. This binding induces a conformational

change of Cdk, which relieves its catalytic cleft, rendering the ATP (adenosine triphosphate) accessible for the protein substrate [41]. However, for their full activation, a threonine residue localized in the activation loop of Cdks shoud be phosphorylated by Cdk-activating kinase (CAK) [42]. This threonine is only accessible for the CAK when cyclin binds to Cdks, indicating that cyclin binding is prerequisite for CAK phosphorylation [41]. Interestingly, cyclin binding simultaneously prevents the dephosphorylation of this threonine by the protein phosphatase PP2C [43]. The phosphorylation of the threonine by CAK not only further exposes the catalytic cleft of Cdks but also stabilizes their activation loops [44]. Nevertheless, there are also some results showing that phosphorylation by CAK contributes to Cdks substrate recognition or binding rather than catalysis. For example, both CAK phosphorylated and CAK non phosphorylated Cdk2 can phosphorylate cyclin B [45]. Moreover, both CAK phosphorylated and CAK non phosphorylated Cdk2 can phosphorylate one synthesized substrate to a similar degree; whereas for the phosphorylation on another synthesized substrate, unphosphorylated Cdk2 is much less competent than phosphorylated Cdk2 [42]. Thus, those results suggest, *in vivo*, that there may exist not only a set of substrates which can only be recognized by phosphorylated Cdks, but also a set of substrates which can be recognized by both phosphorylated and unphosphorylated Cdks, especially substrates that bind tightly to Cdks [46].

In contrast to the positive regulation by CAK phosphorylation, Cdks activity can also be negatively regulated by phosphorylations on two inhibitory residues –T14 and Y15. Wee1 is a tyrosine kinase which phosphorylates Y15 [47], while Myt1 is a dual-specificity kinase which can phosphorylate both T14 and Y15 [48, 49]. It was shown recently that the phosphorylation of Y15, in contrast to the activation loop phosphorylation, does not induce a major conformational change of Cdks but rather disrupt their substrates recognition [50]. These two inhibitory phosphorylations on Cdks are removed by Cdc25 phosphatases [51-53]. These proteins kinases and phosphatases provide an additional mechanism of fine tuning of Cdks activity at precise time and in specific cellular compartments. For example, during interphase, Wee1 principally localizes in the nucleus to prevent cyclinB─Cdk activation in this compartment, which ensures the completion of DNA replication prior to mitosis [54]. However, upon mitotic entry, Wee1becomes inactive and relocalizes to the cytoplasm [55, 56], whereas Cdc25C becomes active and enters nucleus [57-59]. Concurrent inhibition of Wee1 and activation of Cdc25C are required for Cdk1complete activation. In turn, the activity of Wee1 and Cdc25C are regulated by

Cdk1 itself. Cdk1 is able to activate its own activator-Cdc25C and inactivate its inhibitor─Wee1 by phosphorylation [57, 60]. These mechanisms contribute to an abrupt, switch-like activation of Cdk1 upon mitotic entry [61] (Figure 1.5). More details about the Cdk1-Wee1-Cdc25 network will be described in section 1.2.4.

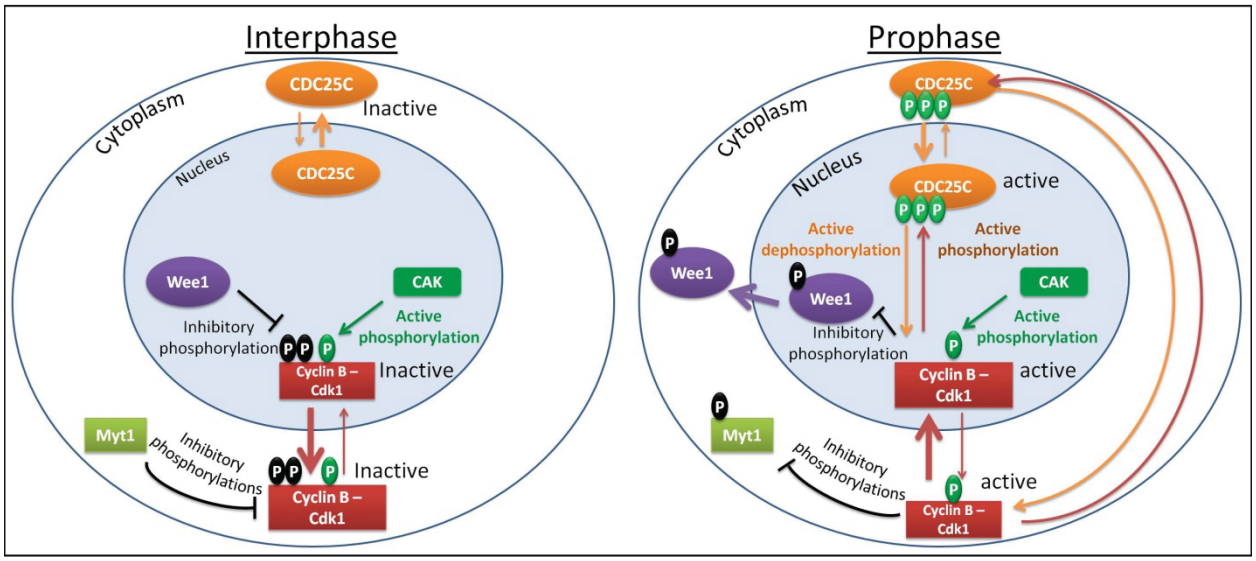


Figure 1.5. The main reversible phosphorylation mechanisms required for Cdk1 activation upon mitotic entry. The relative rate of nuclear import and export of each protein are represented by arrows of varying thickness. Black "P" and Green "P" indicate respectively the inhibitory and activating phosphorylation. During interphase, gruadually increased cyclin B binds to Cdk1 to form the cyclin B – Cdk1complex in the cytoplasm. Despite the fact that CAK phosphorylates Cdk1 at its activating site, the phosphorylations at inhibitory sites by Wee1/Myt1 block Cdk substrates access to the Cdk. CDC25C is sequestered in the cytoplasm and inactive in interphase. At the onset of mitosis, CDC25C is activated by upstream kinase and subsequently remove the inhibitory phosphorylations on Cdk1 in the cytoplasm. In turn, Cdk1 activates CDC25C and inactivates Wee1/Myt1 by phosphorylation. Then, Wee1 is translocated into the cytoplasm, meanwile, activated CDC25C and Cdk1 begin to accumulate in the nucleus to initiate nuclear events such as nuclear envelope breakdown.

1.2.1.5 Other mechanisms regulating Cdks activity.

The kinase activity of cyclin─Cdks complexe can also be tightly regulated by a plethora of Cdk inhibitor proteins (CKIs), which bind and inactivate Cdks. They primarily serve as a

brake for the G1 and S phases. For example, in response to unfavorable conditions, INK4, one familly of CKI, is induced to bind to Cdk4, 6 to prevent their assembly with cyclin D. Simultaneously, Cip/Kip, the other family of CKI, is released to inhibit cyclin E, A-Cdk2 complexes by directly blocking their ATP binding site. Therefore, the progression into S phase is efficiently prevented and the cell cycle is halted in G1 phase [62].

In addition to the activation by cyclins as mentioned above, Cdks can be activated through binding to Speedy/RINGO, which yet has no sequence homology to known cyclins [63, 64]. This family of proteins was originally described to promote G2/ M transition during meiotic maturation in *Xenopus* oocyte. Later, one human Speedy homolog was shown to promote the G1/S transition through activation of Cdk2 [65]. Different from cyclin─Cdks complexes, Speedy─Cdk2 activity is neither CAK dependent nor susceptible to inhibitions by CKIs [66]. Furthermore, structural analysis indicates that Speedy binds to Cdks not through Cdks' cyclin binding domain but rather their CKI binding region, suggesting that Speedy binding may prevent CKIs binding to Cdks [67]. Moreover, as Cdk2 has been shown to phosphorylate CKI leading to its proteasome mediated degradation, it is therefore assumed that Speedy─Cdk2 may promote G1/S transition through enhanced CKI degradation [68]. However, in vitro assay revealed that Speedy─Cdk2 has low enzymatic activity toward conventional cyclin─Cdk2 substrates [69], which suggests that Speedy family may have untraditional roles in the regulation of mitotic cell cycle and other roles beyond cell cycle control. Further studies are required to uncover the exact functions of Speedy in cell cycle control.

In summary, Cdks could be described as the engine which drives the cell cycle progression and can be adjusted at multiple levels. It cooperates intimately with the accelerators (CAK) and the brakes (CKI, Wee1/Myt1) to halt the cell until the fuel (cyclins) is enough accumulated. Once brake block is released through the degradation of CKI and the dephosphorylation of Wee1/Myt1 sites on Cdks by CDC25, the cell dashes into the next phase.

1.2.2 The cell cycle control system is cyclical proteolysis dependent.

In addition to cyclin─Cdk complexes dependent phosphorylation mechanism, protein degradation is another crucial mechanism in cell cycle control. Many key cell cycle regulators, such as cyclins, Wee1 and CKIs, are targeted by ubiquitination complexes for degradation by the

26S proteosome. Ubiquitination is a process of generation of a polyubiquitin chain on target proteins, which is carried out through an enzymatic cascade. The process begins when an ubiquitin molecule is activated by an E1 ubiquitin activating enzyme. The activated ubiquitin is then transferred to an ubiquitin conjugating enzyme E2. Finally, E2 binds to the ubiquitin ligase E3 which recruits a substrate and guides the transfer of activated ubiquitin to the substrate (Figure 1.6) [70]. Therefore, the E3 enzymes play an important role during the ubiquitination cascade where they are responsible for substrate specificity. It is noteworthy that there are more than 600 different E3 ligase genes in the human genome, providing a high specificity in the ubiquitination of target proteins [71].

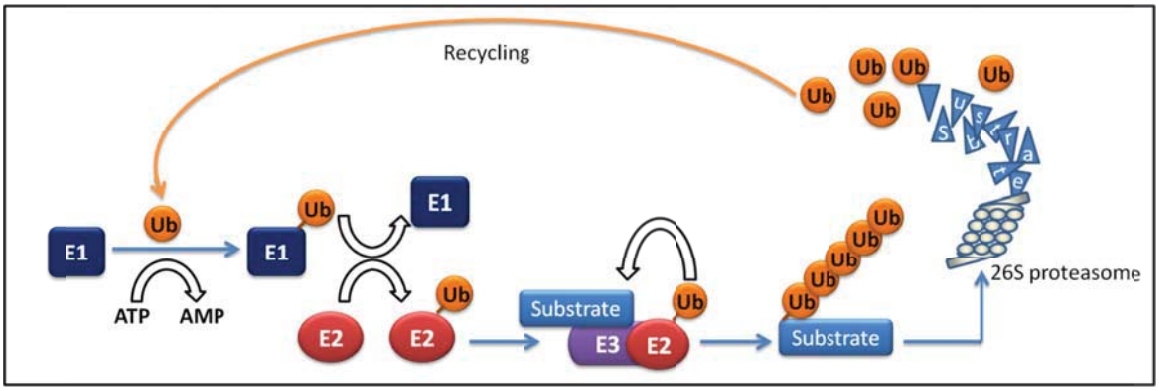


Figure 1.6. The ubiquitination pathway. Ubiquitin is activated in an ATP dependent manner by E1 ubiquitin-activating enzyme. Then it is transferred to E2 ubiquitin-conjugating enzyme. With the help of E3 ubiquitin-protein ligase, E2 catalyzes the transfer of ubiquitin to the target protein. Finally, the polyubiquinated substrate is degraded by the proteasome 26S.

Basic cell cycle control requires the activity of two ubiquitination complexes, the Skp1─cullin─F-box─protein complex (SCF) and the anaphase─promoting complex/cyclosome (APC/C). SCF plays pivotal role in G1/S and G2/M transitions, while APC/C is crucial for metaphase-to-anaphase transition and the establishment of a stable G1 phase.

Once assembled, SCF is constitutively active through the cell cycle. However, SCF substrate recognition depends on the phosphorylation state of their targeting proteins. For example, Sic1, a G1phase CKI in *S.cereviae*, has to be phosphorylated at multiple sites to be recognized by SCF. Then SCF induces the degradation of Sic1, which is required for G1/S transition [72]. In addition, substrates recognition of SCF also depends on its variable component

— F-box protein (also known as receptor protein). There are about 70 F-box proteins, among which only SKP2, FBW7, and β-TRCP are thought to play critical role in cell cycle control, however the functions of most other F-box proteins remain unknown [73]. It is well known now that SCF^{SKP2} is specifically responsible for the degradation of G1 cyclin and CKI, therefore promotes G1/S transition [73, 74].Whereas, the $SCF^{\beta\text{-}TRCP}$ mediates the degradation of Wee1, which is required for mitotic entry [75].

In contrast to SCF, APC/C activity is restrained to certain phase of the cell cycle. Its activation depends on the binding of its co-activator (also known as receptor protein) — Cdc20 or Chk1. Cdc20 binding is regulated by phosphorylation. In prophase, Cdks phosphorylate APC/C at multiple sites, where these phosphoyrlation are prerequisite for the Cdc20 binding from prophase to anaphase [76-79]. Different from Cdc20, Cdh1 activates APC/C regardless of the APC/C's phosphorylation statue. However, from late G1 to anaphase during which Cdks activity is high, Cdks phosphorylate Cdh1 to prevent its association with APC/C [80-82]. In yeast, Cdh1 remains phosphorylated until anaphase where it is dephosphorylated by CDC14, then associates with and activates APC/C. Subsequently, APC/C^{Cdh1} complex mediates the degradation of M phase cyclins leading to mitotic exit [83]. Both Cdc20 and Cdh1 confer substrate specificity in the same way as F-box proteins do in the SCF complex. APC/C^{Cdc20} triggers anaphase by mediating the degradation of securin and Shugoshin, both of which are necessary for disrupting sister chromatid cohesion. Moreover, APC/C^{Cdc20} promotes the mitotic exit by inducing the degradation of cyclin B. In contrast to APC/C^{Cdc20}, APC/C^{Cdh1} is a key regulator in maintenance a stable G0 or G1 by targeting various proteins involved in DNA replication, cell cycle progression and mitosis. Moreover, in contrast to SCF which only recognizes phosphorylated substrates, APC/C recognizes its substrates containing degradation motifs such as the D-box or Ken-box [84, 85]. It was demonstrated that APC/C^{Cdc20} prefers the D-box containing substrates, whereas APC/C^{Cdh1} can target both D-box and Ken-box containing substrates [81, 86].

In addition, it was demonstrated that there is also a high level of crosstalk and interdependency between SCF and APC/C. For example, in order to maintain a stable G0 or G1 phase, APC/C^{Cdh1} mediates the degradation of Skp2 to prevent premature G1/S transition [87, 88]. Whereas, in order to ensure the metaphase/anaphase transition, $SCF^{\beta\text{-}TRCP}$ induces the degradation of Emi1 which is an inhibitor of APC/C.

In conclusion, collaborating with cyclin-Cdks complexes, SCF and APC/C dependent degradation of key regulators ensures the arrest or initiation of a specific event or a specific cell cycle phase (Figure 1.7). Importantly, in addition to the reversible post-translational regulation such as phosphorylation, SCF and APC/C mediated proteolysis provides an irreversible mechanism that assures the strict unidirectional progression of the cell cycle.

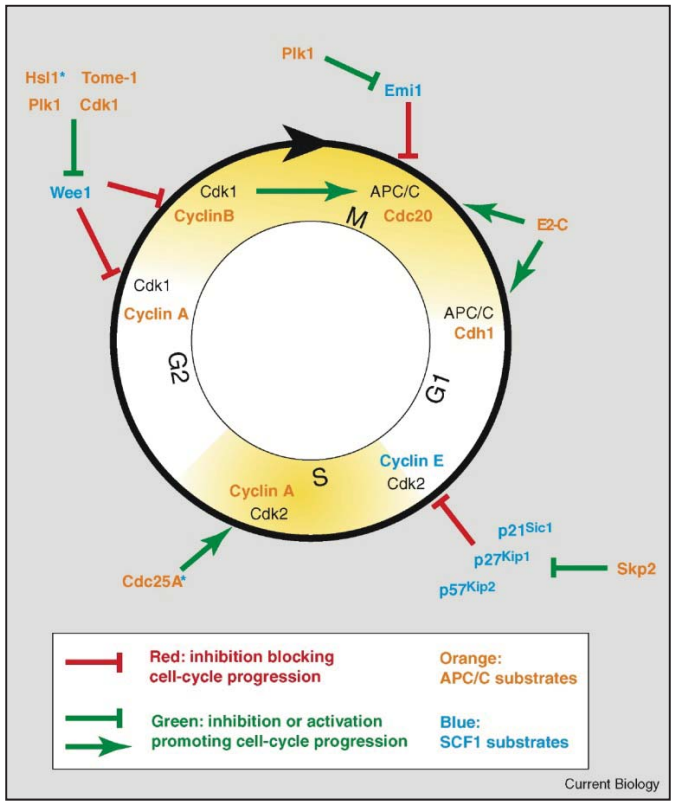


Figure 1.7. Global roles for APC/C and SCF1 in the core cell cycle. Green denotes activation or inhibition that promotes cell cycle progression, whereas red indicates activities that block progression. APC/C substrates are in orange letters and SCF1 substrates in blue. Note that most cell-cycle-advancing activities are inactivated by the APC/C, whereas blocking activities are removed by SCF substrates. Blue asterisks indicate proteins that become SCF1 targets during checkpoint or stress responses. This figure was adapted from Hartmut C. Vodermaier (2004) [89], with permission from publisher Elsevier.

1.2.3 Cell cycle checkpoints

In addition to the different components of cell cycle control system mentioned above, the cell also sets a series of checkpoints to monitor the quality of each phase of the cell cycle. If previous event was not successfully completed, the cell cycle can be arrested at a specific checkpoint until conditions are suitable for the cell to proceed to the next stage. This arrest can provide time to fix problems and also prevents catastrophic consequence from the premature progression of the cycle into the next phase. In higher eukaryotes, multiple conserved checkpoints exist to ensure that if one fails, others will take in charge of the genome integrity and cell survival. For example, two DNA damage checkpoints are separately positioned before the

cell enters S phase (G1/S checkpoint) and after DNA replication (G2/M checkpoint). In addition, it was shown that there appears to be DNA damage checkpoints during S and M phases [90]. In the following paragraphs, I will describe briefly four major checkpoints:

1.2.3.1 G1/S checkpoint. To prevent entry into S phase with damaged DNA, the apical signal transducing kinase ATM (**A**taxia-**T**elangiectasia **M**utated)/ATR (**A**taxia-**T**elangiectasia Mutated and **R**ad3 Related), which 'recognize' DNA damage, are activated in G1 cells to trigger the checkpoint signaling cascade [91]. ATM/ATR subsequently phosphorylate and activate the distal signal transducing kinases Chk1 (**Ch**eckpoint **K**inase **1**)/Chk2 (**Ch**eckpoint **K**inase **2**) [92] which, in turn, target two critical effectors involved in distinct branches of the G1 checkpoint: the protein phosphatase CDC25A and the transcriptional factor p53.

Phosphorylation of CDC25A by Chk1 and Chk2 leads to its nuclear exclusion and its SCF mediated degradation by the proteasome [93-95]. Consequently, the inhibitory phosphorylations on Cdk2 by Wee1 can't be removed [96], and the inactive Cdk2 can't phosphorylate Cdc45 whose phosphorylation is required for the initiation of DNA synthesis [97]. Although the CDC25A cascade is implemented rapidly, it is relatively transient and capable of delaying cell cycle progression only several hours [96, 97].

Compared to the CDC25A cascade, the p53 pathway is much slower and may require up to several hours due to the fact that this pathway needs the transcription and accumulation of newly synthesized proteins [98]. For its activation, the P53 can be phosphorylated not only by Chk1/Chk2, but also directly by the upstream kinase ATM/ATR [99]. Morevoer, in response to DNA damage, ATM/ATR also target the ubiquitin ligase Mdm2 to prevent p53 degradation [100, 101]. Both mechanisms contribute to the stabilization and accumulation of p53 which, in turn, induce the transcription of p21— a CKI (**Cdk i**nhibitor) of Cdk2. This pathway complements and eventually replaces the transient acute inhibition of Cdk2 through the CDC25A degradation pathway, leading to the maintenance of the cell cycle arrest.

1.2.3.2 Intra-S phase checkpoint. Unlike the G1/S or G2/M DNA damage checkpoint, intra-S phase checkpoint can only delay the initiation of DNA replication, but cannot maintain the arrest of the cell cycle, and is independent of p53 [91, 98, 99]. This checkpoint is mediated by two pathways. The first pathway is the ATM/ATR — Chk1/Chk2 — CDC25A — cyclin E (or A)-

Cdk2 ─ CDC45 described above. The second pathway involves Nbs1, a component of complex Mre11─Nbs1─Rad50 [102]. ATM mediated Nbs1 phosphorylation leads to the initial DSB (**D**ouble-**S**trand **B**reak) recognition by the complex Mre11─Nbs1─Rad50, which not only contributes to the delay the cell cycle, but also to activate the repair processes [95]. However, the precise molecular mechanism of this pathway remains elusive.

1.2.3.3 G2/M checkpoint. The G2/M checkpoint prevents cells from entering mitosis when they encounter DNA damage in G2, or then they have unrepaired damage produced in S phase or G1 phase. The G2/M checkpoint mediated cell cycle arrest is due to a combination of several mechanisms.

One of those mechanisms is the ATM/ATR─Chk1/Chk2─CDC25A pathway described above. So far, it is also the only mechanism known to be shared by all the three DNA damage checkpoints. This pathway prevents the initial activation of cyclin B-Cdk1 by CDC25A in the nucleus, which appears to be required for chromosome condensation [103]. Phosphorylation of CDC25A by Chk1 also induces its binding to the protein 14-3-3, which specifically inhibits its ability to interact with cyclin B-Cdk1 [104].

Different from CDC25A, CDC25B activates cyclin B-Cdk1 in the cytoplasm to trigger the centrosome separation [103]. In response to UV induced DNA damage, phosphorylation of CDC25B by p38 (a Mitogen Activated Protein Kinase) induces its binding to14-3-3, which blocks the access of Cdk1 to its catalytic site, thus impairing the cytoplasmic activation of Cdk1 [105, 106].

In addition to CDC25A and CDC25B, CDC25C is essential for the full activation of Cdk1 and mitotic entry. Its phosphorylation by Chk1/Chk2 creates a site for 14-3-3 binding, which sequesters CDC25C and blocks its interaction with cyclin B- Cdk1 [107-109]. Hence, both ATM/ATR─Chk1/Chk2 and p38 pathways, which involve different CDC25 isoforms, finally cooperate to inhibit the key target of G2/M checkpoint ─ cyclin B-Cdk1. In addition to prevent the CDC25-dependent Cdk1 activation, G2/M checkpoint also up-regulates Wee1 to sustain its inhibitory effect on Cdk1[110].

Moreover, unlike G1/S checkpoint, the G2/M checkpoint mediated maintenance of cell cycle arrest is partly p53 dependent. Many sorts of cells lacking functional p53 still tend to

accumulate in G2 in response to DNA damage, suggesting supplementary mechanisms may function with p53 cascade to maintain G2 arrest [111].

1.2.3.4 M phase checkpoint. The M phase checkpoint, also known as **s**pindle **a**ssembly **c**heckpoint (SAC) or mitotic checkpoint, is a quality control mechanism which prevents anaphase onset until all the chromosomes are stably and correctly attached to microtubule spindle via their kinetochores [112]. Note that one considers the name 'spindle assembly checkpoint' inappropriate, because SAC does not monitor spindle assembly but rather the microtubule ─ kinetochore attachment.

The M phase checkpoint targets Cdc20, a co-activator of Anaphase Promoting complex/Cyclosome (APC/C), which was described in previous section (1.2.2). As long as kinetochores do not attach to microtubules, it recruits SAC module to greatly facilitate the formation of **m**itotic **c**heckpoint **c**omplex (MCC) [113]. Cdc20 itself is also a component of MCC [114]. The incorporation of Cdc20 into MCC prevents its substrates recruitment thus inhibiting APC^{Cdc20} mediated proteins (including securin and cyclin B) degradation, and as a result preventing the cell to progress into anaphase [115].

Once chromosomes are bi-oriented through correct attachment of kinetochore ─ microtubules, the inhibition on APC^{Cdc20} is rapidly released by the checkpoint silencing process, which is ensured by several mechanisms. First, upon microtubule attachment, the conformation and composition of kinetochores dramatically change [116]. This leads to the removal of some MCC components from kinetochore, thus preventing new MCC assembly [116]. Additionally, the disassembly of existing MCC is carried out by $p31^{comet}$ and APC15 [117-119]. However, the regulatory mechanism of these proteins in response to microtubule attachment is still not clear. Second, checkpoint silencing depends on the phosphatases which counterbalance mitotic kinase. It was demonstrated that Aurora B kinase and Polo-like-kinase 1(Plk1) destabilize the microtubules at unattached kinetochores and eliminate erroneous attachments [120-123]. Once chromosomes are bi-oriented, the phosphatase PP2A-B56 functions towards the substrates phosphorylated by Aurora B and Plk1 to stabilize the correct microtubule ─ kinetochore interactions [124]. Moreover, chromosome bi-orientation stretches kinetochores, thus separating Aurora B from its kinetochore substrates [125]. Some of these substrates create docking sites for large recruitment of phosphatase PP1 to kinetochore to ensure the dephosphorylation of

checkpoint proteins [126]. However, the switch mechanisms of these kinases and phosphatase activity upon bi-oriented attachment remain largely unclear and their substrates at kinetochores stay to be identified.

In addition to the four major checkpoints described above, other checkpoints have been reported to be involved in the cell cycle control. For example, it was proposed that there exist of a checkpoint which monitors the integrity of centrosome. This checkpoint controls the G1-S progression through a p53 mediated pathway [127]. Moreover, in addition to DNA damage checkpoint induced DNA synthesis arrest, a distinct DNA replication checkpoint is trigged to stabilize stalled replication forks and prevent the formation of new forks by suppressing late replication origin firing [128]. Furthermore, it was demonstrated that there are also nutrient-sensing cell growth checkpoint which regulates G1 cell cycle progression[129], and antephase checkpoint which can reversibly delay mitotic entry in response to stress agents [130], etc.

Altogether, different checkpoints monitor specific event at specific phase of the cell cycle. They convey the run or stop signals to the cell cycle engine. Therefore, the checkpoints provide the control system a more sophisticatedly regulatory ability.

1.3 *Drosophila melanogaster* as a model

Drosophila, also known as the fruit fly, is a powerful model organism for cell cycle research. In *Drosophila melanogaster*, there are only four chromosome pairs compared to twenty-three in human. However, about 75% of human genetic disease genes are conserved in *Drosophila* [131]. Moreover, *Drosophila* has a completely sequenced genome, a short generation time of approximately ten days and a high reproduction rate, all making it an interesting and cost effective model organism for the functional study of human genetic disease genes. In addition, a plethora of approaches, such as RNAi screen, live-cell imaging and classical genetics are available in *Drosophila*. Here, I will only describe some of these approaches that I have applied in my PhD work.

Genetic approaches, such as enhancer and suppressor screen, have led to a large number discovery of novel genes. Both chemical and transposable element mediated methods have been applied for such screens. P transposable element for example, which is exclusively present in

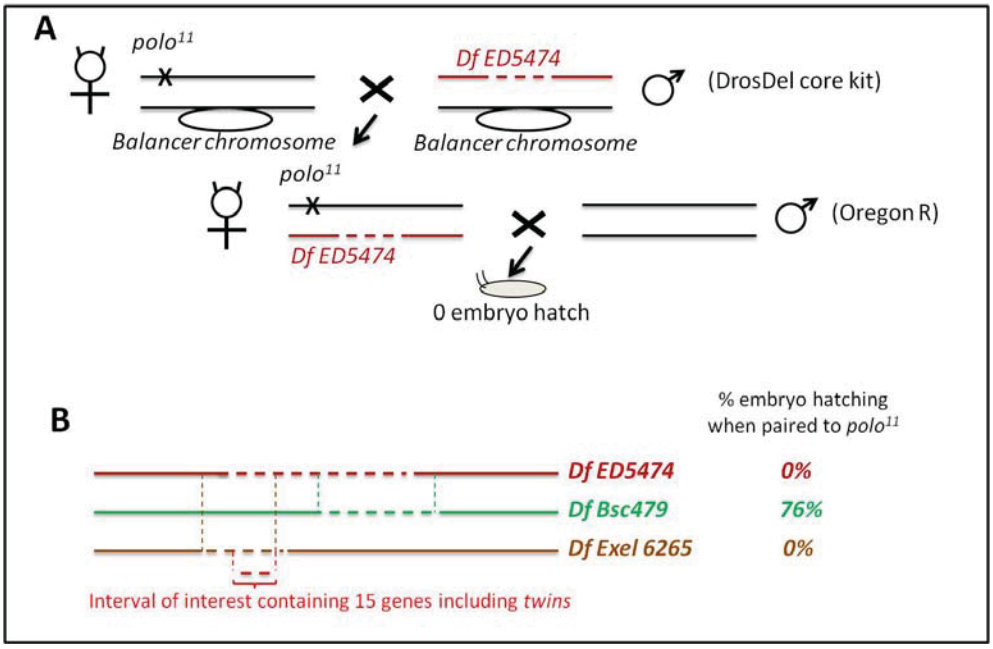
Drosophila melanogaster, is widely used for gene disruption. P element is even more commonly used than chemicals in mutagenesis, since the mutation can be more easily localized in the genome[132]. The technique of single P element insertion has been successfully used to mutate more than 25% of vital *Drosophila* genes in the Berkeley Drosophila Genome Project (BDGP) [133]. Some mutants frequently used in my PhD work, such as $twins^{p}$ and gwl^{6a}, were generated by P element insertion induced mutations [134, 135].

Diverse P element based vectors integrated with the GAL4/UAS system have been largely applied for gene/protein function analysis. The yeast GAL4 protein can specifically bind and activate an Upstream Activation Sequence (UAS) which is an enhancer of transcription. Thus, the GAL4 gene under the control of native gene promoter can induce the expression of a cloned target gene placed downstream from UAS. This system confers the *Drosophila* genetic system the ability to express genes *in vivo* in a time and tissue specific manner [136].

In addition, P element based techniques are also used for generating deficiency chromosomes which each lack defined chromosomal regions. Deficiency chromosomes are an invaluable tool for mapping mutations and identifying interacting loci. A loss-of-function mutation can be mapped to a specific chromosome region where a deletion fails to complement this mutation. In the Bloomington *Drosophila* stock center, a large-scale deficiency chromosomes collection covers about 98.4% of the euchromatic genome, which is considerably useful for fine-scale gene mapping [137]. Furthermore, deletions that enhance or suppress mutant phenotype are advantageous for identifying sets of genes involved in the same biological process. In our laboratory, we have taken advantage of this method in the identification of *twins* as a functional interactor of *polo* and *scant* (Figure 1.8) [138].

Figure 1.8. A screen for genes interacting with *polo* identified PP2A regulatory subunit *twins*. Deficiencies of the DrosDel Core kit were combined with one allele of $polo^{11}$ or gwl^{Scant}. Doubly heterozygous females were taken and their embryos were tested for the ability to hatch. A. The shema of genetic hybridization which identified the strongest genetic interactor of *polo* and gwl^{Scant}(not shown): deficiency (*Df*) *ED5474* which is, as *polo*, located on chromosome 3. B. Identification of twins as a genetic interactor. Testing deficiencies overlapping with *Df ED5474* for their effect on embryo hatching rate when introduced in the maternal $polo^{11}$ /+ background allowed to restrict the interval of interest to15 genes which included *twins*. The dotted line

schematically represents the deleted chromosomal region. The ellipse in the balancer chromosome schematically represents multiple inversions.



Balancer chromosomes, another most used tool for genetic manipulation, are broadly used for maintaining the mutations in flies through preventing meiotic recombination. Multiple invesions have been generated in balancer chromosomes which decrease dramatically the possibility of homologous recombination. Even though in some rare cases it could still happen, such recombination will be lethal for flies due to duplication or deletion of some genes. Balancer chromosome also carries some kind of visible phenotype markers and contains a lethal recessive allele. Thus, a fly carrying a homozygous lethal mutation can always be maintained in its heterozygous state with a balancer chromosome and can be easily recognized.

In addition to genetic methods, different biochemical approaches have been developed to investigate protein networks in *Drosophila*. Most biological processes are integrated system requiring protein-protein interaction. Identification of the components of a protein complex can provide an additional indication of the interacting partners identified from genetic interaction analysis. In our laboratory, a single step affinity purification method has been developed. We take advantage of a high affinity between the protein A (PrA) tag and the rabbit IgG which is coupled to paramagnetic bead. Using Gateway^{TM} vectors, the PrA tag can be easily fused to either the N terminal or the C terminal of the protein of interest. Then, the PrA tagged protein, serving as bait, can be expressed either in cultured cells or in syncytial embryos. Following affinity purification coupled to mass spectrometry analysis, associated proteins can be identified

(Figure 1.9). This method has been validated by the purification of several known protein complexes in our laboratory [139].

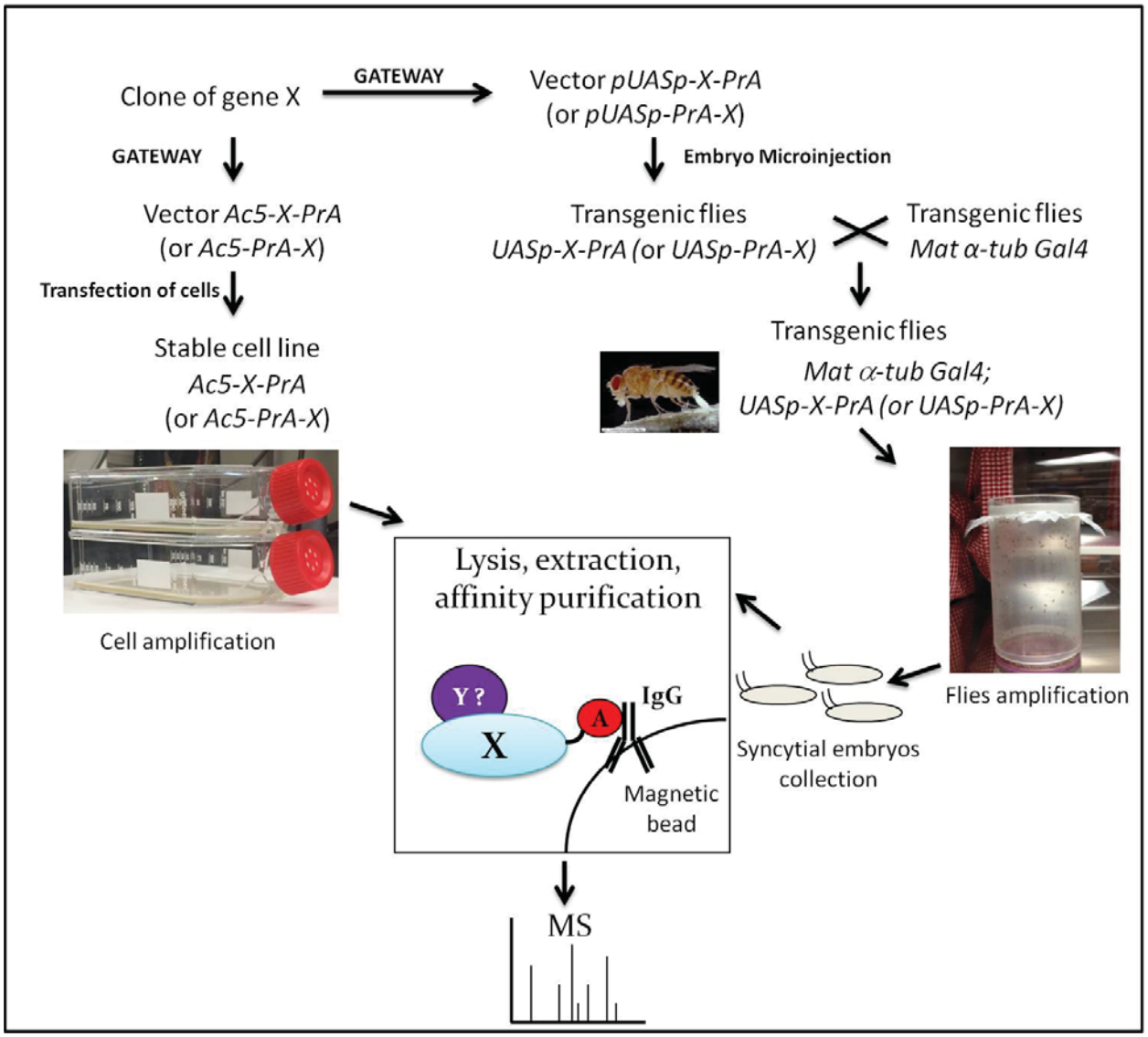


Figure 1.9. The principle of PrA affinity purifucation coupled to mass spectrometry (MS) of protein complex from *Drosophila* cultured cells and syncytial embryos. The main steps of cell line and fly line preparations are illustrated in the flowchart. Both cells and flies can be largely amplified in appropriate containers illustrated in the figure. Follwing the collection of a huge amount of cells and syncytial embryos (laid for less than two hours), PrA tagged protein X can be purified with IgG coupled to magnetic bead. Protein Y co-purified with the bait protein X can be identified subsequently by MS.

During my PhD work, *Drosophila* syncytial embryos were frequently used for the cell cycle study. These embryos provide an unparalleled opportunity for mitosis regulation study through the combination of biochemical, genetic and real-time imaging approaches. Embryonic syncytial cell cycles are non canonical cycles consisting of 13 rapid rounds (about 10 min per cycle) of synchronous S and M phases. Such an S/M cycle, which lacks of the gap phases of G1 and G2, relies on stockpiles of mitotic protein complexes in the embryo. In our laboratory, by using syncitial embryos, we succeeded to purify several novel mitotic protein-protein complexes which have been missed when cultured cells were used. Moreover, with regards to genetic manipulation, extremely rapid S/M cell cycle is also an advantage since such cycle is more sensitive to the dose of mitotic factors. For example, heterozygous *polo* (*polo*^{11}) and *twins* (*twins*^{p}) double mutants are viable and normal, but a dramatic centrosome detachment phenotype has been discovered in syncytial embryos laid by such mothers [138]. Moreover, syncytial cycle is independent of zygotic gene expression but is completely driven by the gene products of the mother. Thus, gene function in syncytial embryos can be investigated by performing simple modification of the genotype of the mother. Also, syncytial cell cycles are nuclear division cycles running in a common cytoplasm, so gene function can be dynamically analyzed with real-time microscopy in animals expressing fluorescently labeled marker proteins, and perturbations can be applied with mutations, microinjection of dsRNA or chemical inhibitors of specific proteins directly into the embryo.

1.4 Mitotic progression is tightly regulated by reversible phosphorylations.

In previous sections, I have described briefly some of the main cell cycle control mechanisms. Among these mechanisms, timely phosphorylation and dephosphorylation of proteins are crucial for faithful progression of the cell cycle. Kinases and phosphotases involved collaborate to ensure the switch-like activation of Cdks which, in turn, trigger abrupt transitions into the next phases. In addition, as described previously, reversible phosphorylation events are also implicated in the control of proteolysis and checkpoints. Here, I will focus on some of the main reversible phosphorylation events which regulate mitotic progression, and which are also the main interests of my PhD work.

As mentioned in the section 1.2.1.4, the abrupt interphase ─ M phase transition depends on the Cdk1-Wee1-Cdc25 mitotic control network. During S and G2 phases, gradually

accumulating cyclin B binds to Cdk1. However, Wee1 and Myt1 kinases phosphorylate the T14 and Y15 on Cdk1 to prevent its activation [47-49]. Upon mitotic entry, the activated Cdc25 phosphatase removes these two inhibitory phosphates, leading to the activation of Cdk1 [51-53]. Activated Cdk1 in turn phosphorylates and inhibits its inhibitors ─ Wee1/Myt1, and simultaneously phosphorylates and activates its activator ─ Cdc25 [57, 60]. These two positive feedback loops ensure a rapid autoamplification of Cdk1 activity [140, 141].

In order to trigger a switch-like activation of Cdk1, additional biochemical mechanisms are required. One important mechanism is that both Wee1 inhibition and Cdc25 activation are ultrasensitive in response to phosphorylation catalyzed by Cdk1 [142, 143]. Therefore, even with a low activity, Cdk1 can very quickly inactivate its inhibitor ─ Wee1 and activate its activator ─ Cdc25 by directly phosphorylating them at multiple sites [144]. This mechanism embedded in the positive feedback loops ensures the abruptness of Cdk1 activation and increases the robustness of the 'bistability' of the Cdk1-Wee1-Cdc25 system. The 'bistability' is an early mathematical concept delineating the mitotic onset [140]. It indicates that the cell either stays steadily in interphase or progresses into M phase without being able to rest in intermediate states. In other words, it describes a character of all-or-none transition into mitosis, which relies on the whole-or-very low activity of Cdk1without any intermediate possibilities [140, 145, 146].

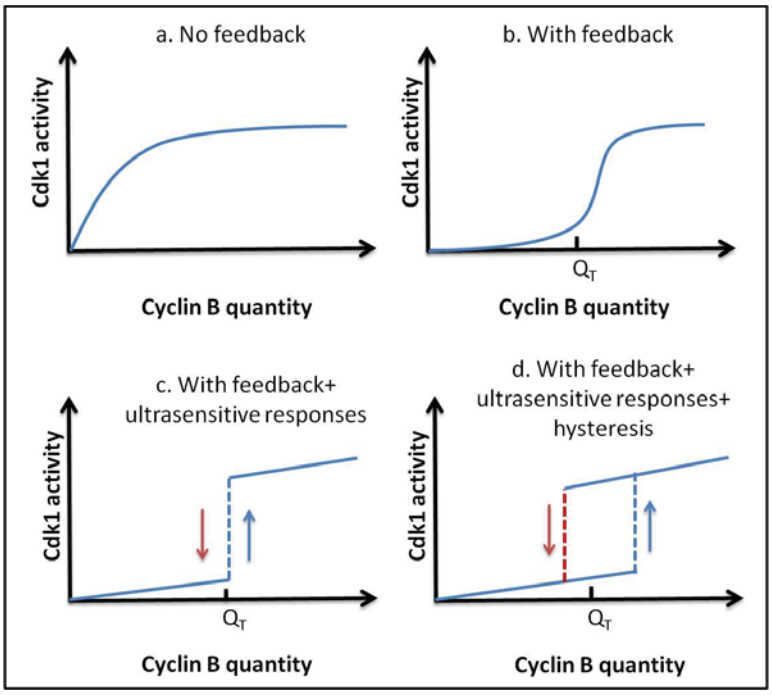


Figure 1.10. Theoretical prediction of the modes of Cdk1 activation controlled by different mechanisms**.** Q_T represents the threshold quantity of cyclin B required for Cdk1activation [147]. The blue arrow indicates the transition from interphase to phase M and the red arrow indicates the reverse transition from phase M to interphase. The blue and red dashed lines represent the switch-like activation and inactivation of

Cdk1, respectively. **a.** This model expects that gradually increased cyclin B directly activates Cdk1. Hence, the threshold quantity of cyclin B ─ Q_T is not required in this model. **b.** In this model, once Q_T is satisfied, Cdk1 activation can be greatly speeded up through the positive feedback mechanism, whereby Cdk1 activates its activator Cdc25 and inhibits its inhibitor Wee1. **c.** This model allows a swith-like activation of Cdk1 through combination of positive feedback and ultrasensitive mechanisms. However, the activation and inactivation threshold quantity of cyclin B are identical, which may lead to an unstable and reversible phase transition. **d.** When hysteresis is applied to the system, cyclin B quantity is elevated above its threshold quantity. Therefore, the cell will transit irreversibly from interphase into M phase. The reverse transition from phase M back to interphase can occur only when the quantity of cyclin B drops below the threshold quantity ─ Q_T.

In addition to the mechanism of ultrasensitive response, the bistable Cdk1-Wee1-Cdc25 system also creates a relatively high Cdk1 activity threshold for switching from interphase to M phase, and a lower Cdk1 activity threshold for switching from M phase back to interphase, a phenomenon is known as hysteresis. Indeed, Cdk1is kept inactive until cyclin B accumulates to the level in excess of the requirement for mitosis [145]. Thus, this mechanism was thought to ensure an irreversible transition into mitosis [146, 148] (Figure 1.10). This model is still broadly accepted today, yet it appears insufficient to account for the "irreversibility". A recent paper showed that retracting Cdk1 activity in prophase does lead to a reversal of mitotic entry [149]. In contrast, when Cdk1 was inhibited at any point in prometaphase or metaphase, cells still progressed forward through the cell cycle [149]. The authors further demonstrated that the phosphorylation of Cdk1 substrates dramatically increases in prophase and continues to rise in prometaphase. Therefore, to be exact, the irreversibility of interphase ─ M phase transition depends on the status of the phosphorylation of Cdk1 substrates rather than Cdk1's own activity. Interestingly, the authors also found that cells, which had been treated simultaneously with both Wee1/Myt1 and Cdc25 inhibitors at the end of S phase, did enter mitosis prematurely but much slower [149]. Even the rounding up of cells, which is a characteristic for mitotic entry and can be triggered by low activity of Cdk1, was noticed to slow down [149, 150]. In accordance with the theoretical prediction of the mode of Cdk1 activation in the absence of positive feedback loops (Figure 10a), this delay is probably due to the relatively slow activation of Cdk1. In addition, they demonstrated that cells, which have been treated with inhibitors of Wee1/Myt1

and Cdc25 at the end of S phase, were unable to maintain their mitotic state after nuclear envelop breakdown (NEBD), a phenotype termed "mitotic collapse" [149]. In those cells, the dephosphorylation of mitotic substrates was detected without the inactivation of Cdk1 and the proteolysis of cyclin B [149]. Moreover, mitotic collapse can be suppressed by inhibiting protein phosphatase 2A (PP2A) with okadaic acid (OA) [149]. Thus, it indicates clearly that PP2A counteracts Cdk1 by dephosphorylating its mitotic substrates. Moreover, when Cdk1 activity is compromised by abrogating the positive feedback loops, PP2A can apparently override the relatively low Cdk1 activity and lead to the dephosphorylation of mitotic substrates. Indeed, the early mathematical model has demonstrated that PP2A counterbalances the action of Cdk1 on Wee1 and Cdc25. [147, 151, 152]. Up to date, we still not know which form of PP2A directly dephosphorylates the Cdk1 phosphorylation sites on Wee1 and Cdc25, but we do know that it is the B55α and B55δ forms PP2A (PP2A-B55α, δ) that counteract Cdk1 on many mitotic substrates in higher eukaryotes [153-155]. Thus, upon mitotic entry, Cdk1 activity should be able to overcome the activity of PP2A-B55, leading to the substantial phosphorylation of mitotic substrates. Indeed, it was demonstrated that Greatwall kinase (Gwl) mediates the inhibition of PP2A-B55 through α-Endosulfine (Ensa or Endos) and cyclic AMP-regulated phosphoprotein (Arpp-19) at mitotic onset, which contributes to the abrupt increase of Cdk1-phosphorylated substrates and the irreversible transition into mitosis [153, 156-160].

In contrast to mitotic entry, timely dephosphorylation of mitotic phospho-proteins as well as the APC/C mediated cyclin B degradation are both required for the initiation of anaphase and mitotic exit [161]. Indeed, several recent papers shed light on the mechanisms of reactivation of key phosphatases and inactivation of Gwl and Endos upon mitotic exit. I will describe these mechanisms in detail in the next section.

In summary, the progression through mitosis involves a large number of reversible phosphorylation events. Phosphorylation has been proven to be the most rapid and versatile reversible post-translational modification of proteins affecting activity, localization, stability and protein-protein interaction, whose changes are usually required for triggering downstream mitotic events [162]. Thus, the interplay between mitotic kinases and phosphatases must be orchestrated to ensure the irreversible mitotic entry and exit and thus faithful mitotic progression.

It is important to note that in addition to the kinases mentioned above, there are other kinase families such as Aurora kinases and Polo-like kinases (Plks), which are also crucial regulators for mitotic progression. Correspondingly, other phosphatases such as PP1 and other members of PP2A family have been demonstrated to play important role in mitotic control. For example, as mentioned previously, PP2A-B56 counterbalances Plk1 and Aurora B to initiate spindle checkpoint silencing when chromosomes are correctly bound to microtubules [124]. Another example, upon mitotic entry, both PP1 and Plk1 are required for the activation Cdc25 [163-166]. Here, I won't describe the roles of all these kinases and phosphatases in detail, the following section will focus on the Greatwall kinase ─ PP2A-B55 phosphatase pathway in mitotic progression control, which has been the major research project of my PhD training during the past five years.

1.5 Greatwall kinase – PP2A-B55 phosphatase axis in mitotic progression control.

1.5.1 The multifunctional phosphatase PP2A-B55 is implicated in a broad variety of cellular processes.

In the paragraphs below, I will describe the role of the protein phosphatase PP2A-B55 in mitotic progression as well as its multiple functions apart from mitotic control. Those non mitotic functions had perplexed me at the beginning of my PhD training by complicating PP2A-B55 pathways. However, they also inspired me to formulate several hypotheses for novel roles of PP2A-B55 which I recently discovered. Here, I will take advantage of the redaction of my thesis to present a mini-review of PP2A-B55.

1.5.1.1 PP2A structure

PP2A is a very abundant and remarkably conserved serine/threonine phosphatase, which is implicated in a multitude of cellular functions. Unlike Cdc25, PP2A does not exist as a free catalytic unit but rather a heterotrimetric enzyme. PP2A contains a core enzyme, which is a dimer ($PP2A_D$) composed of a structural subunit A (also known as PR65) and a catalytic subunit C ($PP2A_C$). This core enzyme associates with a variable regulatory B-type subunit to constitute the holoenzyme. There are 4 families of B-type subunits: B (also known as B55 or PR55), B'

(B56 or PR61), B" (PR48/PR72/PR130) and B"' (PR93/PR110). Moreover, each family consists of multiple isoforms, some of which have multiple splice variants. For example, the B55 family contains 4 isoforms (α, β, γ, δ) and the B56 family has five isoforms (α, β, γ, δ and ε). Because A and C subunits also exist in two isoforms, more than 70 PP2A holoenzymes can be assembled by combination of different subunit isoforms [161]. These B subunits are expressed in a tissue specific and cell type specific manner. In this regards, the regulatory subunits determine the substrate specificity as well as the subcellular localization of PP2A holoenzyme [167, 168]. Therefore, the assembly of a core enzyme with the appropriate B type subunit is important for the function of PP2A [169].

1.5.1.2 PP2A-B55 assembly regulation and substrate recruitment

It was demonstrated that distinct modifications of the C-terminal tail of catalytic subunit C ($PP2A_C$) underlies the selectivity for B-type subunits for the holoenzyme assembly [170]. For example, there is evidence that the methylation of leucine 309 in C-terminal tail of $PP2A_C$ is necessary for the B55 but not the B56 subunit recruitment [170]. Interestingly, there is a differential methylation of $PP2A_C$ during the cell cycle, suggesting a role of the methylation in cell cycle regulation [171]. However, some recent papers showed that methylation of $PP2A_C$ is not necessary for *in vitro* assembly of PP2A-B55, but only for facilitating the assembly by enhancing B55 binding affinity [172-174]. In contrast to $PP2A_C$ methylation, the direct phoshorylation at Ser167 on B55α in mitotic cells was demonstrated to prevent the holoenzyme assembly [155]. This site corresponds to the phosphorylation motif of Cdk1, suggesting that Cdk1 may directly inhibit PP2A-B55α by preventing its assembly. However, up to date, there are no further results to prove this possibility. Altogether, these results suggest that both the methylation of $PP2A_C$ and the phosphorylaiton of B55α contribute to a cell cycle-dependent regulation of PP2A-B55 holoenzyme assembly.

In addition to the intrinsic regulations mentioned above, PP2A-B55 assembly is also affected by external stimulus. For example, upon glutamine but not glucose deprivation, a $PP2A_C$ interacting protein named α4 promotes the assembly of PP2A-B55, which supports cancer cells survival in a p53 dependent manner [175]. Moreover, in response to ionising radiation, B55 dissociates from the core enzyme in the nucleus through an ataxia-telangiectasia-mutated (ATM) dependent mechanism [176]. Furthermore, it was demonstrated that both the

polyoma and simian virus (SV40) small t antigen displaces the B55 from the PP2A holoenzyme by competing for the same binding sites on the structural subunit A, leading to the disorganisation of cytoskeleton [169, 177, 178].

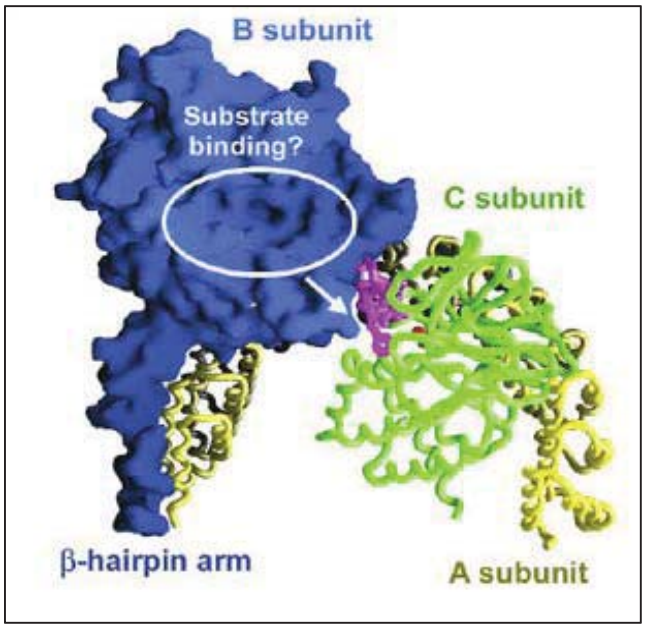


Figure 1.11. Overall structure of the PP2A holoenzyme involving the Bα subunit.The scaffold (Aα), catalytic (Cα), and regulatory B (Bα) subunits are shown in yellow, green, and blue, respectively. The mycrocystin-LR (MCLR), a potent inhibitor of PP2A, is shown in magenta. The putative substrate-binding groove on the top face of the Bα propeller is located in close proximity to the active site of the C subunit of PP2A. This figure was adapted from Yanhui Xu *et al.* (2008) [174], with permission from publisher Elsevier.

Once assembled into a holoenzyme, the PP2A-B55 complex recruits different substrates through a putative substrate-binding groove localized in the B55 subunit, at a position proximal to the active site of $PP2A_C$ [174] (Figure 1.11). This substrate-binding groove may also mediate the binding of Endos, which is both a substrate and a competitive inhibitor of PP2A-B55 blocking the active site of $PP2A_C$ [179]. In addition, adenovirus E4orf4 protein, whose overexpression induces p53-independent apoptosis in cancer cells but not in normal human cells, was suggested to modify PP2A-B55 activity through direct interaction with B55 [180-182]. Interestingly, a recent paper demonstrated that E4orf4 binds to B55 across its putative substrate binding groove [183]. Therefore, it was assumed that E4orf4 induced cancer cell death is a result of the prevention of binding of some PP2A-B55 key substrates, which are essential for cancer cell survival. Indeed, it was demonstrated that the interaction between E4orf4 and B55 prevents the binding of retinoblastoma protein p107 [183], which plays an important role in apoptosis [184]. However, during a typical adenovirus infection, the E4orf4 expression level is not high enough to induce efficient cell killing. Hence, it was suggested that E4orf4 binding may target PP2A-B55 to some substrates favoring the virus infectious cycle. Indeed, it was demonstrated

that the dephosphorylation of splicing factor ASF/SF2/SRSF1 by PP2A-B55, which is necessary for late viral protein production, is E4orf4 dependent [183].

In conclusion, these results indicate that the activity of PP2A- B55 is not only regulated by Endos and Arpp19, which directly bind to and inhibit its activity, but also determined by the mechanisms interfering in the B55 type holoenzyme assembly or substrate binding.

1.5.1.3 PP2A-B55 and cancers

The B55 subunit is thought to be the master regulator of PP2A and has been demonstrated to play important role in different signaling pathways, and its deregulations was associated with different cancer types [185]. For example, the level of expression of the B55α subunit was found to be inhibited in acute myeloid leukaemia (AML) patients, and accompanied with an increased phoshorylation of the Akt kinase at Thr308 [186]. Moreover, PP2A-B55α was previously shown to be required for the dephosphorylation of Thr308 of Akt [187]. Thus, B55α suppression leads to the consistent activation of Akt, which subsequently leads to the enhanced proliferation and survival of cancer cells [187]. Indeed, the PP2A activator FTY720 treatment led to an over-expression of B55α and leukaemia cells death [188]. In addition to AML, it was demonstrated that PP2A-B55 is also involved in Alzheimer's, and the deletion of the gene encoding for B55 was observed in luminal B breast cancer, ovarian cancer and prostate cancer [189-193].

1.5.1.4 PP2A-B55 and cytoskeleton stability

As mentioned above, both the polyoma and the simian virus (SV40) small t antigen form stable complexes with the PP2A core enzyme by displacing B55 from the holoenzyme [194-196]. Later, it was demonstrated that in small-t expressed MDCK (Matin-Darby canine kidney) cells, the B55 subunit dissociated from the membrane and redistributed in the cytoplasm and nucleus[178]. This redistribution of the B55 subunit consequently leads to the deregulation of cytoskeleton organization and tight junction protein localization, both of which are likely to be required for the development of small t induced tumors [178]. Moreover, it is now well known that PP2A-B55 dephosphorylates the neuronal-specific microtubule associated protein ─ Tau and mammalian fibroblast intermediate filament ─ vimentin, both of which are essential for the stabilization of the cytoskeleton [197-199]. Recently, it was shown that PP2A-B55

dephosphorylates and activates PRC1, which is a highly conserved component of the anaphase central spindle and is required for the stabilization and antiparallel organization of microtubules [200]. Taken together, PP2A-B55 appears to play a key role in regulating cytoskeleton stability at multiple levels in different cell types. Indeed, PP2A-B55 was demonstrated to associate with and regulate microtubules function in a cell-cycle dependent manner [201]. In agreement with these results, recently I found that PP2A-B55 activity is required for microtubules organization during cytokinesis in *Drosophila* cells (data not shown). Nonetheless, further studies are required to shed light on the precise molecular mechanism of the role of PP2A-B55 in cytoskeleton regulation.

1.5.1.5 PP2A-B55 and centrosome maturation

In addition to its roles in cancer genesis and cytoskeleton stability, PP2A-B55 is also involved in centrosome maturation. A genome-wide RNAi screen in *Drosophila* S2 cells revealed that PP2A-Tws, which is the sole B55 type PP2A in *Drosophila*, is essential for an efficient PCM (pericentriolar material) recruitment [202]. Later, PP2A-Tws was shown to dephosphorylate and stabilize the Plk4 (Polo-like-kinase 4) kinase, which is a key regulator for centriole amplification [203]. Such regulation was also found to be conserved in the roundworm *C.elegans* [204]. In the same year, we reported that PP2A-Tws collaborates with Polo kinase to ensure centrosome ─ nucleus attachment and cell cycle progression in syncytial embryos of *Drosophila* [138]. All these results indicate a convincing role of PP2A-B55 in the regulation of centrosome maturation. However, the precise molecular mechanism of this regulation and the substrates involved remain elusive.

In addition to the various functions described above, it was also demonstrated that PP2A-B55 (Tws) is implicated in the Wnt signaling through regulation of β-catenin phosphorylation in *Drosophila* [205]. Moreover, PP2A-Tws also regulates the self-renewal of *Drosophila* neural stem cells and circadian clock through regulation of a periodic protein PER [206, 207]. Furthermore, PP2A-B55 may be involved in the developmental and translational control by dephosphorylating potential substrates such as $p70^{S6K}$ and 4E-BP1 [208]. Therefore, PP2A-B55 is a veritable multifunctional phosphatase involved in vast cellular processes. In the following section, I will focus on its role in mitotic progression.

1.5.1.6 PP2A-B55, as a major opponent of cyclin B-Cdk1 in higher eukaryotes, must be inhibited at mitotic onset and reactivated upon mitotic exit.

Since a long time, people have tried to find Cdk1 opposing phosphatases which must affect the phosphorylation level of Cdk1 substrates. In1992, B55 type PP2A was shown to dephosphorylate a synthetic peptide pre-phosphorylated by Cdk1 *in vitro* [209]. The next year, PP2A was identified as the major phosphatase targeting several Cdk1 substrates in vertebrate cell extracts [210]. Meanwhile, in *Drosophila*, it was demonstrated that a B55 loss-of-function mutant led to an abnormal anaphase with perturbed chromosome segregation [211]. Just one year later, it was discovered that such mitotic defects induced by B55 loss-of-function mutant was likely due to the dramatic reduction in PP2A ability to dephosphorylate Cdk1 substrates upon mitotic exit [212]. This is the first study suggesting that PP2A-B55 is a main Cdk1 counteracting phosphatase *in vivo*.

In 2009, it was demonstrated that 65% immunodepletion of the subunit B55δ reduced about 65% phosphatase activity targeting Cdk1-phosphorylated peptide, whereas the depletion of other phophatases or other B subunits of PP2A did not [213]. Soon afterwards several articles showed in various ways that B55 associated PP2A is the major phosphatase against Cdk1 activity on mitotic substrates in *Drosophila* (Tws), in human cells (B55α/δ), and in *Xenopus* egg extracts(B55δ) [138, 153-155, 214]. Thus, it makes sense that PP2A-B55 should be inhibited to protect Cdk1-phosphorylated substrates upon mitotic entry and then reactivated to reverse those phosphorylations at mitotic exit. Indeed, in 2007, Mochida and Hunt found that a phosphatase 'X' (later identified as PP2A-B55δ) activity, which targeted mitotic phospho-proteins, disappeared at mitotic entry and reappeared at mitotic exit in *Xenopus* egg extracts [213, 215]. Such cyclical regulation of PP2A-B55 activity during cell division is extensively supported by experimental results.

The first results revealing the importance of PP2A-B55 inhibition upon mitotic entry came from biochemical studies performed in *Xenopus* egg extracts. First, when recombinant PP2A- B55δ was added to *Xenopus* egg interphase extracts to increase 50% the endogenous PP2A-B55δ concentration, entry into mitosis was delayed, and when this concentration was doubled, mitotic entry was prevented completely [213]. Second, the depletion of B55δ in interphase extracts induced premature mitotic entry with a lower amount of cyclin B [213]. Third,

in the interphase extracts, the depletion of a PP2A-B55 mitotic inhibitor ─ Greatwall kinase, blocked mitotic entry, but it was rescued by B55δ removal [153]. Later, it was clearly demonstrated that depletion of Ensa or its close relative ─ Arpp19, which are the sole known substrates of Gwl in animals and the specific inhibitors of PP2A-B55, did prevent mitotic entry even in the presence of Gwl [158, 159]. Conversely, the addition of Gwl-thiophosphorylated Ensa or Arpp19 into interphase extracts promoted mitotic entry even in the absence of Gwl [158, 159]. To date, the Ensa (Endos) or Arpp19 mediated inhibition of PP2A-B55 upon mitotic entry has been demonstrated to be conserved in *Xenopus*, *Drosophila*, starfish and human [158, 159, 200, 214].

In addition to the importance of its inhibition at mitotic entry, the necessity of PP2A-B55 reactivation at mitotic exit is also demonstrated by different groups. It was showed that Gwl-thiophosphorylated Ensa or Arpp19 blocked mitotic exit in *Xenopus* CSF extract [158]. Moreover, we found that in *Drosophila* syncytial embryos, overexpressing Gwl and simultaneously reducing *tws* (B55) prevented mitotic exit [138]. Furthermore, human cells arrested in metaphase by proteasome inhibition can be forced to exit from mitosis by adding Cdk1 inhibitor, but simultaneous B55 depletion by RNAi delayed Cdk1 substrates dephosphorylation, and consequently a significant delay of nuclear reassembly was observed [155]. However, the molecular mechanism of PP2A-B55 reactivation during mitotic exit is still controversial. Recently, Michael Goldberg's group showed that PP2A-B55 dephosphorylates Endos after Gwl inactivation upon mitotic exit [179], whereas Helfred Hochegger's group proposed that the RNA polymerase II C-terminal tail domain phosphatase Fcp1 dephosphorylates Endos to release PP2A-B55 which subsequently dephosphorylates Gwl during M phase exit [216]. Therefore, further studies are required to clarify the contributions of specific phosphatise to Gwl and Endos inactivation.

In budding yeast, the main Cdk1 counteracting phosphatase is Cdc14 instead of PP2A-B55 [217]. Cdc14 is also regulated in a cell cycle dependent manner through periodic association with its competitive inhibitor Cfi1 (also known as Net1) [218]. From interphase to metaphase, Cfi1/Net1 binds to and inhibits Cdc14 activity by sequestering it in the nucleolus [219]. During anaphase, the FEAR (Cdc **f**ourteen **e**arly **a**naphase **r**elease) pathway and the MEN (mitotic exit network) pathway sequentially release Cdc14 from its inhibitor and relocalize Cdc14 to the

nucleus and cytoplasm, allowing the dephosphorylation of Cdk1 phosphorylated substrates [220]. So, what does PP2A-B55 (B55 is known as Cdc55 in yeast) do in budding yeast? It was demonstrated that PP2A-Cdc55 negatively regulates the FEAR and MEN pathways, thereby functioning as an inhibitor of mitotic exit [221]. However, a recent paper delineated that in early anaphase, PP2A-Cdc55 activity was indeed downregulated to promote the FEAR, whereas in late anaphase its activity was required for the full activation of the MEN to contribute to complete mitotic exit [222]. Moreover, in contrast to its role in animals, PP2A-Cdc55 activity was required for mitotic entry in budding yeast [223]. Recently, it was demonstrated that Zds1 and Zds2 promote the cytoplasmic localization of PP2A-B55, where PP2A-B55 targets Swe1 kinase (Wee1 homologue) and Mih1 phosphatase (Cdc25 homologue) to promote mitotic entry [224, 225]. Furthermore, Endos family proteins Igo1/Igo2 also exist in budding yeast and can directly inhibit PP2A-B55 *in vitro* [226, 227]. In addition to their direct inhibition, Rim15 (yeast Gwl) phosphorylated Igo1/Igo2 proteins also contribute to PP2A-B55 nuclear export, thus promoting mitotic entry [227]. However, a very recent article indicated that compared to Zds proteins, Igo1/Igo2 have only a minor effect at mitotic entry [228]. Further investigations are required for clarifying the precise role of Zds proteins and Endos family proteins in PP2A-B55 regulation in budding yeast.

1.5.2 The Greatwall kinase, a defense system, is set up by Cdk1 upon mitotic entry and disabled during M phase exit.

1.5.2.1The role of Greatwall in mitotic progression

During the last decade, Greatwall (Gwl) kinase has been proven to be a crucial mitotic kinase in many organisms. Even the process of identification of PP2A-B55 as the major counteracting phosphatase of Cdk1, in fact, was initiated from the functional analysis of Gwl kinase. In 1996, *greatwall* was originally discovered in *Drosophila* as *scant* (**Sc**ott of the **Ant**arctic) which, when homozygous, led to mitotic defects in syncytial embryos and to female sterility [229]. *Scant* was later mapped to the *greatwall* gene and found to encode a mutant, hyperactive form of the kinase (K97M) in *Drosophila* [135]. Interestingly, in *Xenopus* eggs, the Scant (K71M) form of Gwl promotes M phase entry, regardless of its much lower expression than that of wild type Gwl [230]. The first recessive allele of *gwl* was identified by the Goldberg group, who gave the gene the name "*greatwall*" (*gwl*) as it appeared to be important for

protecting the structure of mitotic chromosomes. They demonstrated that in larval neuroblasts from *gwl* loss-of-function mutants, the mitotic progression was dramatically slowed down from late G2 phase through nuclear envelope breakdown (NEBD) until anaphase onset, and was accompanied by chromosome condensation and segregation defects [231]. They suggested that Gwl is required for establishing high cyclinB-Cdk1 activity at mitotic entry. However, later in the same year, in *Drosophila* S2 cells, it was shown that depletion of Gwl by RNAi did not induce chromosome condensation defect but rather chromosome congression and segregation defects [232]. Therefore, these results suggest that in different cell types, Gwl might be needed at different time points to ensure the progression of different mitotic processes. Later, it was demonstrated that in *Xenopus* egg extracts, Gwl depletion prevents cyclinB-Cdk1 activation and mitotic entry, whereas activated Gwl promotes both mitotic entry and oocyte meiotic M phase entry [233, 234]. Gwl depletion from mitotic extracts induced a rapid loss of cyclinB-Cdk1 activity due to the accumulation of inhibitory phosphorylation (pTyr15) on Cdk1 [233]. Remarkably, the inhibitor of phosphatase, okadaic acid (OA), as activated Gwl, promotes mitotic entry and is able to remove the mitotic entry blockage in Gwl depleted CSF extracts [234]. Thus, Gwl might negatively regulate an OA ─ sensitive phosphatase to ensure sufficiently high MPF activity in mitosis and maintain the phosphorylations state of mitotic proteins including Cdc25 and Wee1/Myt1 [156, 234, 235]. Soon afterwards, as described in the previous section, this OA ─ sensitive phosphatase was identified as B55 type PP2A, which is the major phosphatase against Cdk1 phosphorylated substrates in *Xenopus* [153, 213]. Strikingly, concurrent depletion of Myt1, Wee1 and Gwl still can't prevent mitotic exit of metaphase arrested *Xenopus* CSF extract, despite the presence of high cyclinB-Cdk1 activity, suggesting that the fully activated Cdk1 alone is not sufficient to defeat its counteracting phosphatase PP2A-B55 to maintain mitotic state [156]. Therefore, Gwl functions as a counterpoise to ensure the declination of the balance towards cyclinB-Cdk1 by inhibiting PP2A-B55, thus promoting mitotic entry and maintenance [153, 154, 156].

1.5.2.2 Gwl's substrates and non-mitotic functions.

Although Gwl was demonstrated to associate with $PP2A_D$ in human cells, a substantial phosphorylation of PP2A-B55 components by Gwl has not been detected [153, 156]. The two small homologous proteins ─ α-Endosulfine (Ensa/Endos) and the cyclic adenosine

monophosphate regulated phospho-protein 19 (Arpp19) provided the missed link between Gwl and PP2A-B55 [159, 214, 236]. Gwl phosphorylated Ensa and Arpp19 strongly bind to and inhibit PP2A-B55 but not any other B type PP2A or PP1 [159]. From these findings, it appears that Gwl ─ Endos/Arpp19 mediated PP2A-B55 inhibition promotes MPF (Mitotic Promoting Factor) activation at G2/M transition by increasing the activatory phosphorylation on Cdc25 and the inhibitory phosphorylation on Wee1/Myt1, and further ensures the mitotic maintenance by preventing the dephosphorylation of MPF substrates. So far, in animals, no substrates of Gwl other than Endos/Arpp19 were reported. However, there might be other Gwl substrates because Gwl was demonstrated to promote the recovery of G2/M DNA damage checkpoint in *Xenopus* egg extract and regulate transcription in yeast [237-239]. Depletion of Gwl from *Xenopus* egg extract increased the DNA damage response (DDR) and impaired recovery from checkpoint arrest and entry into mitosis, whereas addition of wild-type Gwl inhibits the DDR [237]. Moreover, through the control of Aurora A kinase activation, Gwl also indirectly regulates the activity of Plx1 (Polo-like-kinase1 in *Xenopus*), which is a key regulator of cell recovery from the G2/M DNA damage checkpoint [240]. Although these studies can be partially explained by an indirect effect of PP2A inhibition mediated by Gwl, unidentified Gwl substrates were strongly suggested. In contrast to Gwl in animals, yeast Gwl ─ Rim15 plays important role in several biological processes other than mitotic progression and its substrates other than Endos/Arpp19 were recently identified. Upon nutrient starvation, Rim15 directly phosphorylates and activates the heat shock transcription factor Hsf1 and zinc finger transcription factor MSN2 to ensure proper cellular adaptation or survival [239]. So far in higher eukaryotes, Gwl functions have not been examined under nutrients deprivation, thus further studies under stressed conditions may allow uncover new Gwl substrates and new regulatory mechanism of Gwl.

1.5.2.3 The regulation of Gwl

In contrast to its well studied functions, the mechanisms regulating Gwl itself are still less understood. Gwl is a member of the ACG kinase family but is distinguished from the others by containing a long poorly conserved insert, which splits its kinase domain into N-terminal and C-terminal sub-domains [231]. This long insert appears not to be essential for its kinase activity [241, 242]. To date, two Gwl activation models have been proposed. Thierry Lorca and Anne Castro's group suggests that Gwl is first phosphorylated on its C-terminal tail-linker residue

(S883) to promote the association of this residue with an N-terminal tail-linker binding site, thus stabilizing Gwl in a partially activated state. Then, the Gwl N-terminal hydrophobic pocket binds to the phosphorylated hydrophobic motif of another AGC kinase to induce a full activation of Gwl [242] (Figure 1.12). However, the Goldberg group proposed that Gwl is initially phosphorylated by MPF at two sites in the presumptive activation loop. Subsequently, Gwl can auto-phosphorylate its C-terminal tail-linker residue (S883), which binds to some basic residues in its N-terminal domain to stabilize active Gwl [241] (Figure 1.13).

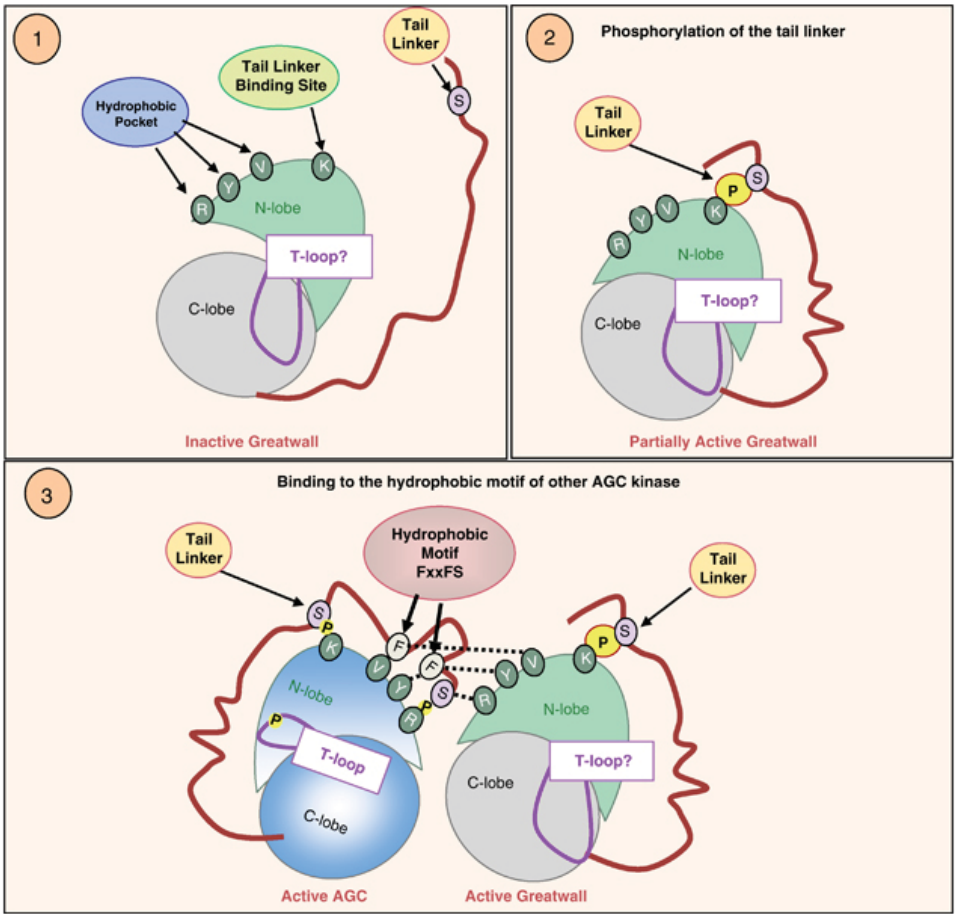


Figure 1.12. Mechanisms of activation of the Gwl kinase. Activation of Gwl involves two different steps. In the first step, Gwl is phosphorylated at its C-terminus in the Tail/Linker site (2). This phosphorylation results in the binding of the Tail/Linker site with the Tail/Linker binding site at the N-terminus of Gwl, promoting the partial activation of this kinase. However, complete Gwl activation requires the association of a phosphorylated hydrophobic motif of another unknown AGC kinase with the hydrophobic pocket of Gwl (3). This figure was adapted from Thierry Lorca and Anne Castro (2013) [243], with permission from publisher Nature Publishing Group.

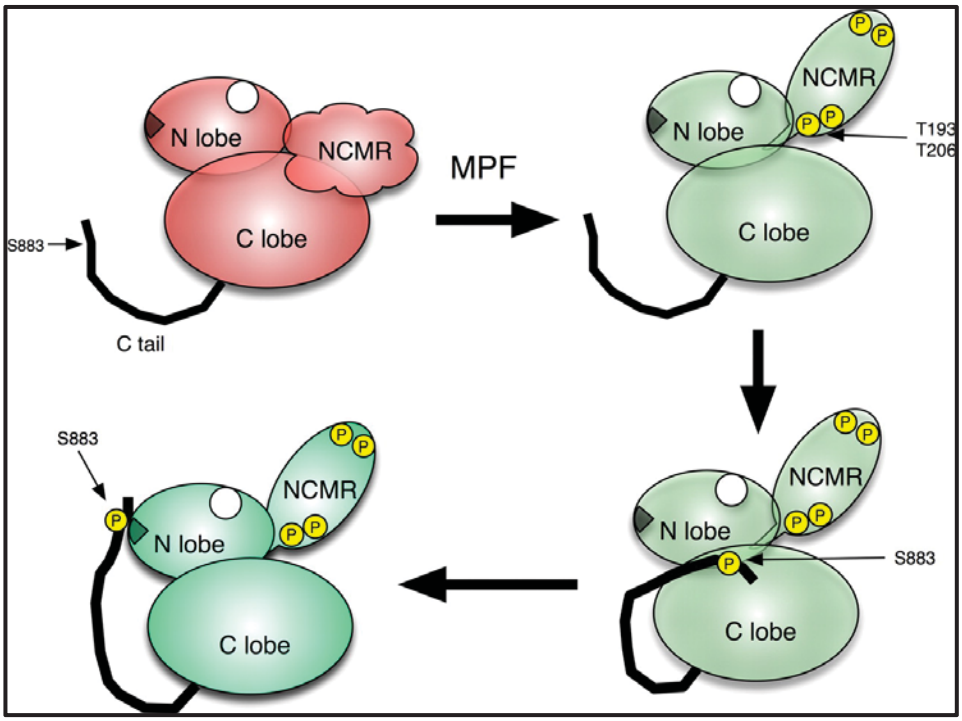


Figure 1.13. A model for Gwl activation. Gwl is depicted with four domains: the N-terminal lobe (N lobe), the C-terminal lobe (C lobe), the nonconserved middle region (NCMR), and the C-terminal tail (thick black line). During M phase entry, MPF phosphorylates Gwl at the presumptive activation loop sites T193 and T206. MPF, and perhaps other kinases, may also target several phosphosites within the NCMR that could have redundant functions. NCMR phosphorylations could conceivably change its conformation to allow substrates access to Gwl's active site between the N- and C-terminal lobes. After Gwl is primed by MPF, S883 in the C-terminal tail can be autophosphorylated at the active site. It is likely that pS883 can subsequently interact with a patch of basic residues in the N lobe (dark triangle) to help stabilize active Gwl (40). The round hole in the N lobe depicts the conserved HF binding pocket, whose role is not yet clear. This figure was adapted from Blake-Hodek *et al.* (2012) [241], with permission from publisher American Society for Microbiology.

Regardless of the controversy of the two Gwl activation models, there is strong evidence indicating that MPF is a critical activator of Gwl. First, Gwl is activated and highly phosphorylated in M phase [233, 244]. Second, MPF phosphorylates and activates Gwl in vitro [233, 241, 242]. Third, Gwl is activated downstream of Cdk1 in starfish oocytes [244]. Finally, Gwl activation is abolished by adding Cdk1 inhibitor Roscovitine and $p21^{Cip1}$ in CSF extract and frog oocyte [234]. Incidentally, if Gwl is a substrate of Cdk1 and PP2A-B55 dephosphorylates Cdk1 substrates, Gwl could be one of PP2A-B55 targets. Indeed, in *Xenopus* egg extracts, both $PP2A_D$ and B55 are able to associate with Gwl in the same complex [156, 230]. Moreover, the

PP2A catalytic subunit dephosphorylates and inactivates Gwl *in vitro* [230]. Furthermore, the depletion of Arpp19 from CSF extracts leads to a rapid dephosphorylation of Gwl and mitotic exit due to the reactivation of PP2A [158]. A recent article demonstrated that during mitotic exit, PP2A-B55 dephosphorylates Gwl at a previously reported Cdk1 site and essential for Gwl activation upon mitotic entry [216, 241]. In this case, then how is Gwl itself activated by Cdk1 at mitotic entry in presence of high activity of PP2A-B55 and to subsequently protect other Cdk1 substrates against PP2A-B55 activity?

One possible answer to this question is that PP2A-B55 and Gwl are physically sequestered when Cdk1 activates Gwl. Thus, I carefully studied the spatiotemporal dynamics of these mitotic progression regulators during my PhD training. We showed that, both in *Drosophila* cultured cells and syncytial embryos, Gwl is spatially regulated during the cell cycle [245]. Gwl is prominently nuclear in interphase, whereas it appears to be excluded from nucleus and turns to be principally cytoplasmic just before nuclear envelope break down (NEBD) [245]. Similar dynamic localization of Gwl was also observed in human cells [246]. Interestingly, at mitotic entry, cyclin B is imported into the nucleus before Gwl is delocalized into the cytoplasm, while B55 appears to be predominantly cytoplasmic both in *Drosophila* and in human cells [155, 206, 212, 245, 246]. Indeed, it was recently demonstrated using a Gwl active site phospho-specific antibody that active Gwl was first detected in the nucleus of human cells [216]. Moreover, I did find that a Gwl mutant, which localizes constantly in the cytoplasm, appears to be less phosphorylated than wild type Gwl in *Drosophila* ovaries [245]. Therefore, in accordance with our hypothesis, the nuclear envelope may serve as a barrier to separate Gwl from PP2A-B55, thus facilitating Gwl activation by CyclinB-Cdk1. However, the earliest Cdk1 cytoplasmic substrates, which are required for cytoskeleton reorganization and cell rounding [150], are totally exposed to the full active PP2A-B55. This might be the reason why Gwl is promptly sent into cytoplasm before NEBD, thus protecting Cdk1 substrates by inhibiting PP2A-B55 (Figure 1.14). Importantly, it was indeed demonstrated that both Gwl nuclear localization during interphase and its nuclear export before NEBD appear to be essential for its function and mitotic progression [245, 246]. However, further investigations are required to show the importance of cytoplamic inhibition of PP2A-B55. The spatiotemporal regulation of PP2A-B55 and Endos relative to each other should also be investigated.

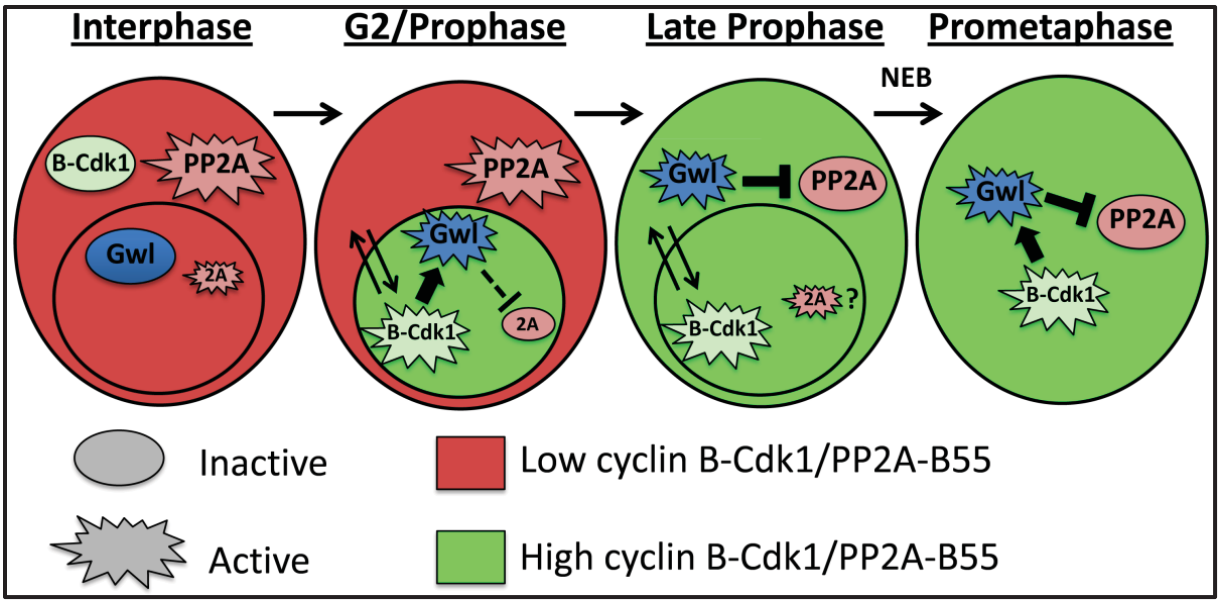


Figure 1.14. Spatial model for the Gwl-PP2A axis in the control of mitotic entry. In interphase, cyclin B-Cdk1 is largely inactive, while PP2A-B55 is active. Both cyclin B-Cdk1 and PP2A-B55 are mostly cytoplasmic, while Gwl is nuclear. In early prophase, cyclin B-Cdk1 begins to shuttle into the nucleus and becomes active through the auto-amplification loop (not shown). In the nucleus, cyclin B-Cdk1 activates Gwl. Gwl then translocates to the cytoplasm, where it is positioned to antagonize PP2A-B55 by phosphorylating endosulfines (not shown). As a result, the cyclin B-Cdk1/PP2A-B55 ratio is high throughout the cell before nuclear envelop breakdown (NEBD). This figure was adapted from Peng Wang *et al.* (2014) [247], with permission from publisher Springer.

In addition to the functional significance of Gwl localization, I have also investigated the regulatory mechanisms of this localization. In the enigmatic long insert region of *Drosophila* Gwl, which previously appeared to be unessential for Gwl activity, two nuclear localization signals (NLS) were identified [245]. Phosphorylation by Polo and Cdk1 in this region of Gwl promotes its cytoplasmic localization in prophase [245]. Phosphorylation by Polo likely promotes the association of Gwl with 14-3-3ε leading to its cytoplasmic retention, whereas Cdk1 regulates Gwl localization through an unknown mechanism [245]. On the other hand, human Gwl nuclear export in prophase has been shown to depend on its own kinase activity and its activation by cyclinB-Cdk1 [246]. Moreover, the existence of nuclear export signals (NES) in both *Drosophila* and human Gwl were suggested but yet to be further characterized [245, 246].

Interestingly, before *gwl* was identified, budding yeast Gwl ─ Rim15 nucleocytoplamic distribution has been already demonstrated to be phosphorylation dependent [248]. Sch9 (functionally analog to S6K in mammals) phosphorylates Rim15 to provide a binding site of Bmh1 and Bmh2 (yeast 14-3-3 proteins), thus promoting Rim15 cytoplasmic retention [248-250]. Therefore, the spatial regulation of Gwl by phosphorylation and 14-3-3 proteins appears to be a conserved mechanism across species.

"We should never ignore the results coming from yeast" ─ Vincent Archambault

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CHAPTER 2

Publication

PP2A-Twins Is Antagonized by Greatwall and Collaborates with Polo for Cell Cycle Progression and Centrosome Attachment to Nuclei in Drosophila Embryos

Article published in PLoS Genetics (2011) Aug; 7(8) e1002227

PP2A-Twins is antagonized by Greatwall and collaborates with Polo for cell cycle progression and centrosome attachment to nuclei in *Drosophila* embryos

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2.1 ABSTRACT

Cell division and development are regulated by networks of kinases and phosphatases. In early *Drosophila* embryogenesis, 13 rapid nuclear divisions take place in a syncytium, requiring fine coordination between cell cycle regulators. The Polo kinase is a conserved, crucial regulator of M-phase. We have recently reported an antagonism between Polo and Greatwall (Gwl), another mitotic kinase, in *Drosophila* embryos. However, the nature of the pathways linking them remained elusive. We have conducted a comprehensive screen for additional genes functioning with *polo* and *gwl*. We uncovered a strong interdependence between Polo and Protein Phosphatase 2A (PP2A) with its B-type subunit Twins (Tws). Reducing the maternal contribution of Polo and PP2A-Tws together is embryonic lethal. We found that Polo and PP2A-Tws collaborate to ensure centrosome attachment to nuclei. While a reduction in Polo activity leads to centrosome detachments observable mostly around prophase, a reduction in PP2A-Tws activity leads to centrosome detachments at mitotic exit, and a reduction in both Polo and PP2A-Tws enhances the frequency of detachments at all stages. Moreover, we show that Gwl antagonizes PP2A-Tws function in both meiosis and mitosis. Our study highlights how proper coordination of mitotic entry and exit is required during embryonic cell cycles and defines important roles for Polo and the Gwl-PP2A-Tws pathway in this process.

2.2 AUTHOR SUMMARY

The development and survival of all living organisms relies on the fine regulation of cell division at the molecular level. This coordination depends on kinases and phosphatases, enzymes that catalyze the addition and removal of phosphate groups on specific target proteins. The genes encoding these enzymes have been largely conserved between species during evolution. In a previous paper published in *PLoS Genetics*, we found an antagonism between the Polo and Greatwall mitotic kinases in the fruit fly model. In this study, we have used fly genetics to identify additional genes that function with *polo* and *greatwall* during early embryogenesis. We have found a specific form of the Protein Phosphatase 2A (PP2A-Tws) to collaborate with the Polo kinase, at a stage when multiple nuclei rapidly divide in a large, single-cell early embryo. We found that Polo and PP2A-Tws are both required for the proper cohesion between nuclei and the centrosomes, which are essential structures for mitosis and embryonic development. We also found that the Greatwall kinase antagonizes the PP2A-Tws phosphatase to promote mitosis and meiosis. Our genetic study sheds new light on cell cycle regulation, and is consistent with recent results from biochemical studies using frog cell extracts.

2.3 INTRODUCTION

The cell cycle is largely driven by networks of kinases and phosphatases that coordinate the sequential events of cell division in addition to regulating each other [1]. Kinases of the Polo, Aurora and cyclin-dependent families play particularly important roles in this process [2-4]. Phosphatases compete with kinases for the same substrates, and the balance between their activities is subjected to a fine regulation through the cell cycle [5,6]. While in budding yeast the Cdc14 phosphatase plays a crucial role in promoting mitotic exit by dephosphorylating Cdk1 substrates and promoting its inactivation [7], it is becoming increasingly clear that a form of Protein Phosphatase 2A (PP2A) bound to a B-subtype adaptor subunit fulfills this function in vertebrates [8,9]. Yet, a clear picture of the dynamic interplay between specific kinases and phosphatases during the cell cycle is still missing.

The developmental program of a complex organism requires that the cell cycle machinery adapt to the situation and contribute to the integration of cell divisions in various tissue contexts

and cell types. Early embryogenesis typically involves rapid cell cycles where S-phases and M-phases alternate rapidly with little or no gap phases, growth or transcription. In *Drosophila*, the first 13 mitotic cycles occur in a syncytium at around 10-15 min intervals with virtually no zygotic transcription, and are driven by maternally contributed proteins and mRNAs [10]. At that stage, nuclei migrate in the syncytium, first in an axial fashion, and then towards the cortex to form the blastoderm [11]. In addition to organizing mitotic spindles, centrosomes are tethered to nuclei in the syncytium and constitute anchors for the nuclei to a network of anti-parallel astral microtubules (MTs) that push nuclei away from each other and towards the cortex [11]. Because of the absence of G1 at that stage, DNA replication and centrosome duplication occur shortly after mitotic exit and as early as telophase [10]. The nuclear envelope does not completely break down during syncytial mitoses, but becomes fenestrated to allow MTs to penetrate nuclei [12-14]. While centrosomes are dispensable for cell division in many cell types, they are absolutely essential for early embryogenesis in *Drosophila* [15,16].

The Polo kinase is a conserved, central regulator of M-phase [2,17,18]. Polo promotes mitotic entry by activating Cdc25 phosphatases that activate Cdk1/Cdc2 [19,20]. Cyclin B-Cdk1 triggers nuclear envelope breakdown and chromosome condensation [21,22]. Polo also plays important roles in centrosome maturation, chromosome attachment to MTs, bipolar spindle assembly and cytokinesis [2,18]. However, how Polo functions contribute to various developmental contexts has been little explored. We have recently identified the Greatwall kinase genetically in an antagonistic functional relationship with Polo in the *Drosophila* syncytial embryo [23]. Decreasing Polo activity and increasing Gwl activity together lead to a failure in early embryogenesis characterized by centrosome detachments from nuclei. Discovered in *Drosophila* as an important mitotic kinase [23,24], Greatwall has rapidly emerged as a crucial regulator of M phase in *Xenopus* extracts [25] and in human cells [26,27]. The precise nature of the functional relationship between Polo and Greatwall that we uncovered genetically remained elusive [23].

Free centrosomes can occur in several distinct ways in syncytial embryos, detaching from mitotic spindles or interphase nuclei, or duplicating independently from nuclei [28]. In response to DNA damage, centrosomes can be inactivated, leading to the loss of the damaged nucleus which sinks into the yolk [29]. This response depends on the Chk2 kinase [30]. Mutations

inactivating *chk2* did not prevent the centrosome detachments observed in embryos where Polo function was decreased and Gwl function was increased [23], suggesting that those events are not due to an activation of the DNA damage checkpoint, but reflect problems in coordinating the early mitotic divisions at another level.

We have conducted a genetic screen to identify additional genes functioning with *polo* and *gwl*. We uncovered two genes encoding subunits of PP2A as the strongest hits in this screen: the catalytic subunit gene (*microtubule star/mts*) and the B-type adaptor subunit gene (*twins/tws*). Phenotypic examination of the nuclear divisions in single mutants shows that Polo promotes the cohesion between centrosomes and nuclei around prophase while PP2A-Tws promotes centrosome attachment to nuclei during mitotic exit. Compromising Polo and PP2A-Tws functions simultaneously strongly enhances centrosome detachments and leads to failures in syncytial embryonic development. Moreover, we show that the Gwl kinase functions to antagonize PP2A-Tws in both meiosis and mitosis. Our results indicate that precise coordination of mitotic entry and exit is crucial during embryonic cell cycles, and implicate Polo, Gwl and PP2A-Tws as key regulators. We discuss our findings in the context of recent studies from the *Xenopus* extract and *Drosophila* systems, which also implicate a pathway linking Gwl and PP2A in regulating M phase.

2.4 RESULTS

2.4.1 Polo function is required for proper cohesion between centrosomes and nuclei.

Halving the levels of Polo kinase in the fly does not cause any obvious problems of development or fertility. However, embryos laid by *polo* heterozygous females are made inviable by a gain of Greatwall (Gwl) function or by overexpression of Map205, a strong physical interactor of Polo [23,31]. In both cases, defective embryos are characterized by a strikingly penetrant phenotype of centrosome detachments from nuclei. These observations prompted us to examine more closely the effects of compromising Polo function in the syncytial embryo. Immunofluorescence in embryos from mothers heterozygous for the null $polo^{11}$ allele [23] shows that several nuclei in prophase have one dislocated centrosome (Figure 2.1A, B left, arrowheads, quantified in Figure 2.5B), consistent with previous results [23]. This phenotype is also observed in embryos from mothers heterozygous for $polo^{9}$, a strong hypomorph (data not shown). Centrosome detachment can also be observed in prometaphase/metaphase (Figure 2.1B right).

Thus, the cohesion between centrosomes and nuclei in early mitosis is sensitive to Polo kinase levels.

The Polo kinase is essential for a wide variety of functions during cell division, including centrosome maturation, spindle assembly and cytokinesis. Yet, these processes do not appear compromised in embryos receiving half their normal amount of Polo, while centrosome attachment to nuclei is partially defective. This suggested that centrosome-nuclei cohesion in the embryo is particularly sensitive to a decrease in Polo activity, while the other functions of Polo are satisfied with a lower Polo level.

We sought to examine the effects of a more severe reduction in Polo function during syncytial embryogenesis. Because Polo is contributed to the embryo maternally, and because it is essential for viability and for meiosis, we could not examine the effects of a complete genetic inactivation of Polo on embryogenesis. To circumvent these limitations, we used chemical inhibition. We found that the Polo-like kinase 1 inhibitor BI2536 [32] is a potent inhibitor of *Drosophila* Polo. Treatment of D-Mel or S2 cells in culture with BI2536 phenocopied RNAi depletion of Polo, leading to a higher mitotic index and an accumulation of prometaphase cells that often displayed monopolar spindles (data not shown). As predicted, treatment of embryos with BI2536 led to a high frequency of centrosome detachment, along with other defects typical of Polo inhibition in cultured cells, including incomplete or absent spindles and misaligned chromosomes (Figure 2.1D, E). Not surprisingly, defective, neighbouring spindles were often fused in the syncytium (Figure 2.1D, E). However, spindle defects were less penetrant than centrosome detachments (Figure 2.1E). All defects were almost never observed (<1%) in control embryos treated with DMSO alone. Altogether, our results suggest that a lower level of Polo activity is sufficient for spindle assembly and function, while proper cohesion between centrosomes and nuclei requires a higher level of Polo activity.

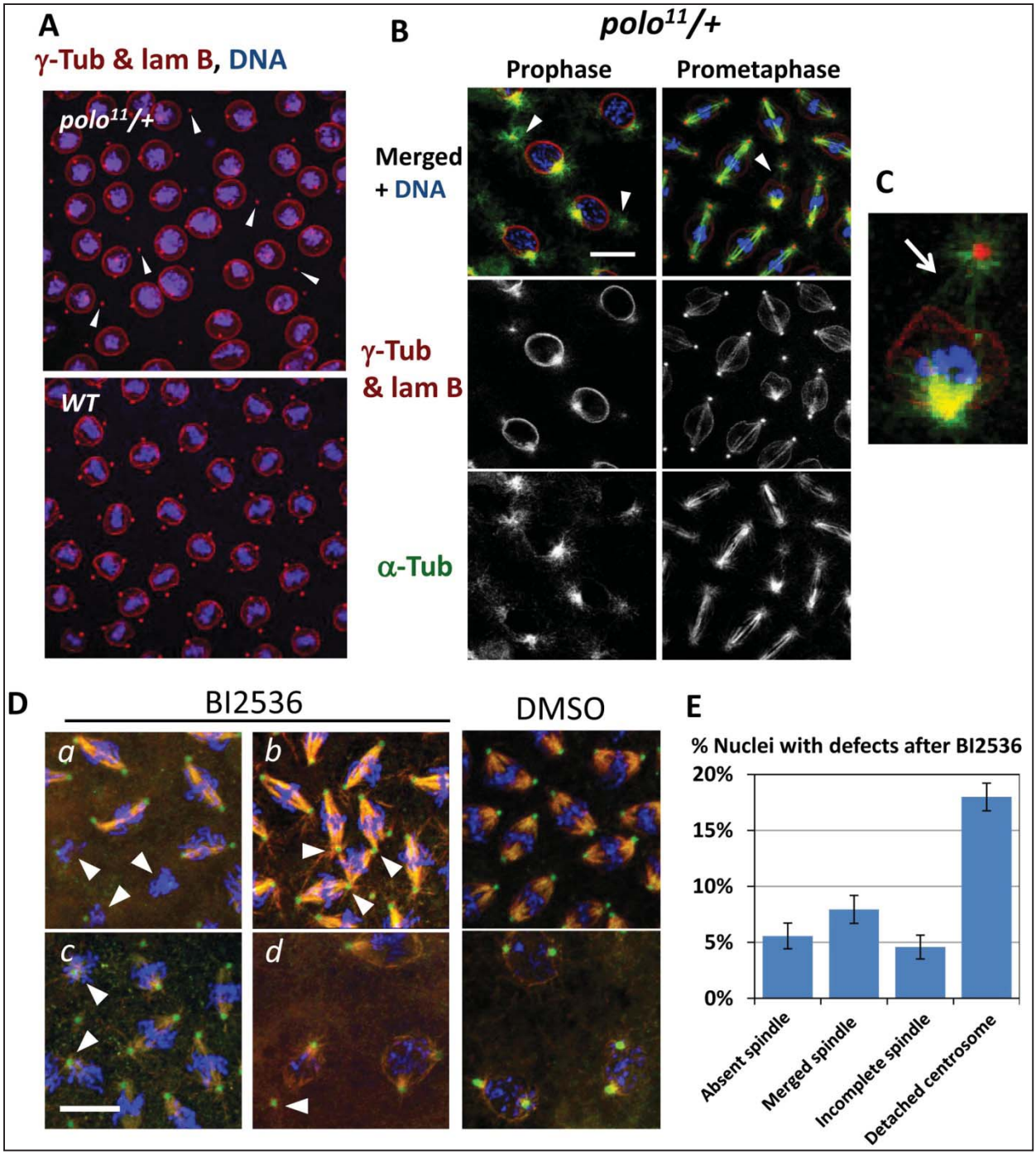
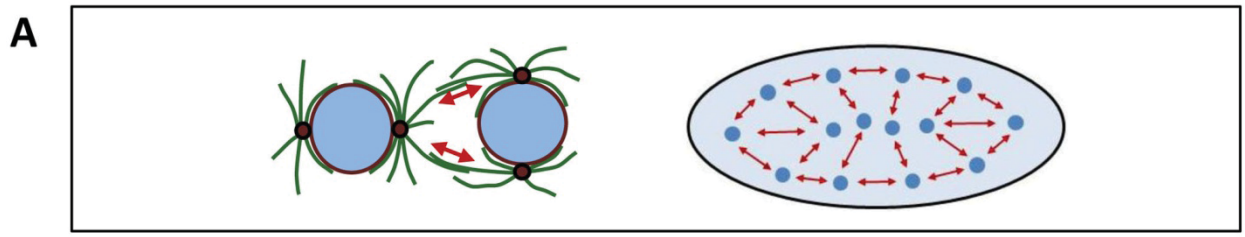


Figure 2.1. The Polo kinase is required for proper centrosome attachment to nuclei in syncytial embryos. A, B. Centrosome detachments observed in prophase in embryos from *polo*^{11}/+ mothers (arrowheads). **B**. Embryos from *polo*^{11}/+ mothers. Left: detachments in prophase; Right: example of a detachment that persists in prometaphase. **C**. Enlargement from the prometaphase image in B. Arrow: MTs from the detached centrosome fail to penetrate the nuclear envelope. **D**. Chemical inhibition of Polo results in centrosome detachments and spindle

defects consistent with known Polo functions: *a*: absent spindles; *b*: fused spindles; *c*: incomplete spindles; *d*: detached centrosomes. Embryos were treated with 1 μM BI2536 for 30 min to inhibit Polo. Red: α-Tubulin; Green: γ-Tubulin, Blue: DNA. **E**. Quantification of the observed defects. N = 19 embryos; Error bars: S.E.M. The frequency of all scored defects in control embryos (DMSO only) was less than 1% (N = 8). Scale bars: 10μm.

2.4.2 Transiently detached centrosomes are recaptured by mitotic spindles.

Centrosome-nuclei cohesion is crucial to embryonic development, since it provides the link between nuclei and a skeleton of anti-parallel astral microtubules (MTs) that pushes the nuclei apart and towards the cortex (Figure 2.2A), although overlapping MTs are difficult to observe [11]. Moreover, centrosomes are essential to the assembly of bipolar mitotic spindles in syncytial embryos [15]. Because the examined *polo*-compromised embryos are able to complete development, the dislocation between centrosomes and nuclei observed either leads to problems that occur at a tolerated rate, or is transient and does not usually lead to mitotic or nuclear migration defects. To examine these possibilities, we used time-lapse microscopy, following GFP-D-TACC as a centrosomal and spindle marker [33], and histone H2A-RFP as a nuclear marker. In embryos from $polo^{11}$/+ mothers, we could readily find several nuclei where one centrosome was dislocated from its nucleus of origin (Figure 2.2B, arrowheads). Yet, dislocated centrosomes were recaptured during spindle assembly (420 s, arrows), and nuclear divisions could then be completed (see also Video S1). This detachment was not observed in embryos from *WT* mothers (Figure 2.2C and Video S2). These observations explain how the centrosome dislocations seen in *polo*-compromised embryos are transient and have no lethal consequences on their own (Figure 2.2D). This centrosome recapture has also been observed in embryos with reduced Polo function and elevated Gwl function, although those embryos were much sicker and inviable, and many centrosomes were not recaptured [23].



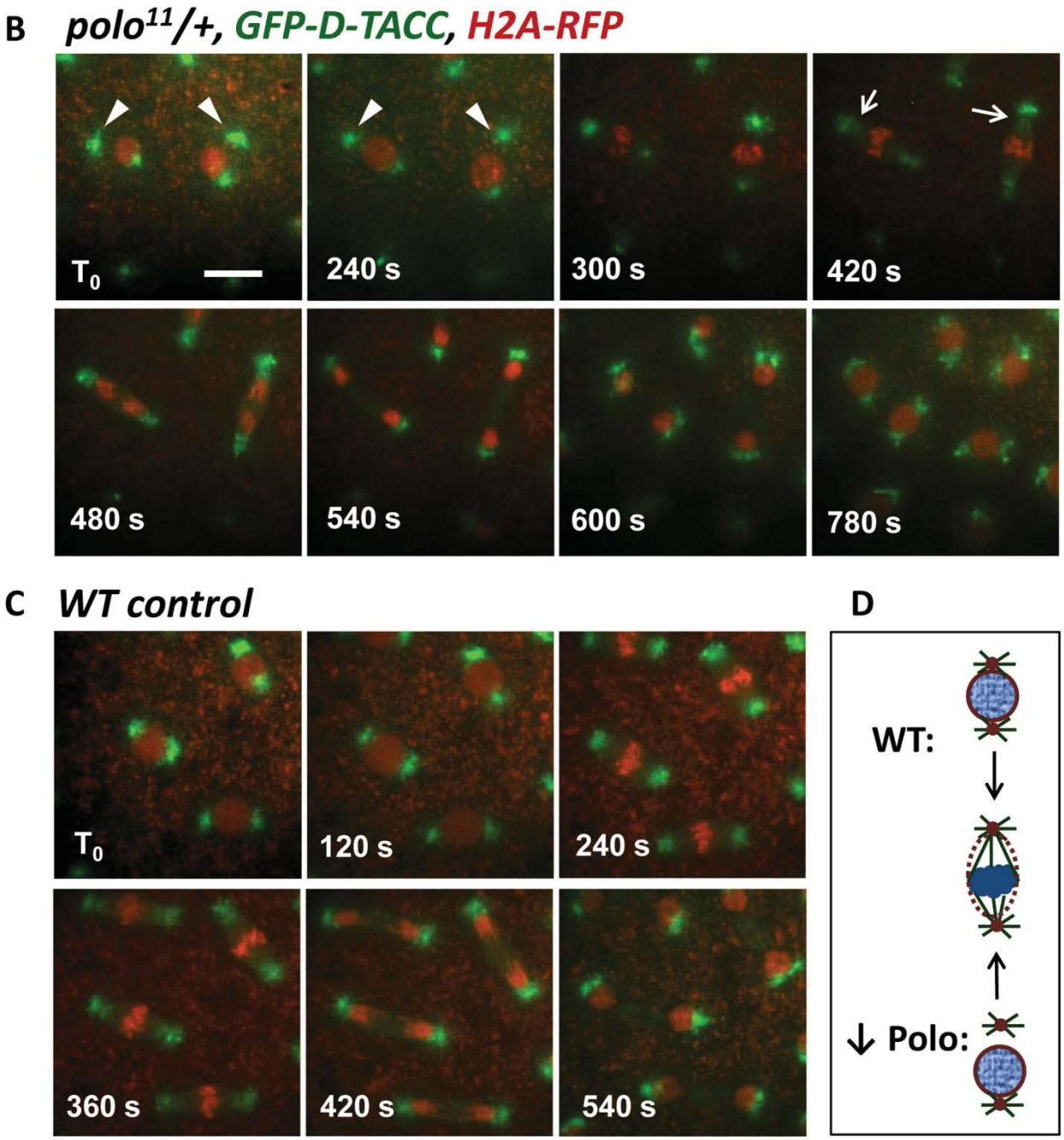


Figure 2.2. Detached centrosomes from nuclei can be recaptured by the mitotic spindles. A. During syncytial divisions, nuclei are linked by anti-parallel astral MTs (left), which push nuclei apart (red arrows) and towards the cortex (right). **B.** Time-lapse imaging of embryos from *polo*^{11}/+ mothers and expressing GFP-D-TACC to mark centrosomes and spindles and H2A-RFP to mark the chromatin. At T_0, detached centrosomes (arrowheads) are clearly away from the nuclei. At 420 s, detached centrosomes are recaptured (arrows) and nuclear division is completed

normally. Scale bar: 10 μm. **C**. Time-lapse imaging of a *WT* embryo as a control. **D**. Schematic illustration of a nuclear division in an embryo with reduced Polo levels, vs a normal, WT embryo. Centrosome detachment in the Polo-compromised embryo is only transient.

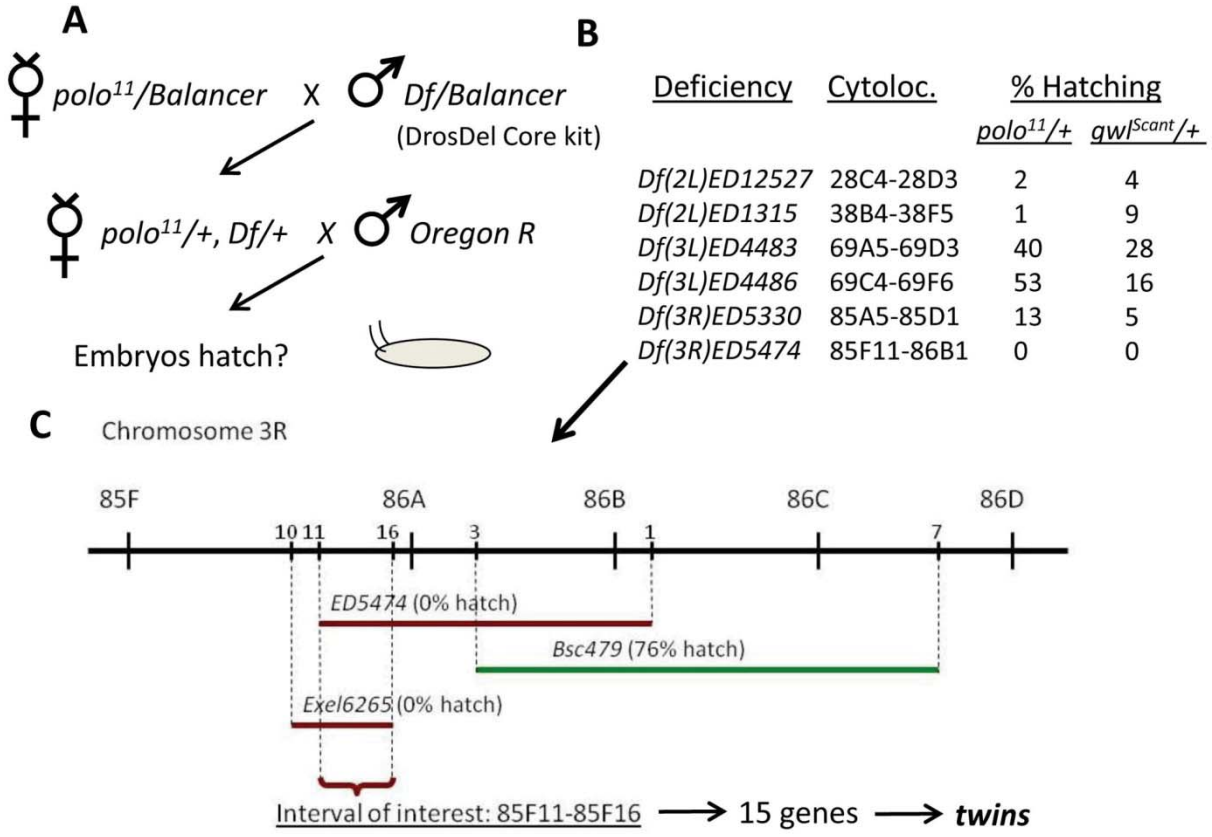
That Polo is required to keep the cohesion between centrosomes and nuclei is consistent with its known functions at mitotic entry, in promoting centrosome maturation and Cdk1 activation [2,20]. Polo also assists Cyclin B-Cdk1 in promoting nuclear envelope breakdown [34,35]. In *Drosophila* syncytial embryos, the nuclear envelope does not completely break down in mitosis, but becomes fenestrated near centrosomes, allowing MTs to penetrate nuclei and reach chromosomes [12-14]. At that stage, centrosome attachment to the nuclear envelope is weakened and is replaced by MT attachments to chromosomes. Thus, the detached centrosomes observed in prophase when Polo activity is decreased could be explained by a failure to coordinate centrosome function with nuclear envelope fenestration at mitotic entry. Consistent with this idea, in *polo*-compromised embryos, MTs emanating from the detached centrosome can be seen as if pressing on the nuclear envelope, which caves in deeply but does not allow MT penetration at the prophase/prometaphase transition, while MTs from the attached centrosomes have already invaded the nucleus (Figure 2.1C).

2.4.3 A genetic screen identifies PP2A-Tws as a strong functional interactor of Polo and Gwl in the syncytial embryo.

The observed centrosome-nucleus cohesion defects in embryos from *polo*-heterozygous females suggested that it could provide a good sensitized background to screen for genes that function with *polo* in the embryo. We conducted such a screen using the DrosDel deficiency core kit, consisting of a sub-collection of large genomic deletions which altogether uncover approximately 60% of the fly genome [36]. Females heterozygous for the $polo^{11}$ null mutation were systematically crossed to males heterozygous for a single deficiency (Figure 2.3A). In F1 progeny, females heterozygous for both $polo^{11}$ and the deficiency were tested for their ability to produce viable embryos, hatching into larvae. The same scheme was applied to test each deficiency in combination with one copy of gwl^{Scant}, a gain-of-function allele of *gwl* identified previously as a dominant synthetic lethal enhancer of *polo* hypomorphic embryos [23,37]. The large majority of deficiencies allowed full fertility when combined with $polo^{11}$ or gwl^{Scant}. Only 6 deficiencies resulted in less than 50% embryo hatching when combined with either $polo^{11}$ *or*

gwl^{Scant} (Figure 2.3B). Interestingly, the deficiencies that interacted with *polo*^{11} tended to also interact with *gwl*^{Scant} (and vice-versa), further suggesting a very close functional link between Polo and Gwl. We reasoned that the deletions identified were likely to uncover genes that function with *polo* and *gwl*.

Among the deficiencies identified in the screen, the strongest genetic interactor with *polo* and *gwl* was *Df(3R)ED5474*, which resulted in complete failure of the embryos to hatch (Figure 3B). Using overlapping deficiencies, we were able to limit the interval of interest to a region containing only 15 genes on chromosome 3R (Figure 2.3C). One of those genes was *twins* (*tws*), which encodes a B-type adaptor subunit of PP2A previously implicated in cell cycle regulation [38-40] and is the sole ortholog of the human B55 group of adaptor subunits [41]. Combining one copy of *tws*^{P} (a strong hypomorphic allele due to a P-element insertion) with one copy of *polo*^{11} in the maternal genotype resulted in a complete failure of embryos to hatch (Figure 2.3D). Similar results were obtained with another allele, *tws*$^{aar-1}$. We did not test if mutations in the other 14 genes uncovered in the interval of interest genetically interact with *polo.* In any case, the genetic interaction between *polo* and *tws* pointed at a functional interdependence between Polo and PP2A in the embryo.



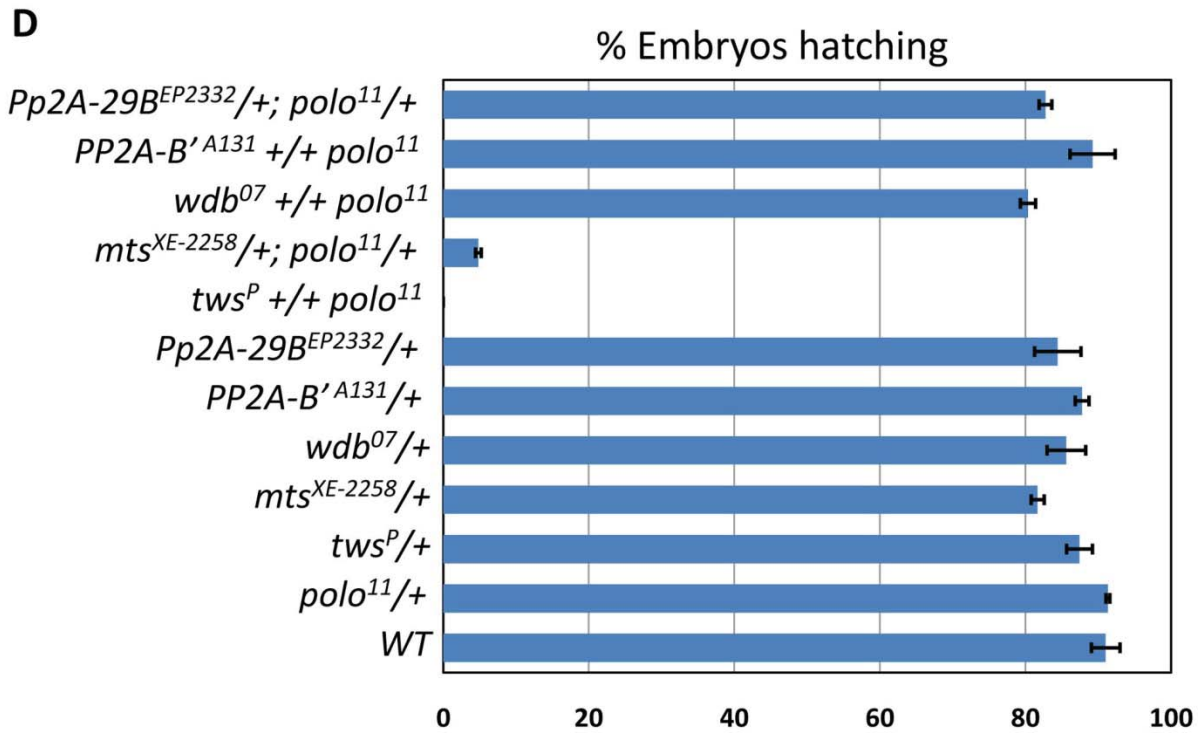


Figure 2.3. A screen for genes functioning with *polo* identifies the PP2A subunit genes *twins* and *microtubule star*. A. Genetic scheme of the screen. Deficiencies of the DrosDel Core kit were combined with one allele of $polo^{11}$. Doubly heterozygous females were taken and their embryos were tested for the ability to hatch. A similar screen was performed with gwl^{Scant} instead of $polo^{11}$. **B.** Deficiencies obtained that yielded less than 50% embryo hatching in the screen when combined with $polo^{11}$ or gwl^{Scant}. For each deficiency, the cytolocation and the percentages of embryos hatching obtained in the screen are indicated. **C.** Identification of *twins* (*tws*) as a genetic interactor. Testing deficiencies overlapping with *Df(3R)ED5474* for their effect on embryo hatching when introduced in the maternal $polo^{11}$/+ background allowed to restrict the interval of interest to 15 genes which included *twins*. **D.** *polo* genetically interacts specifically with *tws* and *mts*. Percentage of embryos hatching for the indicated maternal genotypes. N = 4; error bars: S.E.M.).

We then tested systematically all PP2A subunit genes in *Drosophila* for which we could obtain mutants, for potential genetic interactions with *polo* in the same assay. Of the three PP2A adaptor subunit genes tested (*tws, widerborst, PP2A-B'*) only *tws* showed a genetic interaction with $polo^{11}$ (*CG4733* (*PP2A-B''*) was not tested). Females heterozygous for both $polo^{11}$ and a mutant allele of *microtubule star* (*mts*), the catalytic subunit gene [42], produced semi-viable embryos (Figure 2.3D). Similar genetic interactions were observed between $polo^{9}$, a strong

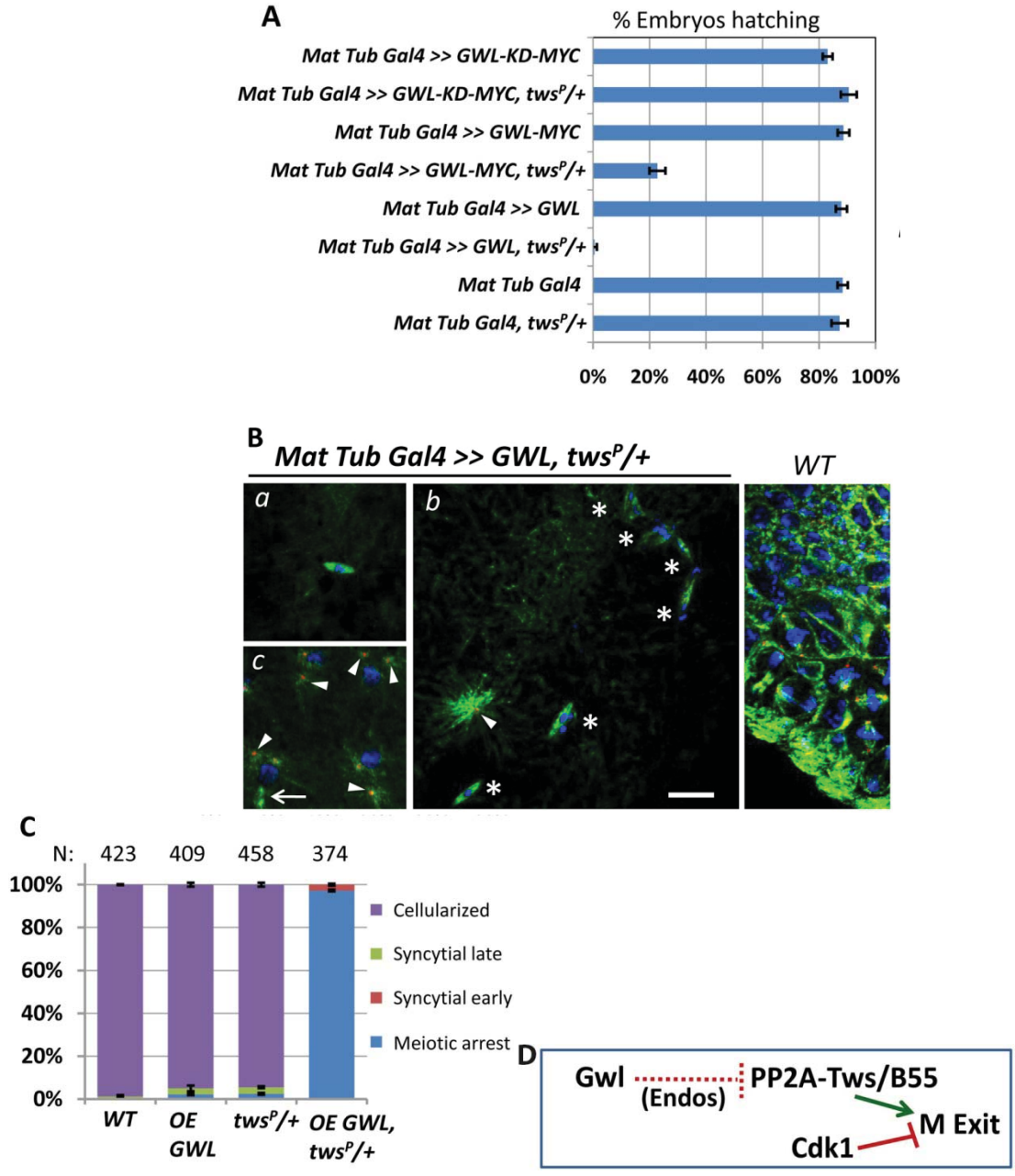
hypomorph, and *tws* or *mts* (data not shown). Interestingly, *mts* is uncovered by *Df(2L)ED12527*, identified as another strong hit in our screen (Figure 2.3B). One copy of a mutant allele of *Pp2A-29B*, which encodes the structural subunit of PP2A had no effect in combination with $polo^{11}$. This could be explained if this subunit were to be present in excess relative to the other subunits of the holoenzyme. No genetic interactions were detected with deficiencies uncovering *widerborst*, *PP2A-B'* or *Pp2A-29B* (data not shown). Strong genetic interactions were also observed between gwl^{Scant} and *tws* or *mts* in the same assay. The percentage of embryos hatching from *mts*/+; gwl^{Scant} /+ and gwl^{Scant} +/+ tws^{P} was zero in both cases (0; N = 4). These results point at PP2A-Tws as a critical functional interactor of Polo and Gwl during syncytial embryo development. The fact that the two strongest hits out of the 60% of the genome that was screened are subunits of PP2A-Tws highlights the specificity of the pathway uncovered here. We note that a strong genetic interaction was also detected between *Df(2L)ED1315* and $polo^{11}$ or gwl^{Scant}; and therefore this deletion could uncover another important gene functioning with Polo, Gwl and PP2A. However, *Df(2L)ED1315* disrupts 94 genes, and the deficiency mapping of the gene of interest is proving challenging.

2.4.4 Greatwall antagonizes PP2A-Tws in M-phase.

The synthetic maternal-effect embryonic lethality between gwl^{Scant} and *tws* or *mts* heterozygous mutations suggested an antagonistic relationship between Gwl and PP2A-Tws. The gwl^{Scant} mutant allele of *gwl* leads to a K97M substitution that makes the kinase hyperactive in vitro [23]. To test directly if embryos with reduced PP2A-Tws function are sensitive to a gain in Gwl kinase activity, we overexpressed Gwl in the egg and early embryos. This was achieved by the late female germline expression of Gal4 under control of the maternal α-tubulin promoter, leading to the Gal4-driven expression of *UASp-GWL* in that tissue. Overexpression of Gwl in eggs/embryos from tws^{P} heterozygous mothers was almost completely lethal, while overexpression of a kinase-dead form of Gwl had no effect on embryonic viability (Figure 2.4A). Therefore, excessive Gwl kinase activity relative to PP2A-Tws activity disrupts either oogenesis and/or embryonic development.

Fertilised eggs or embryos overexpressing Gwl and with a reduced Tws dosage were examined by immunofluorescence. Strikingly, most eggs/embryos were blocked in metaphase of meiosis I, even after 4 to 6 hours post-laying (Figure 2.4B*a*, 2.4C). Embryos overexpressing Gwl

alone or with a reduced dose of Tws alone were almost all cellularized by that time, like *WT* embryos (Figure 2.4B right, 2.4C). These results suggest a role for Gwl in promoting the meiotic arrest by antagonizing PP2A-Tws. Conversely, we previously showed that a loss of Gwl function in oocytes leads to a failure to arrest in meiosis, associated with unstable sister chromatid cohesion [23]. Our results are consistent with recent biochemical studies showing that Gwl promotes M-phase in *Xenopus* egg extracts by antagonizing PP2A-B55δ (ortholog of Twins), which has itself been shown to promote M-phase exit by dephosphorylating Cdk1 substrates (Figure 2.4D, discussed below) [9,43,44].



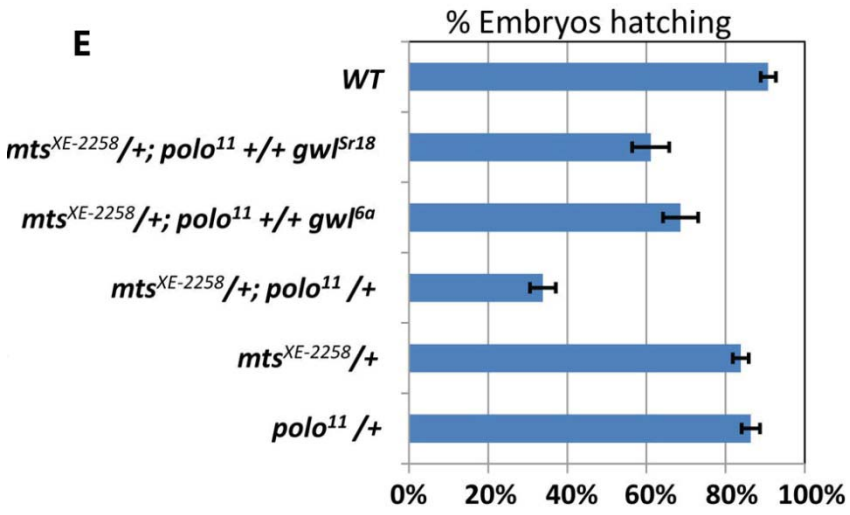
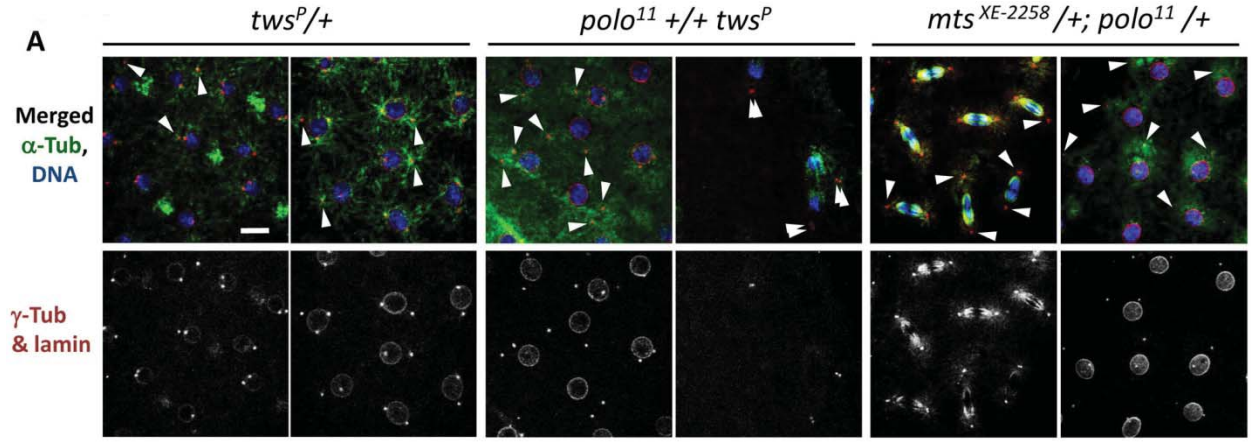


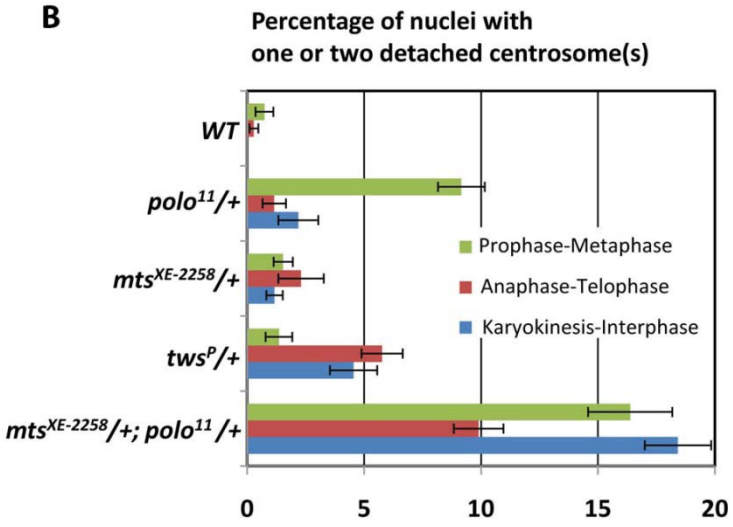
Figure 2.4. Greatwall antagonizes PP2A-Tws in meiosis and mitosis. A. Overexpression of Gwl in the embryo with reduced dosage of Tws leads to a failure to hatch. This effect is dependent on the kinase activity of Gwl (KD: Kinase-dead). Results shown for the transgenic genotypes combine values obtained for 2 independent transgenic lines. N = 5; Error bars: S.E.M. **B.** Examples of embryos between 4 to 6 hrs post-laying. *WT* embryos are cellularized by that time (right). *a*: Most embryos overexpressing Gwl and with reduced Tws fail to exit metaphase of meiosis I, where a single acentrosomal meiotic spindle is observed. *b*: Some embryos attempt mitotic cycles and are mostly blocked with multiple aberrant structures, containing condensed chromatin and MTs (asterisks). *c*: mitotic divisions in karyokinesis (rarely seen). Note the presence of a central spindle (arrow). Centrosomes are detached from nuclei (*b, c*; arrowheads). Scale bar: 10 µm. **C.** Quantitation of the different stages observed for the indicated genotypes in embryos 4 to 6 hours post-laying. *OE GWL*: Overexpression of *UASp-GWL*. The number of eggs/embryos examined is indicated above each column. Error bars: S.E.M. **D.** Model: Greatwall antagonizes PP2A-Tws to prevent M-phase exit (see text for discussion). **E**. Halving the amount of Gwl in embryos from mothers heterozygous for both *polo*^{11} and *mts* $^{XE-2258}$ mutations partially rescues their ability to hatch. N = 4; Error bars: S.E.M.

Although most eggs overexpressing Gwl and with a reduced amount of Tws arrested in meiosis, a few eggs managed to complete meiosis and attempted to initiate embryonic development. However, theses embryos usually aborted in the first mitoses, displaying several small aberrant structures with condensed chromatin at the center of small spindles (Figure 2.4B*b*,

2.4C). The few mitotic nuclei observed showed a very high incidence of detached centrosomes (Figure 2.4B*b*,*c*, arrowheads). These observations suggest that the Gwl-PP2A-Tws pathway can also regulate mitotic divisions.

We reasoned that if normal levels of Gwl regulate mitosis by antagonizing PP2A-Tws, then decreasing the activity of Gwl could rescue the viability of embryos from *mts* $^{XE-2258}$/+; *polo*^{11}/+ mothers, which are semi-viable and show severe mitotic defects (Figure 2.5A, 2.5B). Indeed, those embryos were partially rescued by the introduction of one copy of the null allele *gwl*^{6a}, or one copy of *gwl*^{Sr18}, which abolishes the only maternally contributed splice variant of *gwl* (Figure 2.4E) [23]. This result strongly suggests that Gwl normally negatively regulates PP2A-Tws during the mitotic divisions in addition to meiosis *in vivo*.





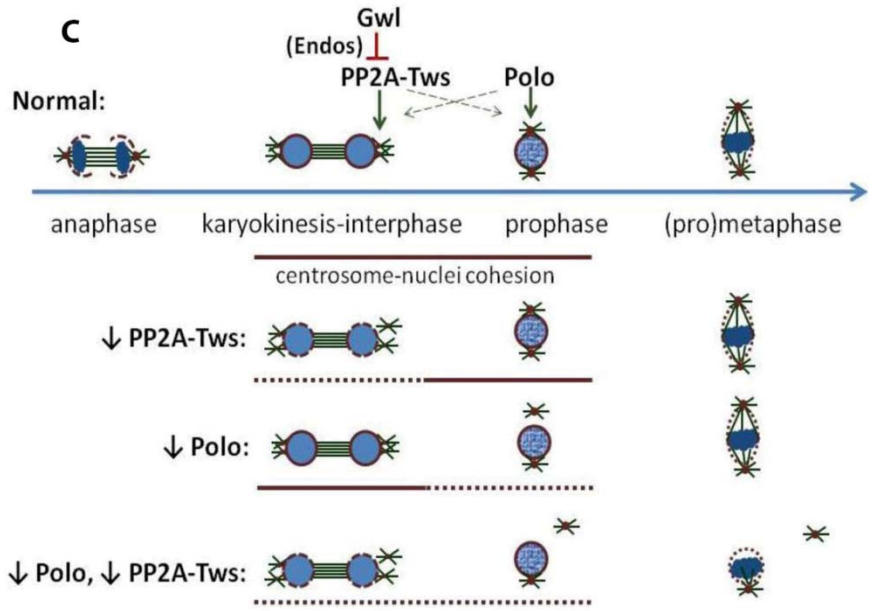


Figure 2.5. PP2A-Tws collaborates with Polo to promote cell cycle progression and centrosome cohesion to nuclei. A. Images of embryos from mothers of the indicated genotypes. Left: Reduced levels of Tws lead to centrosome detachments in late M-phase that can persist in interphase. Middle and right: reducing both Polo and Mts or Tws strongly enhances centrosome detachments. Scale bar: 10 μm. **B.** Quantitation of centrosome detachments at different cell cycle stages for the indicated genotypes. Between 5 and 18 embryos were scored for each category. Error bars: S.E.M. **C.** Model: Polo and PP2A-Tws collaborate to ensure centrosome attachment during the syncytial cell cycles. See text for details.

2.4.5 PP2A-Tws collaborates with Polo to ensure centrosome cohesion to nuclei and nuclear divisions in the syncytial embryo.

Phenotypic examination of embryos from *polo*-heterozygous mothers revealed an elevated frequency of transient centrosome dislocations from nuclei in prophase (Figure 2.1, Figure 2.5B). Because of the strong genetic interaction between *polo* and *tws* or *mts*, we examined embryos from mothers heterozygous for *tws* and *mts* mutations to reveal any potential defects. Interestingly, we detected a significant incidence of centrosome detachments in both cases. However, unlike those observed in *polo*-compromised embryos which occurred mostly in prophase, centrosome dislocation tended to occur in late M-phase (between anaphase and karyokinesis) in embryos with reduced PP2A-Tws (Figure 2.5A, 2.5B).

As expected, embryos combining reductions in Polo and PP2A-Tws levels displayed a strongly elevated incidence of centrosome detachments. Embryos from mothers heterozygous for *polo*^{11} and *mts*$^{XE-2258}$, of which a minority were able to hatch into larvae (Figure 2.3D), showed a high frequency of detached centrosomes at any stage of the mitotic cycles (Figure 2.5A, 2.5B). Embryos from mothers heterozygous for *polo*^{11} and *tws*^{P}, which all failed to hatch (Figure 2.3D), aborted very early during syncytial divisions, with most or all centrosomes detached already in the first few cycles (Figure 2.5A). Therefore, Polo and PP2A-Tws collaborate to ensure proper centrosome cohesion to nuclei and cell cycle progression during early embryogenesis. Since the centrosome detachments that occur upon reduction in Polo function are seen mostly in prophase while those that occur when PP2A-Tws is reduced occur mostly in late M-phase, many centrosomes in double mutants may never be able to recover their attachment and drift away, leading to a failure in nuclear division (Figure 2.5C), as seen in embryos from *polo*^{11}*-Scant* females [23].

2.5 DISCUSSION

In this study, we have explored the functional and molecular relationship between the Polo and Gwl kinases. Our previous study pointed at the importance of proper coordination between these enzymes in the embryonic cell cycles in *Drosophila* [23]. Our genetic screen has lead to the identification of PP2A-Tws as a potent collaborator with Polo in promoting the cohesion between centrosomes and nuclei. Moreover, our results are consistent with a model where Greatwall antagonizes PP2A-Tws to promote M-phase, and where PP2A-Tws promotes exit from mitosis and meiosis. This model is strongly supported by recent biochemical studies using the *Xenopus* extract system.

2.5.1 Regulation of mitosis and centrosome attachment in the rapid embryonic cell cycles.

Our results shed new light on cell cycle regulation and syncytial embryogenesis. We clearly show that high Polo activity is needed to promote the normal cohesion between centrosomes and nuclei, and this is mostly observable around the time of mitotic entry. Interestingly, transiently detached centrosomes can be recaptured by the assembling spindle and nuclear division can then be completed. This centrosome recapture is probably essential for successful development of the syncytial embryo. Our systematic genetic screen unveiled a very

strong and specific functional link between Polo and a specific form of PP2A associated with its B-type subunit Tws. We also show that PP2A-Tws activity is required for centrosome cohesion with nuclei, although in late M-phase, around the time of mitotic exit. This is consistent with a recent study where centrosome defects were observed in late M-phase when the small T antigen of SV40, which binds PP2A, was expressed in *Drosophila* embryos [45]. PP2A-B55δ has been recently implicated in promoting mitotic exit in vertebrates, by inactivating Cdc25C and by directly dephosphorylating Cdk1 mitotic substrates [43,46]. The closely related isoform PP2A-B55α has been shown to promote the timely reassembly of the nuclear envelope at mitotic exit [8]. Thus, the failure to reattach centrosomes to nuclei during mitotic exit in PP2A-Tws compromised embryos could be due to problems or a delay in nuclear envelope resealing.

Our results indicate that the proper regulation of the events of mitotic entry and exit by Polo and PP2A-Tws is crucial. This may be particularly true in the syncytial embryo due to the rapidity of the cycles, where one mitosis is almost immediately followed by another, and because of the obligatory cohesion between centrosomes and nuclei for their migration towards the cortex of the syncytium. Combining partial decreases in the activities of Polo and Tws strongly enhances the frequency of centrosome detachments observed (Figure 2.5). This suggests that when centrosomes fail to attach properly for too long between mitotic exit and the next mitotic entry, they become permanently detached from nuclei, leading to failures in mitotic divisions (Figure 2.5C).

The differences in timing between the detachments observed in *polo* and *tws* hypomorphic situations lead us to propose that the two enzymes act in parallel pathways, of which the disruption can lead to a failure in centrosome-nucleus cohesion. This is also supported by the prominent roles of Polo in regulating centrosome maturation and mitotic entry [2], and the specific requirements of PP2A-Tws/B55 at mitotic exit. However, we cannot exclude that Polo, Gwl and PP2A-Tws could function on a common substrate, or even in the same linear pathway, where the different players of the pathway could become more or less influential at different times of the cell cycle. In has been proposed that PP2A promotes full expression of Polo in larval neuroblasts and in S2 cells [47]. It has also been shown that depletion of Tws by RNAi leads to centrosome maturation defects in S2 cells [48], which could be explained by a reduction in Polo levels. However, we have repeatedly failed to detect a significant difference in Polo levels in embryos from gwl^{Scant}/+ or *tws*/+ females, compared to wild-type controls by Western blotting

(data not shown). Deeper genetic and molecular dissection of those pathways should lead to a clearer understanding of the regulation of centrosome and nuclear dynamics during mitotic entry and exit.

2.5.2 A conserved pathway controls M-phase entry and exit.

Our results add strong support to an emerging model for a pathway that controls entry into and exit from mitosis and meiosis in animal cells. It is increasingly clear that a form of PP2A associated with a B-type regulatory subunit plays a crucial and conserved role in competing with Cdk1. In *Xenopus* egg extract, PP2A-B55δ activity is high in interphase and low in M phase [9]. PP2A-B55δ must be down-regulated to allow mitotic entry, and conversely, it appears to promote mitotic exit both by inactivating Cdc25C and by dephosphorylating Cdk1 substrates [9,46]. In human cells, depletion in B55α delays the events of mitotic exit, including nuclear envelope reassembly [8]. Already some years ago, mutations in *Drosophila tws* were found to lead to a mitotic arrest in larval neuroblasts [38], and extracts from *tws* mutants were shown to have a reduced ability to dephosphorylate Cdk substrates [49]. Mutations in *mts* resulted in an accumulation of nuclei in mitosis in the embryo [42]. The budding yeast now appears to be a particular case, as its strong reliance on the Cdc14 phosphatase to antagonize Cdk1 may reflect the need for insertion of the anaphase spindle through the bud neck prior to mitotic exit [50], a constraint that does not exist in animal cells. Nevertheless, additional phosphatases to PP2A, including PP1 are likely to play conserved roles in promoting mitotic and meiotic exit, and this remains to be dissected.

Our identification of PP2A genes as functional interactors of *polo* and *gwl* is the result of an unbiased genetic screen. We found that an elevation in Gwl function combined with a reduction in PP2A-Tws activity leads to a block in M phase, either in metaphase of meiosis I or in the early mitotic cycles. However, our positioning of Gwl as an antagonist of PP2A-Tws was facilitated by reports that appeared subsequent to our screen, proposing that the main role of Gwl in promoting M-phase was to lead to the inactivation of PP2A-B55δ in *Xenopus* egg extracts [43,44]. Results consistent with this idea were also obtained in mammalian cells [26].

More recently, two seminal biochemical studies using *Xenopus* egg extracts showed that the antagonism of PP2A-B55δ by Gwl is mediated by α-endosulfine/Ensa and Arpp19, two small, related proteins which, when phosphorylated by Gwl at a conserved serine residue, become able

to bind and inhibit PP2A-B55δ [51,52]. By this mechanism, Gwl activation at mitotic entry leads to the inhibition of PP2A-B55δ, which results in an accumulation of the phosphorylated forms of Cdk1 substrates. Depletion of human Arpp19 also perturbs mitotic progression in Hela cells [51], suggesting a conserved role among vertebrates.

In an independent study, the group of David Glover has recently identified mutations in *Drosophila endosulfine* (*endos*) as potent suppressors of the embryonic lethality that occurs when gwl^{Scant} (the gain-of-function allele) is combined with a reduction in *polo* function, in a maternal effect (see accompanying paper by Rangone et al [53]). *endos* is the single fly ortholog of *Xenopus* α-endosulfine and Arpp19. That the identification of *endos* by Rangone et al came from another unbiased genetic screen testifies of the specificity and conservation of the Gwl-Endos-PP2A pathway in animal cells. The authors went as far as showing that the critical phosphorylation site of Gwl in Endos is conserved between frogs and flies, and is critical for the function of Endos in antagonizing PP2A-Tws in cultured cells. These findings are consistent with a previous report showing that mutations in *endos* lead to a failure of oocytes to progress into meiosis until metaphase I [54]. Moreover, loss of Gwl specifically in the female germline also leads to meiotic failure, although in that case oocytes do reach metaphase I but exit the arrest aberrantly [23]. Although the meaning of those phenotypic differences is not yet understood, Gwl and Endos are both required for meiotic progression in *Drosophila*. Conversely, we show here that excessive Gwl activity relative to PP2A-Tws prevents exit from the metaphase I arrest, suggesting that the inhibition of PP2A-Tws by Gwl and Endos must be relieved to allow completion of meiosis. Moreover, Rangone et al show that the Endos pathway also regulates the mitotic cell cycle in the early embryo, in larval neuroblasts and in cultured cells [53].

Together, the systematic and unbiased identifications of mutations in PP2A-Tws subunit genes as enhancers (this paper), and of mutations in *endos* as suppressors [53] of gwl^{Scant} provide strong evidence for a pathway connecting these genes to control M phase in flies. Our studies provide a convincing genetic and functional validation of the recent biochemical results from *Xenopus* extracts, and show that the Gwl-Endos-PP2A-Tws/B55 pathway is conserved and plays a key role in regulating both meiosis and mitosis in a living animal.

2.6 MATERIALS & METHODS

2.6.1 *Fly husbandry, genetic screen and fertility tests*

Flies were kept at 25°C on standard food. The wild-type strain used was Oregon R. For the genetic screen, the DrosDel Core deletion kit [36] (obtained from John Roote, Cambridge, UK) was used and uncovered approximately 60% of the genome with 200 deficiencies. For fertility tests, 3 to 5 well-fed, 1 to 4 days-old virgin females were given 3 to 5 Oregon R males per tube and allowed to mate for one day. Flies were then transferred on grape juice-containing agar and yeast. After one day, flies were removed (usually transferred to a new tube). Between 24 and 30 hours later, the percentage of hatched embryos was counted. At least 100 embryos were counted. $polo^{11}$, gwl^{Scant} and gwl^{Sr18} alleles were previously published [23,37]. tws^{P} (tws^{J11C8}) *and* tws^{aar-1} were from David Glover. $mts^{XE-2258}$, $Pp2A\text{-}29B^{EP2332}$, $PP2A\text{-}B^{tA131}$, and wdb^{07} were from Bloomington stock center. *GFP-D-TACC* and *H2A-RFP* stocks were kindly provided by Pat O'Farrell and Jordan Raff. *UASp-GWL-MYC* and *UASp-GWL-KD-MYC* (K87R) expressed the long splice variant of Gwl, and were made in pPWM (*Drosophila* Genomics Resource Center). Transgenic flies were generated using the P-element-based method by BestGene Inc (Chino Hills, CA, USA). *UASp-GWL* flies were reported previously [23]. Overexpression in the early embryo was driven by maternal α-Tubulin Gal4, which was obtained from Adelaide Carpenter.

2.6.2 *Immunofluorescence and confocal microscopy*

For immunofluorescence, embryos were collected on grape juice agar, and dechorionated and fixed as described [23]. Antibodies used for stainings are: α-Tubulin: YL1/2 (1:50; Serotec), γ-Tubulin: GTU-88 (1:50; Sigma) and lamin B/Lamin Dm_0 (1:100; Developmental Studies Hybridoma Bank). Secondary antibodies were coupled to Alexa-488 (1:200; Invitrogen) or Texas red (1:200; Invitrogen). DNA was marked with DAPI. Images were acquired on a Laser scanning confocal microscope LSM 510 Meta (Zeiss), using a 100X oil objective.

2.6.3 *Time-lapse microscopy*

Embryos from *WT* or $polo^{11}$/+ females and expressing *GFP-D-TACC* and *H2A-RFP* were dechorionated and imaged on a Swept-Field confocal microscope (Nikon Eclipse Ti), using a 100X oil objective.

2.6.4*Chemical inhibition of Polo*

Chemical treatments of embryos used a protocol modified from Sibon et al [29]. Embryos were dechorionated and incubated for 30 min in a 1:1 mixture of Express Five *Drosophila* cell culture medium (Invitrogen) and heptane with 1 μM of BI2536 (from a DMSO stock solution) or DMSO alone.

2.7 ACKNOWLEDGMENTS

We thank David M. Glover in whose lab this work was started. We are also grateful to Adelaide Carpenter, Pat O'Farrell and Jordan Raff for fly stocks, and Ana Roque for technical assistance. We are grateful to Christian Charbonneau and Paul Maddox for help with the microscopy. We thank Marc Therrien, Sébastien Carréno and Jean-Claude Labbé of IRIC for comments on the manuscript.

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CHAPITER 3

Publication

Cell Cycle Regulation of Greatwall Kinase Nuclear Localization Facilitates Mitotic Progression

Article published in the Journal of Cell Biology (2013) Jul 22; 202(2); 277-93.

Cell Cycle Regulation of Greatwall Kinase Nuclear Localization Facilitates Mitotic Progression

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Running title: Spatial Regulation of Greatwall

eTOC Summary Statement – Suggested by authors, different from the one suggested by editors:

Greatwall kinase relocation from the nucleus to the cytoplasm is required at mitotic entry and is mediated by a phosphorylation-dependent mechanism targeting its central region.

Keywords: Mitosis, Cell cycle, Greatwall, Kinase, *Drosophila*

Number of characters: 39,757

3.1 ABSTRACT

Cell division requires the coordination of critical protein kinases and phosphatases. Greatwall (Gwl) kinase activity inactivates PP2A-B55 at mitotic entry to promote the phosphorylation of cyclin B-Cdk1 substrates, but how Gwl is regulated is poorly understood. We found that the subcellular localization of Gwl changed dramatically during the cell cycle in *Drosophila*. Gwl translocated from the nucleus to the cytoplasm in prophase. We identified two critical nuclear localization signals in the central, poorly characterized region of Gwl, that are required for its function. The Polo kinase associated with and phosphorylated Gwl in this region, promoting its binding to 14-3-3ε and its localization to the cytoplasm in prophase. Our results suggest that cyclin B-Cdk1 phosphorylation of Gwl is also required for its nuclear exclusion by a distinct mechanism. We show that the nucleo-cytoplasmic regulation of Gwl is essential for its functions *in vivo* and propose that the spatial regulation of Gwl at mitotic entry contributes to the mitotic switch.

3.2 INTRODUCTION

The molecular events driving the cell cycle are regulated by a complex network of kinases and phosphatases with cyclically ordered and specific activities. The cyclin-dependent kinase 1 (Cdk1) in complex with cyclin B triggers mitosis, by promoting nuclear envelope breakdown, chromosome condensation and spindle assembly [1]. Many effector and regulatory proteins of mitosis are targets of cyclin B-Cdk1, and their phosphorylation must be reversed to allow mitotic exit. This dephosphorylation is thought to be largely achieved by Protein Phosphatase 2A (PP2A) in complex with its B-type regulatory subunits known as B55 in vertebrates and Twins (Tws) in *Drosophila* [2-4]. In addition to Cdk1, several other kinases are required to coordinate the events of mitosis and cytokinesis. These include members of the Polo-like kinase and Aurora kinase families [5, 6].

Greatwall (Gwl; MASTL in humans) was first discovered in *Drosophila* as an essential mitotic kinase [7-9]. *gwl* mutants show delays in chromosome condensation and chromosome segregation defects in larval neuroblasts, and similar phenotypes were observed in Gwl-depleted S2 cells [7, 8, 10]. Gwl was then showed to be essential for mitosis by its participation in the positive feedback loop leading to full cyclin B-Cdk1 activation in *Xenopus* extracts [11]. Strong evidence now indicates that Gwl antagonizes PP2A-B55 in its ability to dephosphorylate Cdk1

substrates in frogs, flies and humans [12-16]. This function of Gwl was shown to be mediated by the endosulfine and Arpp19 homologous proteins in vertebrates, and by their sole ortholog, Endos in *Drosophila*. In *Xenopus* extract, these proteins are phosphorylated by Gwl at mitotic entry to become inhibitors of PP2A-B55δ, thereby promoting the phosphorylated state of Cdk1 substrates [17, 18]. In *Drosophila*, mutations in *endos* rescue maternal-effect embryonic defects induced by a gain of Gwl function, and Gwl regulates Endos at a site conserved with *Xenopus* endosulfine and Arpp19 [16]. Therefore, the Gwl-Endos-PP2A-B55/Tws pathway appears strongly conserved [9, 19].

The current model predicts that in order to mediate the regulation in PP2A-B55/Tws activity through M-phase, Gwl and/or Endos must be active at mitotic entry and inactive at mitotic exit. The molecular mechanisms of this regulation are unclear. Gwl has been shown to become activated and hyperphosphorylated at mitotic entry in *Xenopus* extracts [11]. Recently, the kinase activity of Gwl has been proposed to be regulated by a non-canonical mechanism for the AGC family of kinases to which it belongs. This mechanism is thought to require the phosphorylation of Gwl in its C-terminal tail/linker site and binding of another kinase to a hydrophobic motif in the N-terminal lobe of Gwl [20]. Another study in *Xenopus* identified 3 phosphorylation sites in Gwl that can increase its activity [21]. The identity of the kinases activating Gwl *in vivo* is uncertain, but strong evidence implicates cyclin B-Cdk1 and Gwl itself in this process [11, 20, 21]. Plx1 (*Xenopus* Polo) has been shown to phosphorylate Gwl [11, 20, 22] in *Xenopus* extracts, and this has been proposed to help Gwl drive re-entry into mitosis in recovery from DNA damage [22]. However, only very modest activation of Gwl was detected upon its phosphorylation by Polo, and a recent study failed to detect any effect of Polo phosphorylation on Gwl activity *in vitro* [21]. To what extent specific phosphorylation events contribute to regulate Gwl activity *in vivo* and whether other mechanisms come into play to regulate Gwl function is unknown. In this regard, Gwl possesses an intriguing, uniquely long protein segment *in lieu* of a T-loop within the kinase domain [7]. Any segment of this region can be deleted with little effect on kinase activity *in vitro* [21]. The role of Gwl's central region remains completely unknown.

Here, we have explored how Gwl is regulated at the level of its subcellular localization in *Drosophila*. We show that Gwl is strongly nuclear in interphase, but becomes cytoplasmic and excluded from the nucleus just prior to mitosis. We found that Gwl's central region contains two

nuclear localization signals (NLSs) required for the nuclear targeting of Gwl. Moreover, Polo associates with and phosphorylates Gwl in the same region. Our results suggest that Polo phosphorylation of Gwl allows its binding to 14-3-3ε, which promotes the re-localization of Gwl to the cytoplasm, where PP2A-Tws is largely localized. Cdk1 sites in the central region of Gwl are also required for its efficient nuclear exclusion in prophase. Importantly, we show that this spatial regulation of Gwl is essential *in vivo* and is required for normal mitotic progression.

3.3 RESULTS

3.3.1 The localization of Gwl is cell-cycle regulated.

At the time of its identification as a mitotic kinase, Gwl was found to be a nuclear protein in interphase [7]. However, how Gwl changes localization through the cell cycle was not investigated. We examined the localization of Gwl in *Drosophila* early embryos, where nuclei divide rapidly in a syncytium. By immunofluorescence, we found that Gwl is nuclear during interphase, but becomes mostly cytoplasmic in prophase, appearing excluded from nuclei before nuclear envelope fenestration, which becomes apparent later when microtubules cross the nuclear envelope (Fig 3.1A). Gwl gradually becomes more diffuse through the embryo during mitosis, but appears excluded again from daughter nuclei in telophase. We confirmed this dynamic localization pattern by time-lapse imaging of embryos co-expressing Gwl-GFP and H2Av-RFP (Fig S3.1A).

To examine if this localization pattern of Gwl is generally conserved in other cell types, we generated a D-Mel cell line stably expressing GFP-tagged Gwl. As seen in embryos, GFP-Gwl was nuclear in interphase, and became cytoplasmic and excluded from the nucleus in prophase, for approximately 5 minutes just before nuclear envelope breakdown (Fig 3.1B and Movie S1). In addition, GFP-Gwl was enriched at the nuclear envelope in early prometaphase (Fig 3.1B and Movie S1). Similar results were obtained with C-terminally tagged, Gwl-GFP. Although immunofluorescence results have suggested the presence of MASTL at the centrosomes and the cytokinetic midbody in human cells [14, 23], we did not observe these localizations for Gwl in *Drosophila*.

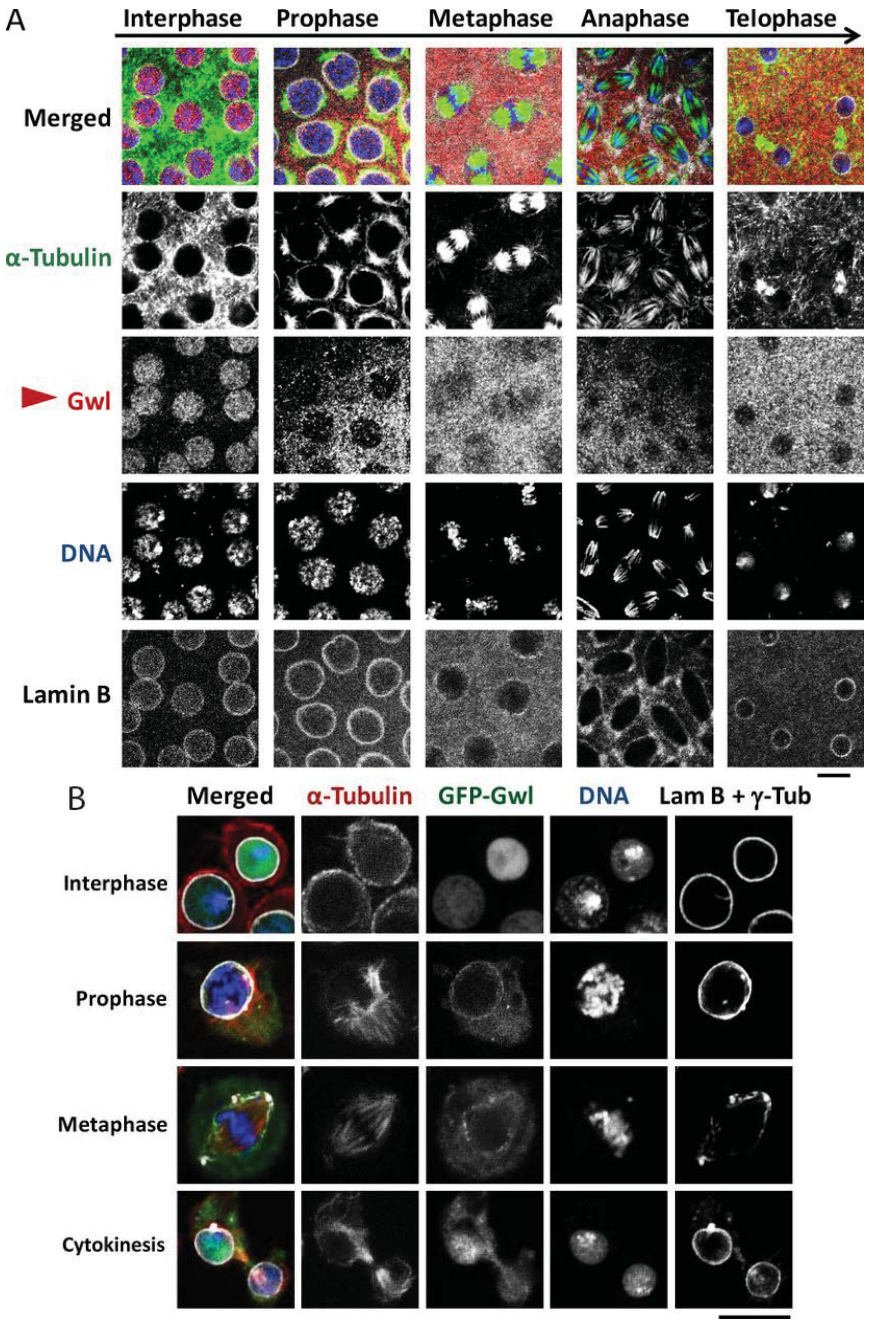
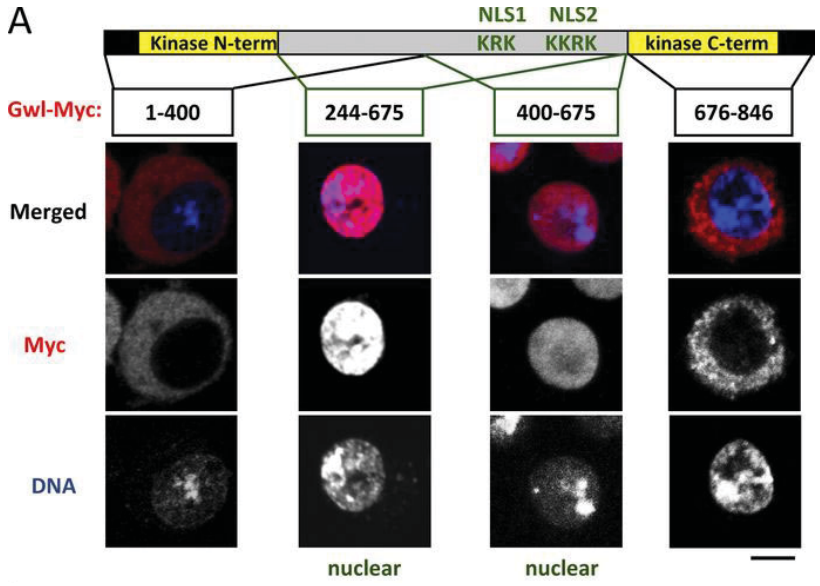


Figure 3.1. The localization of Gwl is cell-cycle regulated. A-B. Immunofluorescence in fixed syncytial embryos (A) and D-Mel cells expressing GFP-Gwl (B) at different stages of the cell cycle. Note that in both panels, Gwl is nuclear in interphase and cytoplasmic in prophase, appearing excluded from nuclei. Scale bars: 10 μm.

3.3.2 Gwl contains two functional NLS motifs in its central region.

We explored the mechanisms responsible for this dynamic localization of Gwl through the cell cycle. Gwl shows a strongly nuclear localization in interphase. To identify the region of Gwl responsible for its nuclear localization, we expressed Myc-tagged truncated forms of Gwl in D-Mel cells in culture and examined their localization. The region containing amino-acid residues 400-675 was necessary and sufficient for nuclear localization (Fig 3.2A). We searched for potential nuclear localization signals (NLSs) in this region of the Gwl protein sequence. NLS motifs are often characterized by the presence of at least 3 consecutive positively charged residues [24]. We found two potential NLS motifs (NLS1 and NLS2) conserved between several related species (Fig 3.2A and S3.2). Whereas mutation of each NLS individually affected Gwl's localization only partially (data not shown), mutation of both of these motifs was sufficient to disrupt the nuclear localization of Gwl in cells (Fig 3.2B) and in embryos (Fig 3.2C). Thus, the nuclear localization of Gwl depends on two NLSs in its central region. Importantly, nuclear localization is the first function assigned to the long central region of Gwl that interrupts its kinase domain.



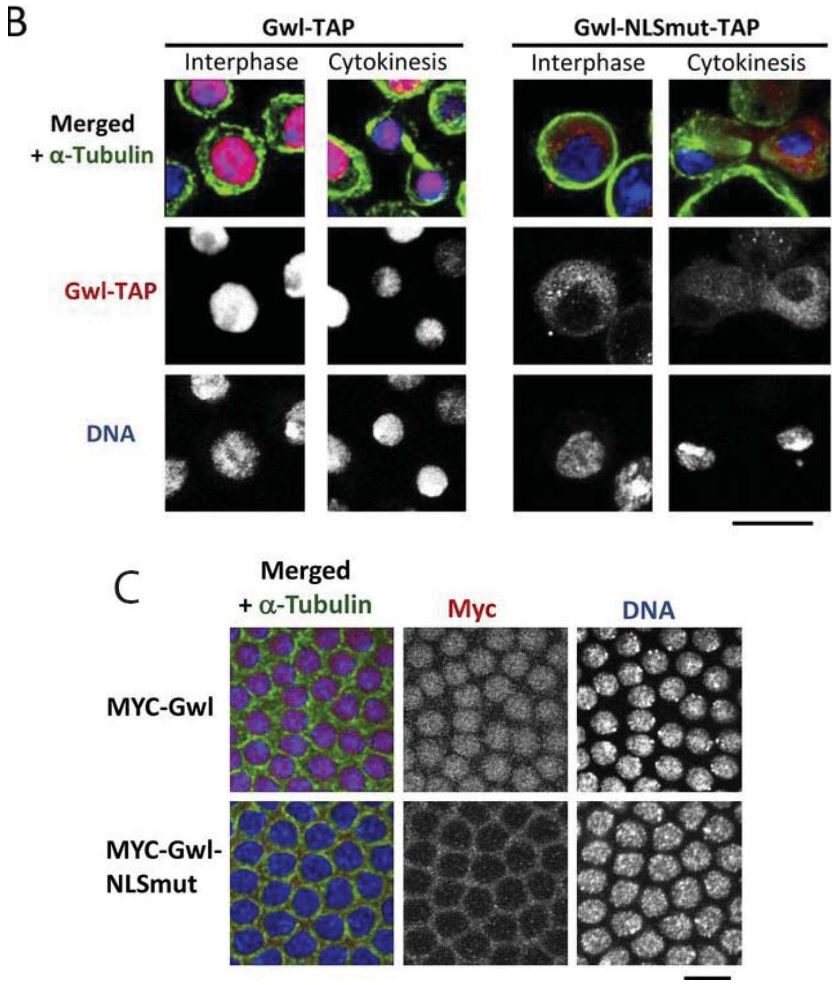
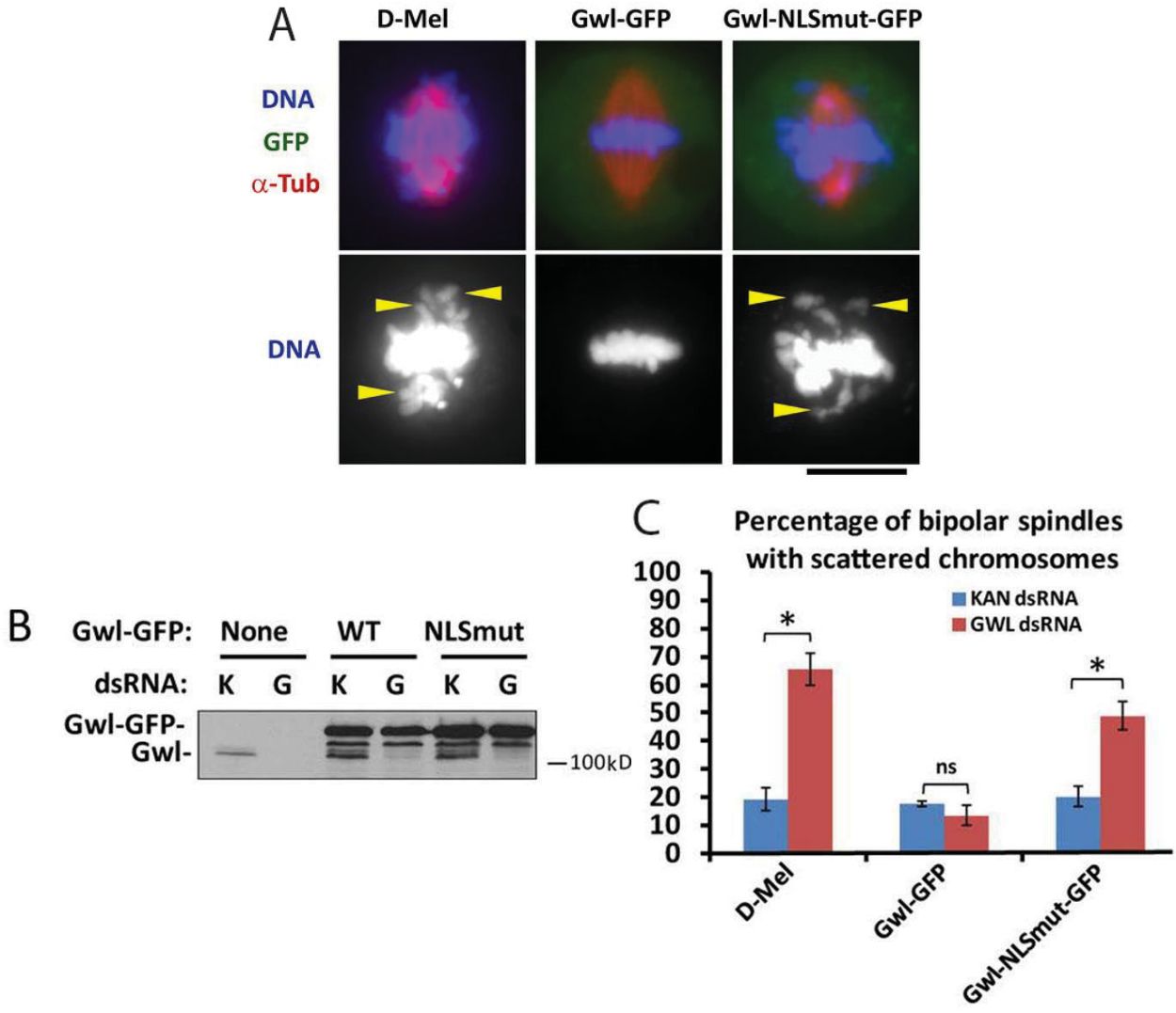


Figure 3.2. Gwl contains two essential NLS motifs in its central region. A. The central region of Gwl is necessary and sufficient for nuclear localization. D-Mel cells expressing the indicated truncations of Gwl tagged with Myc were examined by immunofluorescence on a confocal microscope. Identified NLS motifs are shown. Bar: 5 µm. **B.-C.** Mutation of both NLSs (NLSmut = K518M, K520M (in NLS1) + K564M, R566L (in NLS2; see Fig S3.2)) prevents the nuclear localization of Gwl. Immunofluorescence in D-Mel cells (**B**) and in syncytial embryos (**C**) expressing the indicated proteins. Bars: 10 µm.

3.3.3 Nuclear localization of Greatwall is required for its function.

We next tested the importance of the nuclear localization of Gwl for its biological function. To examine the effect of a mislocalization of Gwl on mitosis, we used cells in culture in which we expressed Gwl-GFP (WT or NLS mutant) under the control of a copper-inducible promoter, while depleting endogenous Gwl. RNAi against the coding sequence of Gwl led to its

efficient silencing, but targeting untranslated regions of endogenous Gwl transcripts did not (data not shown), precluding this potential strategy for the specific silencing of endogenous Gwl. Thus, we created Gwl cDNAs resistant to a dsRNA targeting the coding sequence, by introducing conservative codon replacements. RNAi silencing of Gwl in D-Mel cells resulted in a marked increase in bipolar spindles with scattered chromosomes (Fig 3.3A-C), consistent with previous findings [8, 10, 16]. Expression of Gwl-GFP rescued this phenotype completely (Fig 3.3A-C). However, rescue by expression of Gwl-NLSmut-GFP was only partial, despite a higher expression level than endogenous Gwl (Fig 3.3A-C). Therefore, the nuclear localization of Gwl in interphase is required for its full function in early mitosis.



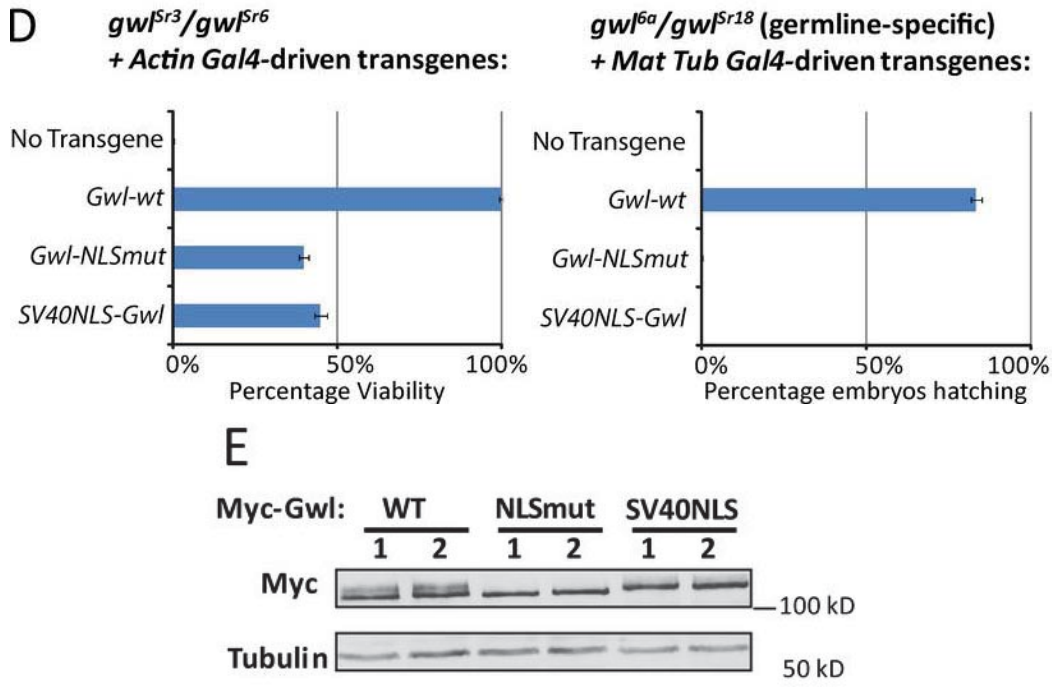


Figure 3.3. Nuclear localization of Greatwall is required for its function. A-C. Nuclear localization of Gwl is required for its mitotic function in cells. Stable cell lines allowing the inducible expression of RNAi-resistant forms of Gwl-GFP (wt or NLSmut) were generated. Simultaneously with induction, D-Mel cells were transfected with dsRNA to deplete endogenous Gwl (G), or with a dsRNA against the bacterial *KAN* gene (K) as a control. After 4 days, cells were analyzed by immunofluorescence (A) and Western blotting for Gwl (B). Scale bar: 10 μm. C. Quantification of cells with bipolar spindle that have scattered chromosomes (arrows in A). Results shown are averages of 3 independent experiments, ±S.E.M. Between 30 and 50 cells were examined for each condition in each experiment. Asterisks: $p < 0.001$ after Student t-test. ns: non-significant. **D.** The regulated localization of Gwl is essential for its functions *in vivo*. *UASp-Myc-Gwl* transgenes were expressed in *gwl* mutant flies as indicated and adult viability (left) and embryo hatch rates (right) were scored. Error bars: S.E.M. For each construction, results from two independent transgenes were combined. E. Western blot of ovaries expressing the indicated forms of *UASp-Myc-Gwl* driven by *Maternal α-Tubulin-Gal4-VP16* from flies used in D. Note that expression levels are similar for all transgenes and was approximately 3-fold higher than endogenous Gwl [8].

We tested the importance of the nuclear localization of Gwl for its biological function *in vivo*. Gwl is an essential gene for *Drosophila* development. Null mutants die mostly as pharate

adults (inside their pupal case), with very rare escapers that hatch with multiple morphological defects [7, 8]. Expression of *UASp-Myc-Gwl* driven ubiquitously by *Actin-Gal4* completely rescued the viability of gwl^{Sr3}/gwl^{Sr6} individuals (strongly hypomorphic mutants [8]). However, expression of *UASp-Myc-Gwl-NLSmut* rescued viability only incompletely in the same mutant background (Fig 3.3D, left). Moreover, flies rescued by Gwl-NLSmut were sterile and dissections revealed a complete lack of ovaries (data not shown). Therefore, the nuclear localization of Gwl is important for its functions during development.

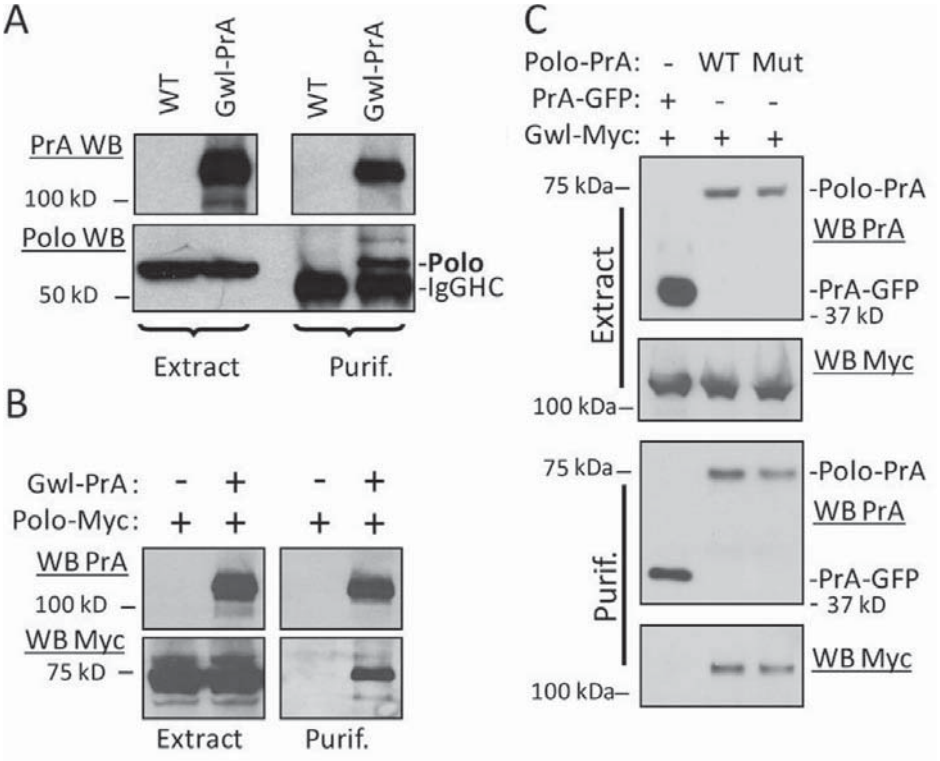
Female germline function and early embryonic development require maternal supplies of Gwl [8]. To test if the nuclear localization of Gwl is also important in this context, we used a mutant allele of *gwl* that completely disrupts Gwl expression in the female germline only (gwl^{Sr18}), which leads to sterility due to defects in oogenesis and meiosis [8]. Expression of *UASp-Myc-Gwl* driven by the *Maternal α-Tubulin-Gal4-VP16* driver largely rescued female fertility assayed by the ability of their embryos to hatch, consistent with previous results [8]. In contrast, *UASp-Myc-Gwl-NLSmut* expression did not rescue fertility (Fig 3.3D right and 3.3E). Therefore, the nuclear localization of Gwl is important for its functions during oogenesis and early embryogenesis. Phenotypic analysis of eggs expressing Gwl-NLSmut was complicated by the fact that the very few eggs laid were abnormally fragile and broke during the fixation procedure.

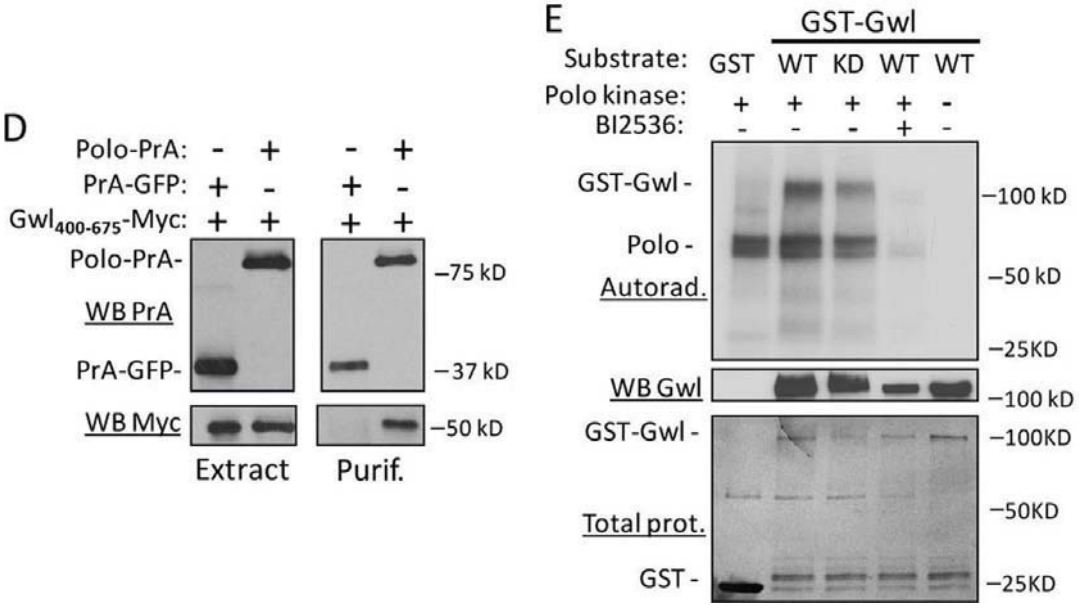
Interestingly, fusing Gwl to a strong NLS from SV40 [25] reduced its ability to rescue the viability and fertility of *gwl* mutants (Fig 3.3D-E). This construction weakened Gwl's ability to relocalize efficiently to the cytoplasm in prophase (Movie S2). These results strongly suggest that Gwl needs to access both the nucleus and the cytoplasm in an orderly and timely manner before NEBD in order to fulfill its essential functions *in vivo*.

3.3.4 Polo kinase interacts with and phosphorylates Gwl.

To identify proteins that could contribute to regulate Gwl directly, we purified Gwl-PrA from transgenic early embryos and analyzed associated proteins by mass spectrometry. We detected peptides from the Polo kinase in the Gwl-PrA purification products, but not in purifications of other bait proteins under the same conditions (data not shown). This result suggested that Polo interacts specifically with Gwl. This was confirmed by anti-Polo Western blot on Gwl-PrA purification products (Fig 3.4A). We also validated this result with the co-purification of Gwl-PrA and Polo-Myc in embryos (Fig 3.4B). The interaction of Polo with

several of its substrates has been shown to require specific residues in its non-catalytic Polo-Box Domain (PBD) that engage in contacts with a pre-phosphorylated motif in the target [26, 27]. Mutation of these residues in Polo did not abolish its interaction with Gwl (Fig 3.4C), suggesting that prior phosphorylation of Gwl is not required for interaction with Polo. Other targets of Polo have been shown to engage in interactions that do not follow the phospho-priming model [27-29].





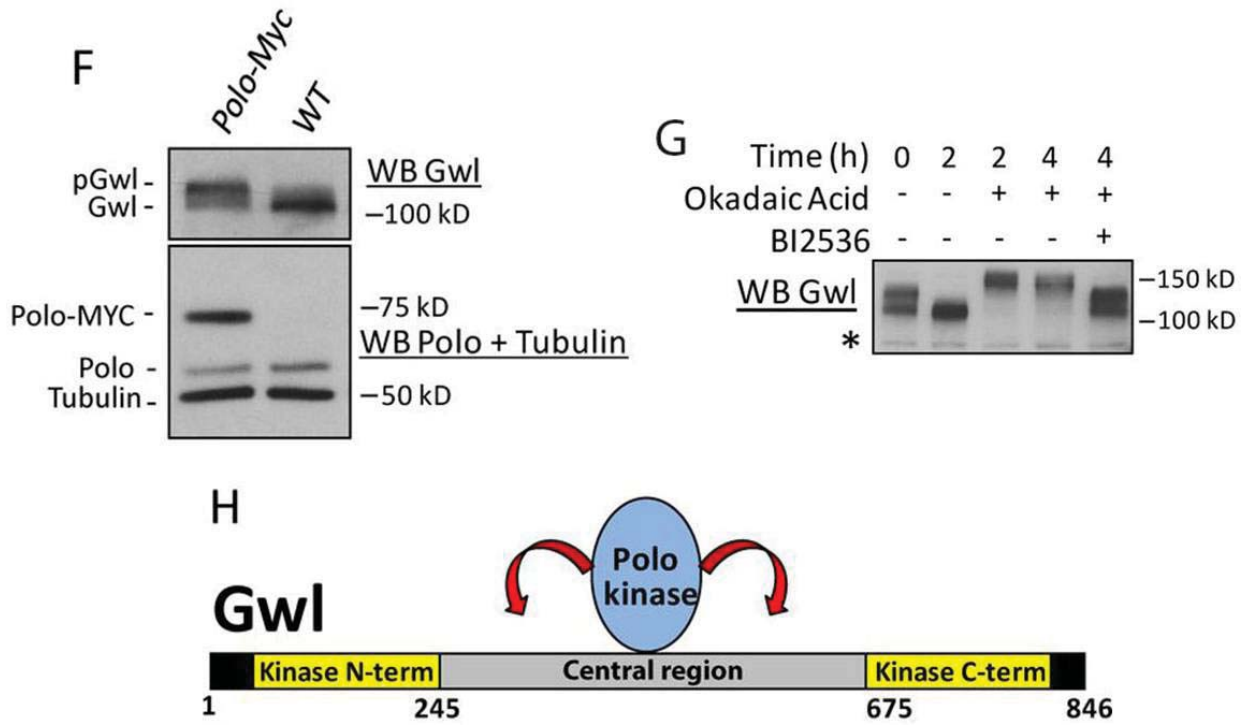


Figure 3.4. Polo interacts with and phosphorylates Gwl. A. Polo is detected by Western blot in a Gwl-PrA purification product from embryos. **B.** Polo-Myc is co-purified with Gwl-PrA from transgenic embryos. **C.** The association of Polo with Gwl does not depend on its canonical phospho-binding activity. D-Mel cells expressing the indicated proteins were used in PrA-affinity purifications followed by Western blots. Mut = Polo W395F, H538A, K540A. **D.** The central region of Gwl associates with Polo. Experiment done as in C. **E.** Polo phosphorylates Gwl *in vitro*. KD: GST-Gwl-K87R, kinase-dead. BI2536 is a Polo inhibitor that was used as a control. **F.** Moderate overexpression of Polo-Myc in embryos induces hyperphosphorylation of Gwl. **G.** Gwl hyperphosphorylation depends on Polo in embryo extracts. After lysis, extracts were incubated as indicated. Okadaic acid induces an upshift in the mobility of Gwl that is abrogated by BI2536 (200 nM). **H.** Model: Polo interacts with Gwl in its central region and phosphorylates multiple sites.

To determine the region of Gwl that mediates its interaction with Polo, we expressed Myc-tagged truncations of Gwl in D-Mel cells, and tested which ones co-purified with co-expressed Polo-PrA. We found that the central region of Gwl, containing the functional NLSs, is sufficient for the association with Polo (Fig 3.4D). Thus, Polo can associate with the central region of Gwl. In addition, we detected a weaker association between Polo and the N-terminal

half of the kinase domain of Gwl (data not shown), consistent with a previous study in *Xenopus* [22]. It is therefore possible that two regions of Gwl can interact with Polo.

We hypothesized that Polo could regulate Gwl. It has been reported that Plx1, the *Xenopus* ortholog of Polo, can phosphorylate *Xenopus* Gwl *in vitro*, but the physiological relevance of this observation was unclear since only a minor effect or no effect on Gwl kinase activity could be detected [11, 20-22]. We found that *Drosophila* Polo can phosphorylate *Drosophila* Gwl *in vitro* (Fig 3.4E). Furthermore, overexpression of Polo in embryos induces an upshift in the electrophoretic mobility of Gwl (Fig 3.4F), suggesting that Polo regulates Gwl phosphorylation *in vivo*. However, the effect of Polo overexpression on Gwl could have been an indirect consequence of alterations in cell cycle dynamics. Thus, we conducted an experiment in non-cycling embryo extracts. We noticed that Gwl gradually collapsed to faster migrating forms in these extracts when incubated on ice (Fig 3.4G). This electrophoretic change of Gwl was partially reversed when extracts were incubated in the presence of okadaic acid, a phosphatase inhibitor, likely reflecting hyperphosphorylation. Addition of the Polo inhibitor BI2536 to the extracts abrogated this upshift, further suggesting that Polo phosphorylates Gwl. The remaining mobility shift observed is likely to be attributable in part to the known phosphorylation of Gwl by cyclin B-Cdk1 [11].

We used a similar assay in cultured cells to map the region of Gwl that is targeted by Polo. The central region alone ($Gwl_{245-675}$) shifted upon okadaic acid treatment, while the N-terminal and C-terminal regions, encoding for the core kinase domain did not shift (Fig 3.5A). Moreover, the mobility shift was reduced when cells were treated with the Polo inhibitor BI2536. Altogether, our results strongly suggest that Polo phosphorylates Gwl in its central region *in vivo*. Mass spectrometry analysis of Gwl after phosphorylation by Polo *in vitro* identified 32 sites, 20 of which are inside the central region of Gwl (Fig S3.3A). Altogether, our results suggest that Polo interacts with Gwl in its central region to phosphorylate it at multiple sites (Fig 3.4H).

3.3.5 Polo activity promotes the cytoplasmic localization of Gwl in prophase.

As shown above, the nuclear localization of Gwl relies on two NLS motifs located in its central region, and this region associates with and is phosphorylated by Polo. We hypothesized that the phosphorylation of Gwl by Polo could regulate its subcellular localization. To test this possibility, we overexpressed Polo in the syncytial embryo and analyzed the localization of Gwl by immunofluorescence. We found that overexpression of Polo or constitutively active $Polo^{T182D}$

partially abrogated the nuclear enrichment of Gwl (Fig 3.5B). Overexpression of Polo^{T182D} in cultured cells also led to an increase in cytoplasmic localization of Gwl (Fig 3.5C). Phosphatase inhibition by short treatments with okadaic acid increased the cytoplasmic localization of Gwl, further suggesting that Gwl phosphorylation promotes its cytoplasmic localization (Fig S3.4). As expected, simultaneous inhibition of Polo with BI2536 partially restored the nuclear localization of Gwl. These results strongly suggest that Polo phosphorylation of Gwl promotes its cytoplasmic localization.

To test if Polo activity is required for the nuclear exclusion of Gwl in prophase, we imaged mitotic entry in GFP-Gwl cells following inhibition of Polo with BI2536. While control cells always excluded Gwl from the nucleus in prophase (in 20/20 cells filmed), cells treated with BI2536 entered mitosis and never showed nuclear exclusion of GFP-Gwl in prophase (in 18/18 cells filmed; Fig 3.5D). Instead, GFP-Gwl gradually spread into the cytoplasm in mitotic cells, when the nuclear envelope breaks down. While control cells completed cell division in less than 2 hours, Polo-inhibited cells failed to divide even after several hours. Therefore, Polo activity is required for the nuclear exclusion of Gwl in prophase.

Our mapping of the Polo phosphorylation sites in Gwl *in vitro* resulted in a large number of sites detected. Interestingly, these sites tended to be clustered in the central region of Gwl, comprising its NLSs (Fig S3.3). We tested the effect of mutating Polo consensus sites in Gwl's central region on the localization of Gwl. Alanine residues were introduced instead of serine or threonine residues at all sites corresponding to the minimal Polo phosphorylation motif (E/N/D)-X-(S/T) [30] in the central region of Gwl, around its NLSs. This Gwl-PoloA mutant was markedly less phosphorylated by Polo compared with Gwl-wt *in vitro* (Fig S3.3B). Remarkably, Gwl-PoloA-GFP failed to be excluded from the nucleus in prophase for 30/30 mitotic entry events examined (Compare Fig 3.6A vs 3.6B, and Movie S3 vs S4), further supporting the idea that Polo phosphorylation of Gwl is required for its nuclear exclusion.

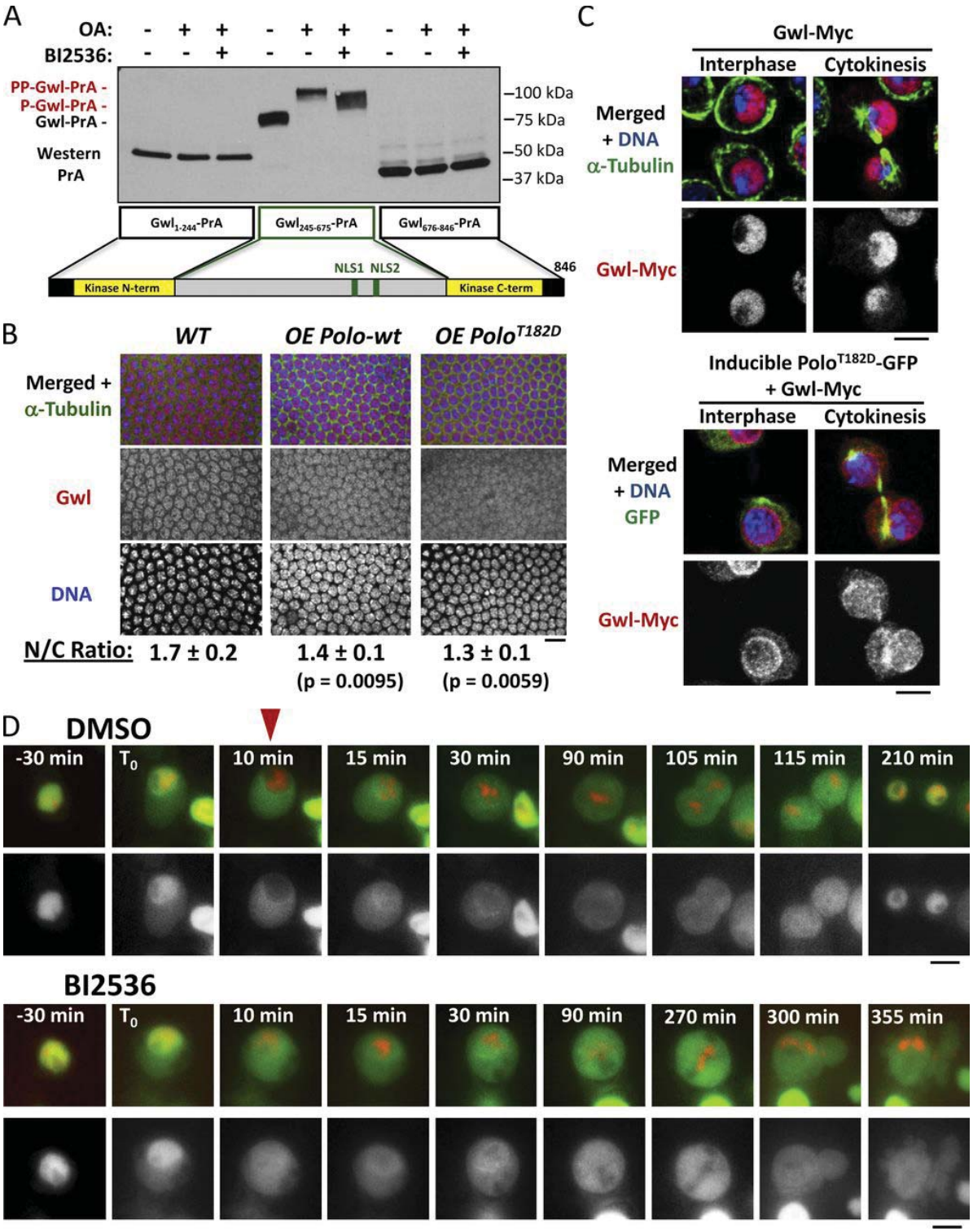


Figure 3.5. Polo activity promotes the cytoplasmic localization of Gwl in prophase. A. Phosphorylation of Gwl in its central region depends on Polo. **B.** Increasing Polo activity induces a more cytoplasmic localization of Gwl in syncytial embryos. *p*-values are from a paired t-test. **C.** Increasing Polo activity induces a more cytoplasmic localization of Gwl in cultured cells. Bars: 5

μm. **D.** Time-lapse microscopy of D-Mel cells expressing GFP-Gwl and H2Av-mRFP. In control, DMSO treated cells, GFP-Gwl is excluded from the nucleus in prophase (red arrowhead). In cells treated with BI2536 (50 nM) to inhibit Polo, GFP-Gwl is never excluded from the nucleus and becomes diffuse throughout the cell in mitosis. T_0 was set as the time when cytoplasmic GFP-Gwl first begins to appear. Bars: 5 μm.

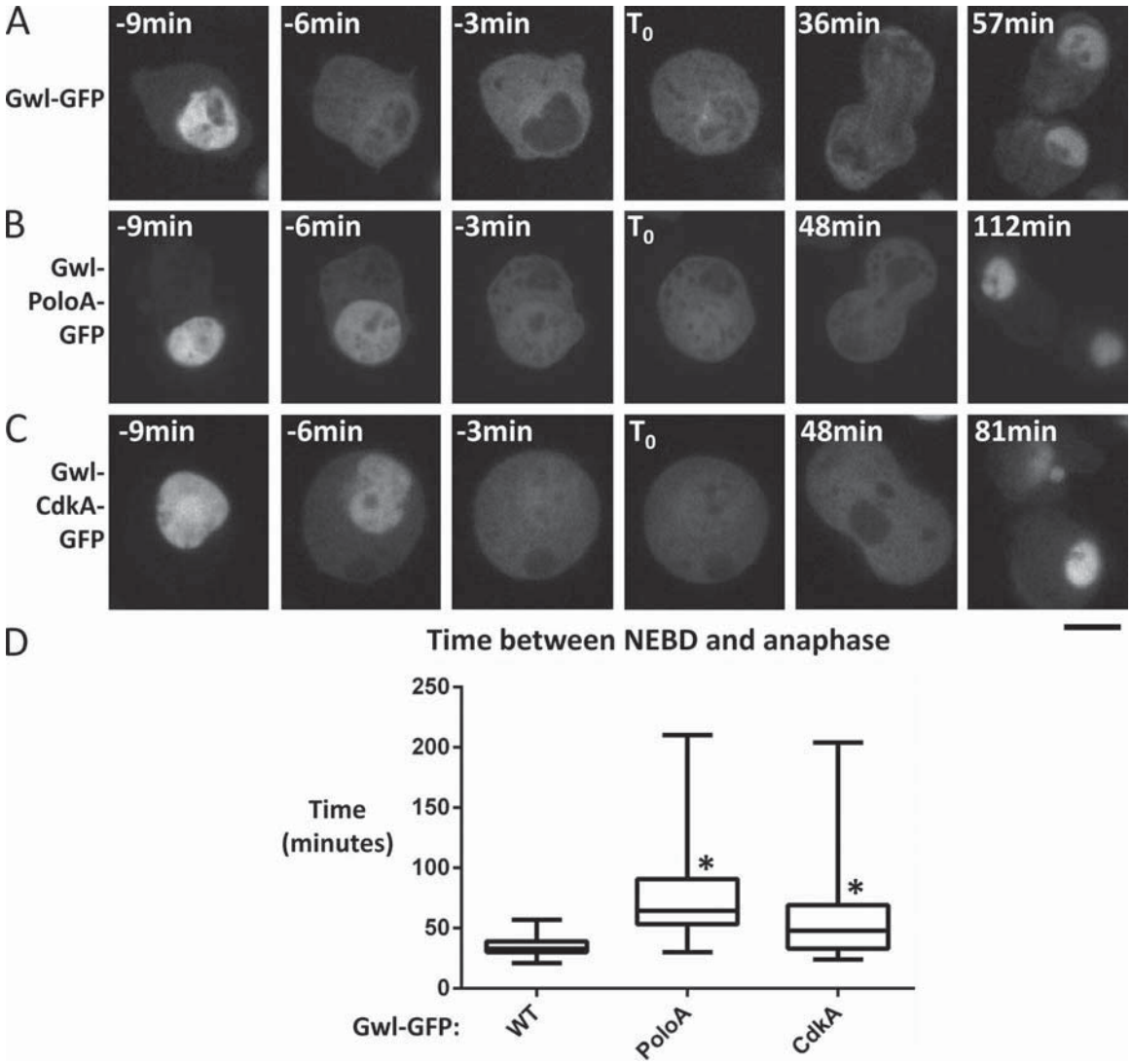


Figure 3.6. Exclusion of Gwl from the nucleus in prophase requires Polo and Cdk consensus sites and is required for timely mitotic progression. Expression of Gwl-GFP WT, PoloA or CdkA was induced and cells were filmed one day later. **A**. Gwl-GFP is nuclear in interphase and becomes cytoplasmic and excluded from the nucleus in prophase. After NEBD (T_0), Gwl-GFP becomes diffuse throughout the cell and returns to nuclei during cytokinesis.

Scale bar: 5 μm. **B-C**. Mutation of Polo or Cdk consensus sites in the central region of Gwl-GFP prevents its nuclear exclusion in prophase. **D**. Expression of Gwl-PoloA-GFP or Gwl-CdkA-GFP extends early mitosis. The time between estimated NEBD (loss of round outline of NE) and anaphase (cell elongation) was measured and shown in a box plot (WT: N=37 cells; PoloA: N=26 cells; CdkA: N=67 cells). Asterisks: $p < 0.001$ after Student t-test.

3.3.6 14-3-3ε collaborates with Polo to promote the cytoplasmic localization of Gwl.

Because a gain of Polo function in interphase led only to a partial relocalization of Gwl to the cytoplasm (Fig 3.5), we hypothesized that other factors were required for this process to occur efficiently in prophase. 14-3-3 proteins are conserved phosphoserine/phosphothreonine-binding proteins that have been implicated in the cytoplasmic retention of several factors including regulators of mitotic entry such as Cdc25C [31]. Two 14-3-3 family members exist in *Drosophila*: 14-3-3ε and 14-3-3ζ. We tested if Gwl could interact with 14-3-3 proteins, as potential regulators of Gwl localization. Recombinant GST-14-3-3ε or GST-14-3-3ζ could pulldown Gwl-Myc from a cell extract (Fig 3.7A). In *Drosophila* cells in culture, Gwl-Myc could be co-purified with both 14-3-3ε and 14-3-3ζ fused to Protein A (Fig 3.7B). In both assays, K to E mutations that weaken the phospho-binding activities of 14-3-3 proteins decreased their association with Gwl [32]. These results suggest that 14-3-3 proteins interact with phosphorylated Gwl.

We tested if 14-3-3 proteins could collaborate with Polo to promote the cytoplasmic localization of Gwl. We found that co-expression of 14-3-3ε and Polo^{T182D} led to a marked increase in cytoplasmic localization of Gwl-Myc in interphase cells, compared with the expression of Polo^{T182D} or 14-3-3ε alone. This effect was abrogated by the K49E mutation in 14-3-3ε, suggesting that its phospho-binding activity is required for its effect on Gwl localization (Fig 3.7C, D). Moreover, the ability of Gwl to interact with 14-3-3ε depended on the presence of the Polo consensus sites in the central region of Gwl (Fig 3.7E). Moreover, cell treatment with the Polo inhibitor BI2536 weakened the interaction of Gwl with 14-3-3ε in a GST pulldown (Fig 3.7F). Together, these results strongly suggest that 14-3-3ε can bind Gwl following its phosphorylation by Polo, leading to the cytoplasmic retention of Gwl.

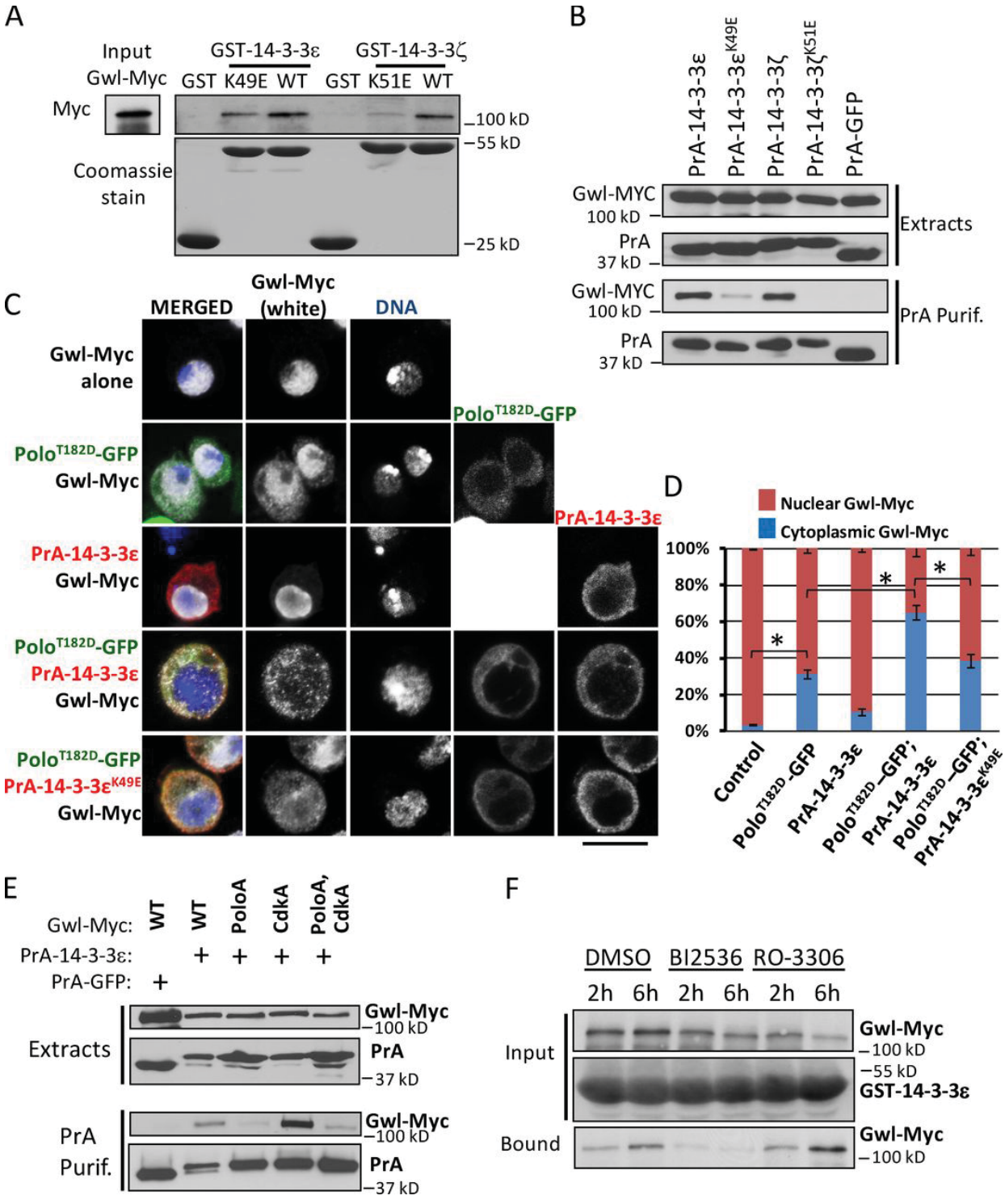


Figure 3.7. 14-3-3ε collaborates with Polo to promote the cytoplasmic localization of Gwl. A. Gwl is co-purified with 14-3-3 proteins in a GST pulldown. An extract from D-Mel cells expressing Gwl-Myc was incubated with GST fusions proteins on sepharose. **B.** Gwl is co-purified with 14-3-3 proteins from cells in culture. Cells co-expressing Gwl-Myc and Protein A

fusion proteins as indicated were used in PrA-affinity purifications. **C-D.** Overexpression of 14-3-3ε enhances the cytoplasmic localization of Gwl induced by a gain of Polo function. **C.** Examples of cells analyzed by immunofluorescence. Scale bar: 10 μm. **D.** Quantification. For each condition, the cytoplasmic and nuclear fluorescence of Gwl-Myc was measured for multiple interphase cells taken randomly. Error bars: S.E.M. Asterisks: $p < 0.001$ after Student t-test. **E**. Polo consensus sites but not Cdk consensus sites in the central region of Gwl are required for its interaction with 14-3-3ε. Cells expressing the indicated proteins were used in PrA-affinity purifications followed by Western blots. **F**. Treatment of cells with a Polo inhibitor (BI2536, 200 nM) but not a Cdk1 inhibitor (RO-3306, 10 μM) abrogated the interaction of Gwl-Myc 14-3-3ε in a GST pulldown.

3.3.7 Cdk1 may contribute to regulate Gwl localization.

Like Polo, Cdk1 plays many important roles in mitosis by targeting several substrates. Moreover, Cdk1 has been shown to activate Gwl kinase activity [11, 20, 21]. To test if Cdk1 could regulate Gwl localization, we mutated into alanine residues 6 minimal CDK consensus sites ((S/T)-P) in the central region of Gwl. Gwl-CdkA was less efficiently phosphorylated by cyclin B-Cdk1 *in vitro*, compared with Gwl-wt (Fig S3C). Interestingly, time-lapse imaging revealed that Gwl-CdkA-GFP is not excluded from the nucleus in prophase (Fig 6C and Movie S5, in 39/39 cells examined). These results suggest that phosphorylation of Gwl by cyclin B-Cdk1 is required for its nuclear exclusion in prophase. However, mutation of the Cdk sites in Gwl did not affect its ability to interact with 14-3-3ε (Fig 3.7E) and cell treatment with a Cdk1 inhibitor did not weaken Gwl's ability to interact with 14-3-3ε in the GST pulldown (Fig 3.7F). These results suggest that Cdk phosphorylation of Gwl affects its localization independently from the Polo/14-3-3 pathway.

3.3.8 Failure to exclude Gwl from the nucleus leads to delays in mitosis.

The failure in nuclear exclusion of Gwl observed for the Polo and CDK phosphorylation site mutants allowed us to ask if the nuclear exclusion of Gwl in prophase was required for normal mitosis. Strikingly, inducing the expression of Gwl-PoloA-GFP or Gwl-CdkA-GFP delayed the onset of anaphase relative to the time of NEBD (approximated by the loss of a visible round nuclear outline) (Fig 3.6D). While this time averaged 34 minutes in cells expressing Gwl-GFP, it extended to averages of 75 minutes in cells expressing Gwl-PoloA-GFP

and 55 minutes in cells expressing Gwl-CdkA-GFP. In addition, this time was much more variable in cells expressing Gwl-PoloA-GFP and Gwl-CdkA-GFP, with some cells incurring delays of over 200 minutes (Fig 3.6D). Since endogenous Gwl was also expressed in those cells, these results strongly suggest a requirement for the nuclear exclusion of Gwl in prophase, rather than a simple requirement for the presence of Gwl in the cytoplasm.

3.3.9 Excessive Polo activity in Gwl-compromised embryos leads to defective mitotic entry.

We asked if Polo or cyclin B-Cdk1 could modify Gwl function in the cell cycle in syncytial embryos. Expression of only half the normal amount of Gwl in embryos laid by mothers heterozygous for a *gwl* null allele (gwl^{6a}) did not prevent embryos from hatching. However, overexpression of Polo in these *gwl*-compromised embryos markedly decreased their hatching rate, while moderate overexpression of Polo alone had only a moderate effect on embryo hatching (Fig 3.8A-B). Thus, Polo appears to modify Gwl activity in this genetic assay.

We examined the development defects in these embryos by immunofluorescence. In embryos expressing half the normal dose of Gwl and overexpressing Polo, we found that a significant fraction of nuclei failed to enter mitosis when the majority of nuclei were already in prometaphase or metaphase (Fig 3.8C). The defective nuclei were characterized by the absence of a mitotic spindle, a very strong lamin staining and undercondensed chromosomes (Fig 3.8C). Such nuclei were also observed, albeit at a lower frequency in embryos from *gwl*-heterozygous mothers. Nuclei either did or did not enter mitosis, and intermediate phenotypes were seldom seen, consistent with a stochastic failure to trigger the bi-stable mitotic switch in which Gwl is implicated [33]. Similar observations were reported following injection of cyclin B dsRNA in syncytial embryos [34]. Expression of constitutively active $Polo^{T182D}$ was already toxic by itself, and was even more harmful to embryos from *gwl*-heterozygous mothers than $Polo^{wt}$, while $Polo^{T182A}$ had no effect on embryonic viability (data not shown). We conclude that Polo kinase activity can regulate the essential function of Gwl in triggering mitotic entry. Overexpression of cyclin B in embryos from gwl^{6a}/+ mothers did not decrease their hatching rate (data not shown), suggesting that Polo levels are more critical than cyclin B levels to regulate Gwl activity during embryonic cell cycles.

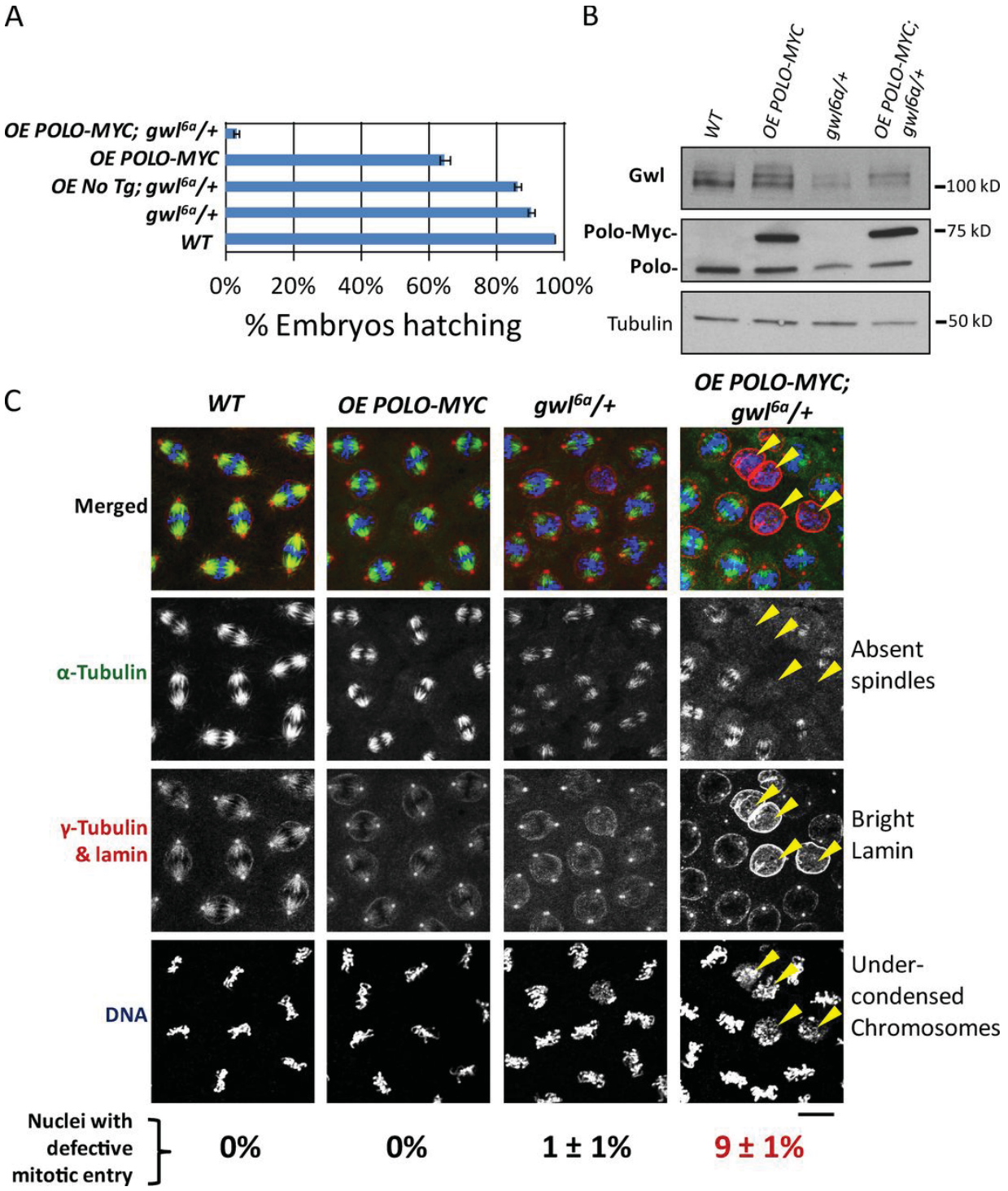


Figure 3.8. Misregulation of Gwl by Polo interferes with cell cycle progression. A. Overexpression of Polo in syncytial embryos compromised for *gwl* is lethal. Expression of *UASp-Polo-Myc* was driven by *Maternal α-Tub Gal4-VP16* (OE: over-expression). No Tg: No Polo Transgene. Error bars: S.E.M. **B.** Western blot analysis of embryos analyzed in A. **C.**

Phenotypes of embryos where a majority of nuclei are in metaphase. Defects in mitotic entry are observed in embryos from mothers heterozygous for *gwl*^{6a} where Polo is overexpressed. Defective nuclei (yellow arrowheads) are characterized by the absence of a spindle, bright lamin staining and undercondensed chromosomes (quantification is at the bottom).

3.4 DISCUSSION

3.4.1 A new level of regulation of Gwl in the cell cycle

We have shown that Gwl is regulated in the cell cycle at the level of its subcellular localization. From a strongly nuclear localization in interphase, Gwl becomes largely cytoplasmic and excluded from the nucleus in prophase, before nuclear envelope breakdown. Our results indicate that Gwl needs to access the nucleus in interphase and translocate to the cytoplasm starting in prophase, for proper cell cycle progression.

Based on our findings and existing knowledge on the factors involved, we can propose a mechanistic model for the role of Gwl's spatial regulation (Fig 3.9). Nuclear sequestration of Gwl could facilitate its activation by cyclin B-Cdk1 in the nucleus at mitotic entry. Phosphorylation of cyclin B by cyclin B-Cdk1 has been shown recently to promote its nuclear import in prophase [35], and increased concentration of cyclin B in the nucleus further stimulates positive feedback loops leading to full cyclin B-Cdk1 activation [35-38]. In *Xenopus*, cyclin B-Cdk1 has been proposed to be at least partly responsible for Gwl activation [11, 20, 21], but a delay has been observed between Cdk1 activation and Gwl activation in starfish oocytes [39] which is consistent with the need to import cyclin B in the nucleus for Gwl activation. In agreement with this model, we find that cyclin B is imported into the nucleus at a time in G2 when Gwl is still in the nucleus (Fig S3.5). Moreover, since PP2A-Tws/B55 is thought to dephosphorylate several cyclin B-Cdk1 substrates, it could potentially inactivate Gwl; therefore the nuclear localization of Gwl could also facilitate Gwl activation by sequestering it away from PP2A-Tws/B55, which is known to be largely cytoplasmic in both *Drosophila* [4] and human cells [35].

Activation of Gwl in the nucleus would be quickly followed by its translocation to the cytoplasm, which depends on its phosphorylation by Polo, followed by 14-3-3ε binding. Our

results suggest that cyclin B-Cdk1 phosphorylation of Gwl in its central region is also required for Gwl's nuclear exclusion, although this phosphorylation is not required for Gwl's binding to Polo or 14-3-3. The requirement for multiple phosphorylation events dependent on two kinases (Polo and Cdk1) may ensure the robustness and proper timing of Gwl's relocalization in prophase, consistent with the physiological importance of this process. Previous studies have established that Gwl promotes mitotic entry by phosphorylating endosulfine proteins, which then become PP2A-Tws/B55 inhibitors [16-18]. Endos is present in both the nucleus and the cytoplasm during the whole cell cycle [16] and therefore, changes in Gwl localization likely dictate where Endos can be phosphorylated. Endos is a small protein that may diffuse freely through nuclear pores if not bound to other proteins, but its localization dynamics have not been studied. Phosphorylation of Endos by Gwl directly in the cytoplasm could help inhibit PP2A-Tws more efficiently in that compartment, and facilitate the accumulation of phosphorylated cyclin B-Cdk1 substrates, and mitotic entry (Fig 3.9).

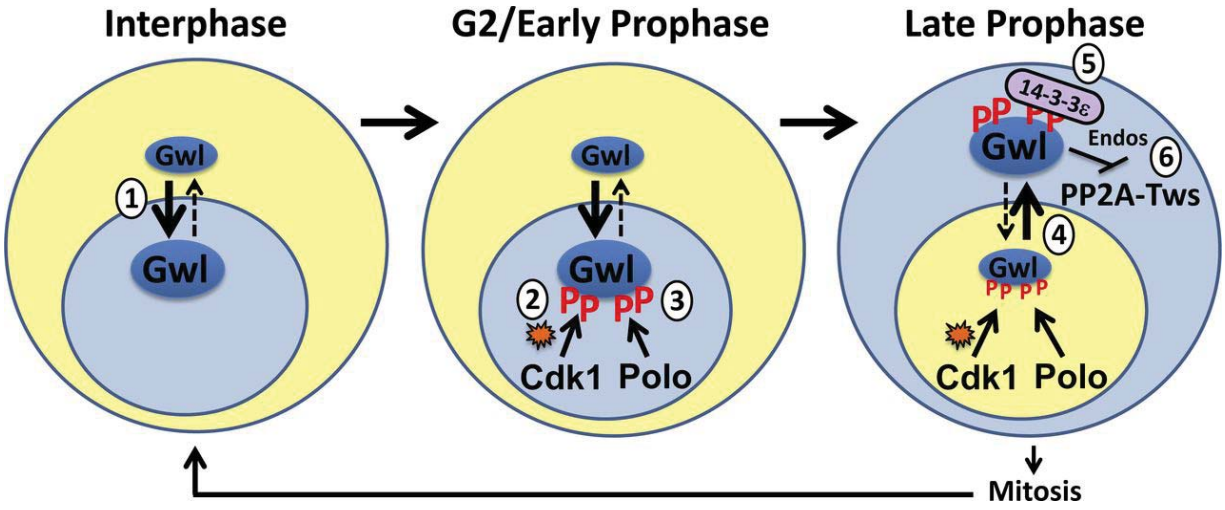


Figure 3.9. An integrative model for the spatial regulation of Gwl. Gwl is actively imported in the nucleus in interphase (1). In early prophase, cyclin B-Cdk1 activates Gwl in the nucleus (2). Following phosphorylation of Gwl in its central region by Polo (3), and possibly by cyclin B-Cdk1 (2), Gwl then re-localizes to the cytoplasm before NEBD (4). Gwl is bound by 14-3-3ε which helps its retention in the cytoplasm (5), where Gwl can inactivate PP2A-Tws to promote orderly mitotic entry (6). See text for details.

Our results suggest that this spatial coordination of Gwl relative to other mitotic regulators is crucial for its function. The fact that the loss of Gwl function altogether does not prevent mitotic entry in many cell types but instead leads to mitotic defects [7-9] already

suggested that failure of the Gwl-PP2A axis in the mitotic switch impacts mitotic events after NEBD. The identity of the PP2A-B55 substrates that must be protected by Gwl from dephosphorylation at mitotic entry first needs to be discovered before we can dissect the importance of their regulation in space and time. These crucial substrates could be nuclear or cytoplasmic. Interestingly, we found that the nuclear retention of Gwl in prophase delays anaphase onset relative to NEBD (Fig 3.6). This result suggests that PP2A-B55 must act on at least one nuclear substrate before NEBD. In yeast, it has recently been shown that PP2A-Cdc55 (B55) protects sister chromatid cohesion in early mitosis by antagonizing Polo phosphorylation of the Scc1 cohesin [40]. A similar mechanism in animal cells could impose a need for Gwl's nuclear exclusion in late prophase. Although Tws (B55) is largely localized to the cytoplasm before NEBD, we have found that it shuttles in and out of the nucleus (our unpublished observations).

3.4.2 A mechanism directly linking Gwl and Polo

We have shown that the spatial regulation of Gwl depends in part on the Polo kinase. Polo interacts with Gwl, phosphorylates it in its central region and promotes its cytoplasmic localization (Figs 3.4-3.5). Consistent with this mechanism, immunostaining in syncytial embryos reveals the presence of active pT182-Polo in prophase nuclei (Fig S3.1B). This is in agreement with a previous study in human cells using a FRET probe that showed that Plk1 activity appears in the nucleus in G2 and increases there during prophase [41]. Moreover, Gwl-NLSmut (which does not enter the nucleus) appears less phosphorylated than Gwl-wt in Western blots (Fig 3.3E). NLSs are often negatively regulated by phosphorylation in their proximity [42]. One classic example is the Cdk1-dependent phosphorylation and nuclear exclusion of the transcription factors Swi5 and Ace2 until mitotic exit in *S. cerevisiae* [43]. Phosphorylation of Gwl by Polo and Cdk1 could partially inactivate its NLSs by reducing the charge in their vicinity. In addition, binding of 14-3-3ε to phosphorylated Gwl could mask its NLSs, thereby preventing its nuclear import. A similar mechanism is known to operate to regulate the Cdk1-activating phosphatase CDC25C in human cells. Binding of 14-3-3 proteins to phosphorylated CDC25C mediates its retention in the cytoplasm until phosphorylation of CDC25C at other sites disrupts its interaction with 14-3-3 to allow the nuclear import of CDC25C that stimulates mitotic entry [44-46].

The current study shows a direct connection between Polo and Gwl and allows clearer interpretations of genetic results. By antagonizing Gwl's nuclear localization in interphase, increased Polo activity leaves less Gwl in the nucleus (Fig 3.5), which would cause Gwl to be less efficiently activated by cyclin B-Cdk1 (Fig 3.9). When *gwl* function is already abrogated, this is associated with lethality, with frequent failures of syncytial nuclei to enter mitosis, as revealed by missing mitotic spindles, undercondensed chromosomes and apparently intact nuclear envelopes (Fig 3.8). In a previous report, we showed that the converse perturbations of the Gwl/Polo balance lead to a very different phenotype; Females heterozygous for a gain-of-function allele of *gwl* (gwl^{Scant}) and for a *polo* null allele lay embryos that fail to hatch, and show a high incidence of mitotic defects with detached centrosomes in the syncytium. Overexpression of Gwl-wt genetically interacts with *polo* hypomorphs in the same way [8]. We subsequently showed that Gwl antagonizes PP2A-Tws in meiosis and mitosis, consistent with the model proposed from biochemical results in frogs extracts [17, 18], and as expected, *polo* hypomorphic mutations strongly interacted with mutations abrogating PP2A-Tws [15]. According to our model (Fig 3.9), lowering Polo activity would lead to an increase in Gwl activity in the nucleus, resulting in the mitotic defects and death observed when Gwl activity is already increased [8] or when PP2A-Tws activity is reduced [15]. Consistent with this model, retention of Gwl in the nucleus in prophase by mutation of Polo consensus sites in the central region of Gwl, leads to long mitotic delays, even in the presence of endogenous Gwl (Fig 3.6). It remains possible that the genetic interactions between Polo, Gwl and PP2A-Tws reflect multiple levels of functional interactions [9].

3.4.3 A mode of regulation that could be generally conserved

Our results allow us to ascribe a first role to the central region of Gwl, which is to regulate its localization in the cell cycle. The large number of Polo phosphorylation sites in Gwl and the fact that they tend to be poorly conserved when considered individually (data not shown) suggests that no single Polo phosphorylation site alone may be responsible for the regulation of Gwl's localization. Indeed, it has been proposed that many physiologically important multi-site phosphorylation events occur in protein segments whose general position and function is conserved, but whose precise amino-acid sequence is not conserved [47, 48]. Although the central region of *Drosophila* Gwl that contains the NLSs is poorly conserved in sequence, its

function in regulating localization in a phosphorylation-dependent manner could be conserved in Gwl orthologs, including MASTL in humans.

The yeast ortholog of Gwl, Rim15 is known to be regulated in its nucleo-cytoplasmic localization in response to phosphorylation [49]. In this case, the TOR and Sch9 (PKB homolog) kinases promote the cytoplasmic localization of Rim15, but the molecular mechanisms have not been fully explored. Although Rim15 and its substrates Igo1/2 have been implicated in the regulation of mRNA metabolism in G0 [50], and not in mitotic entry as for Gwl, the molecular mechanisms regulating Rim15 and Gwl could share general features.

The recent discovery of the Gwl-PP2A axis and of its major role in cell cycle regulation was brought by *Drosophila* genetics and biochemistry in *Xenopus* extracts, but the subcellular spatial dynamics of the pathway had been little explored. Here, we have shown that Gwl is spatially regulated in the cell cycle and that this aspect of its function is essential *in vivo*. Moreover, we have uncovered a molecular mechanism responsible for this regulation of Gwl that targets its central region, for which no function was previously known. Nucleo-cytoplasmic coordination of the main mitotic regulators including Gwl appears like a crucial level of cell cycle control.

3.5 MATERIALS & METHODS

3.5.1 Fly culture and transgenesis. Transgenic flies were made by BestGene Inc. by P-element based insertions in the w^{1118} background. *UASp-GWL-GFP* flies were published elsewhere [8]. The wild-type strain used was Oregon R. Viability and fertility tests in genetic rescue experiments were conducted as described [8]. Briefly, for fertility tests, well-fed females in the presence of males were placed in tubes containing grape juice agar and allowed to lay eggs for 1 day before being removed. The percentage of hatched embryos was scored 24 hrs later. For viability tests, flies were crossed and the number of observed flies of the genotype of interest relative to their expected number in the progeny was expressed as a percentage.

3.5.2 DNA constructs. Most expression vectors were generated in the Gateway system (Invitrogen). Coding sequences were first cloned into the pDONR221λ entry vector. They were then recombined with destination vectors to generate the following expression plasmids: pMT-

GFP-Gwl, pMT-Gwl-GFP, pAC5-Gwl-Myc, pAC5-Gwl-TAP, pAC5-PrA-GFP, pAC5-Gwl-PrA, pMT-Polo^{T182D}-GFP, pAC5-H2Av-mRFP, pUASp-Gwl-PrA, pUASp-Myc-Gwl, pUASp-Polo-Myc, pAC5-Polo-PrA, pAC5-PrA-14-3-3ε, pAC5-PrA-14-3-3ζ, as well as all related truncations and mutants. The pDEST15-GST-Gwl vector was a gift of Hélène Rangone. Point mutants were generated by QuickChange (Stratagene) in entry clones or were synthesized by BioBasic.

3.5.3 Cell culture. All cells were in the D-Mel2 (D-Mel) background and were cultured in Express Five medium (Invitrogen). Stable cell lines expressing pMT-GFP-GWL, pMT-Gwl-GFP (WT and mutants) tagged proteins were generated as described [28]. Briefly, selection was based on resistance to 20 μg/ml blasticidin. Inducible pMT-based vectors already contained the resistance gene, while other vector did not and were co-transfected with pCoBast.

3.5.4 Immunofluorescence and Western blotting. Antibodies used in immunofluorescence and Western blotting were anti-α-Tubulin YL1/2 (Sigma), anti-γ-Tubulin GTU88 (Sigma), anti-lamin and anti-cyclin B (both from DSHB), anti-Myc 9E10 (Santa Cruz), anti-Polo MA294 (gift from David Glover), anti-pT182-Polo (Biolegend), rabbit IgG (for PrA in TAP tag) and anti-Gwl (custom-made against full-length Gwl by Genscript). Immunofluorescence in embryos and in cells was done as described [28]. Briefly, cells were fixed with 4% formaldehyde for 10 min, and permeabilized and blocked in Phosphate Buffer Saline (PBS) containing 0.1% Triton X-100 and 1% BSA (PBSTB). Cells were incubated with primary antibody diluted in PBSTB for 1 h to O/N, washed 3 times in PBSTB, and incubated with secondary antibodies for 1 h to 2 h. Cells were washed several times in PBSTB and once in PBS, before being mounted in Vectashield medium containing DAPI. Zero to 3 h old embryos were dechorionated in 50% bleach before fixation using formaldehyde and methanol. Embryos were then rehydrated, permeabilized, blocked and incubated with antibodies. DAPI was added in the secondary antibodies solution and embryos were mounted in Vectashield.

3.5.5 Microscopy. Cells shown in Fig 3.3 were acquired on a Zeiss AxioImager microscope with a 100x oil objective (NA 1.4 DICIII) and an AxioCam HRm camera, using AxioVision software. All other images of fixed cells and embryos were acquired on a Zeiss 510 Meta confocal microscope with a 100x oil objective (NA 1.4 DICIII), using LSM510 3.2 software. Time-lapse

imaging of embryos was done on a Nikon Eclipse Ti Swept-Field microscope with a 60x oil objective (NA 1.1) and a Photometrics Quantum: 512SC camera, using NIS Element software. Time-lapse imaging of GFP-Gwl or Gwl-GFP expressing cells was performed using an Ultraview Vox spinning disc confocal system (PerkinElmer), a plan Apo 100x oil immersion objective (NA 1.4) and an Orca-R2 CCD camera (Hamamatsu) with 2x2 binning and using Volocity 6.0 software (Improvision/Perkin Elmer) (Movies S3.1 to S3.5, Fig 3.6). Other time-lapse images were acquired on a DeltaVision microscope with a 60x oil objective (NA 1.42) and a Photometrics CoolSNAP HQ^2 camera with binning 2x2, at 25°C using SoftWoRx 5.5 Explorer (Fig 3.5D). The quantifications in Fig 3.5B and 3.7D were done using ImageJ. All images were acquired at room temperature unless otherwise specified.

3.5.6 Affinity purifications. Protein A affinity purifications from cells were carried out essentially as described [51]. Briefly, pelleted cells from confluent 75 cm^2 flasks were resuspended in approximately 10 volumes of lysis buffer and passed through a needle several times using a syringe. Lysates were clarified by centrifugation for 15 min at 14,000 rpm in a tabletop centrifuge. Supernatants were incubated with 25 µl of IgG-conjugated DynaBeads (Invitrogen) for 1 to 2 hours and washed with lysis buffer 4 to 5 times for 5 min. Purification products were eluted by heating at 95°C for 2 min in SDS-PAGE Laemmli buffer (Sigma) and analyzed by Western blotting. Purifications from embryos were performed following very similar protocol. Zero to 3 hrs old embryos were crushed thouroughly in lysis buffer and centrifugated for 15 min at 14,000 rpm in a tabletop centrifuge. Clarified supernatants were taken, without disturbing the fatty layer on top. The centrifugation was repeated and any residual fatty fraction was discarded. The remaining steps of the purifications were as with cells.

3.5.7 Kinase assays. Active HIS-Polo obtained from Sf9 cells was used to phosphorylate GST-Gwl obtained from bacterial expression. Cyclin B-Cdk1 (human) was from Millipore. Reactions were done in kinase buffer (20 mM K-HEPES, 2 mM MgCl2, 1 mM DTT, 1µM ATP, 1 µCi ^{32}P-γ-ATP) at 30°C for 20 min and stopped with the addition of Laemmli buffer. Products were resolved by SDS-PAGE and transferred onto nitrocellulose for autoradiography.

3.5.8 Phosphorylation site mapping. Samples were reconstituted in 50 mM ammonium bicarbonate with 10 mM TCEP and vortexed for 1 hour at 37°C. Chloroacetamide was added for alkylation to a final concentration of 55 mM Samples were vortexed for another hour at 37°C. 1 µg of trypsin was added and the digestion was carried out for 8 hours at 37°C. Samples were dried down and solubilized in ACN 5% formic acid (FA) 0.2%. Samples were loaded on a home-made C18 precolumn (0.3 mm i.d. x 5 mm) connected directly to the switching valve and separated on a homemade reversed-phase column (150 µm i.d. x 150 mm) with a 56-min gradient from 10–60% acetonitrile (0.2% FA) and a 600 nl/min flow rate on an Eksigent nanoLC-2D system (Eksigent,Dublin, CA) connected to an LTQ-Orbitrap Elite (Thermofisher, San Jose, CA). Each full MS spectrum acquired with a 60,000 resolution was followed by 12 MS/MS spectra, where the 12 most abundant multiply charged ions were selected for MS/MS sequencing. Tandem MS experiments were performed using collision-induced dissociation in the linear ion trap. The data were processed using the Mascot 2.4 (Matrix Science, UK) and the Uniprot *Drosophila melanogaster* database (58,894 sequences). Tolerances on precursors and fragments were 15 ppm and 0.5 Da respectively. Variable selected post-translational modifications were carbamidomethyl (C), oxidation (M), deamidation (NQ) and phosphorylation (STY).

3.5.9 RNA interference. dsRNAs corresponding to the *GWL* coding sequence between bp 200 and 625 or to the bacterial kanamycin resistance gene (as control) were synthesized after PCR using T7 sequence-containing primers using the T7 Ribomax kit (Promega). To obtain RNAi-resistant forms of Gwl-GFP, we replaced all codons in the region targeted by the GWL dsRNA with alternative, conservative codons. DNA cassettes were synthesized by BioBasic, were subcloned into pDONR-GWL (WT and NLSmut), allowing the generation of pMT-Gwl^{RES}-GFP and pMT-Gwl-NLSmut^{RES}-GFP, for copper-inducible expression. Stable cell lines were generated with these constructions. The day before RNAi treatment, 500,000 cells were plated in 1 ml of medium in 12-well plates. RNAi was induced with 15 µg of dsRNA transfected with Transfast (Promega). Cells were analyzed after 4 days.

3.5.10 Preparation of GST-fusion proteins. Overnight cultures of BL21 *E. Coli* transformed with pGEX-14-3-3ε or 14-3-3ζ, or corresponding mutants (K49Eε or K51Eζ), were diluted into

500 ml and expression induced with 1 mM IPTG (isopropyl β-D-thiogalactopyranoside) overnight at 25°C. Cells were pelleted and resuspended in 40 ml of PBS, 0.1 M EDTA, 0.1% Triton-X and supplemented with 1 mM PMSF (phenylmethylsulfonyl fluoride), 5 µg/ml pepstatin A, 10 µg/ml leupeptin and 1 mM PMSF. Bacterial cell suspensions were lysed by sonication and extracts were centrifugated at 13,000 × g for 30 minutes, and were stored in 1 ml aliquots at -80°C until used. For preparation of beads, 1 ml of clarified extract was incubated with 200 µl of washed 50% slurry of glutathione–agarose (G Biosciences) for 8 hrs. Beads were washed four times with PBS, 0.1 M EDTA, 0.1% Triton-X and then three times with cell lysis buffer (see below). Prior to use, GST-bound 14-3-3 protein was normalized and quantified using commassie staining of BSA (Bovine Serum Albumin) as a standard. All experiments were performed at 4°C.

3.5.11 GST-pulldown assay. For pulldown assays, D-Mel cells were washed with ice-cold PBS and lysed in 10 mM K_3PO_4, 1 mM EDTA, 5 mM EGTA, 10 mM $MgCl_2$, 50 mM β-glycerophosphate, 0.5% NP-40, 0.1% Brij 35, 0.1% deoxycholic acid, 1 mM sodium orthovanadate (Na_3VO_4), 1 mM PMSF, and Complete protease inhibitors (Roche, Indianapolis, IN, USA). Clarified lysates were incubated with 10 µg of GST-bound 14-3-3 proteins for two hours, following which beads were washed three times with cell lysis buffer prior to SDS-PAGE and immunoblotting.

3.5.12 Online Supplemental Materials. Fig S3.1 shows that the localization of Gwl is cell-cycle regulated in embryos and is a complement to Fig 3.1. It also shows that active Polo is nuclear in prophase. Fig S3.2 shows a sequence alignment between Gwl orthologs from *Drosophila* and mosquito species, where the NLS motifs are conserved. Fig S3.3 documents the phosphorylation site mapping in Gwl. Fig S3.4 shows that Polo phosphorylation of Gwl promotes its cytoplasmic localization as a complement to Fig 3.5. Fig S3.5 shows that cyclin B is imported in the nucleus while Gwl is still in the nucleus. Movies S3.1 to S3.5 show time-lapse imaging of mitosis in a D-Mel cell expressing GFP-Gwl (S1), GFP-SV40NLS-Gwl (S2), Gwl-GFP (S3), Gwl-PoloA-GFP (S4), and Gwl-CdkA-GFP (S5).

3.6 ACKNOWLEDGMENTS

We thank Damien D'Amours and Vincent Boudreau for comments on the manuscript, Benjamin Kwok and Lama Talje for producing active HIS-Polo kinase, and Christian Charbonneau at IRIC's Imaging facility. We thank David Glover for providing Polo antibody, Hélène Rangone for the plasmid for bacterial expression of GST-Gwl, and Pat O'Farrell for H2Av-RFP flies. PW holds a studentship from the Fonds de recherche du Québec – Santé (FRQS), and JAG holds a postdoctoral fellowship from the Canadian Institutes of Health Research (CIHR). This work was supported by a Human Frontier Science Program Career Development Award and by grants from the CIHR to VA and PPR. VA holds a New Investigator Award from the CIHR, and PPR holds the Canada Research Chair in Signal Transduction and Proteomics. IRIC is supported in part by the Canadian Center of Excellence in Commercialization and Research, the Canada Foundation for Innovation and the FRQS.

3.7 SUPPLEMENTARY FIGURES

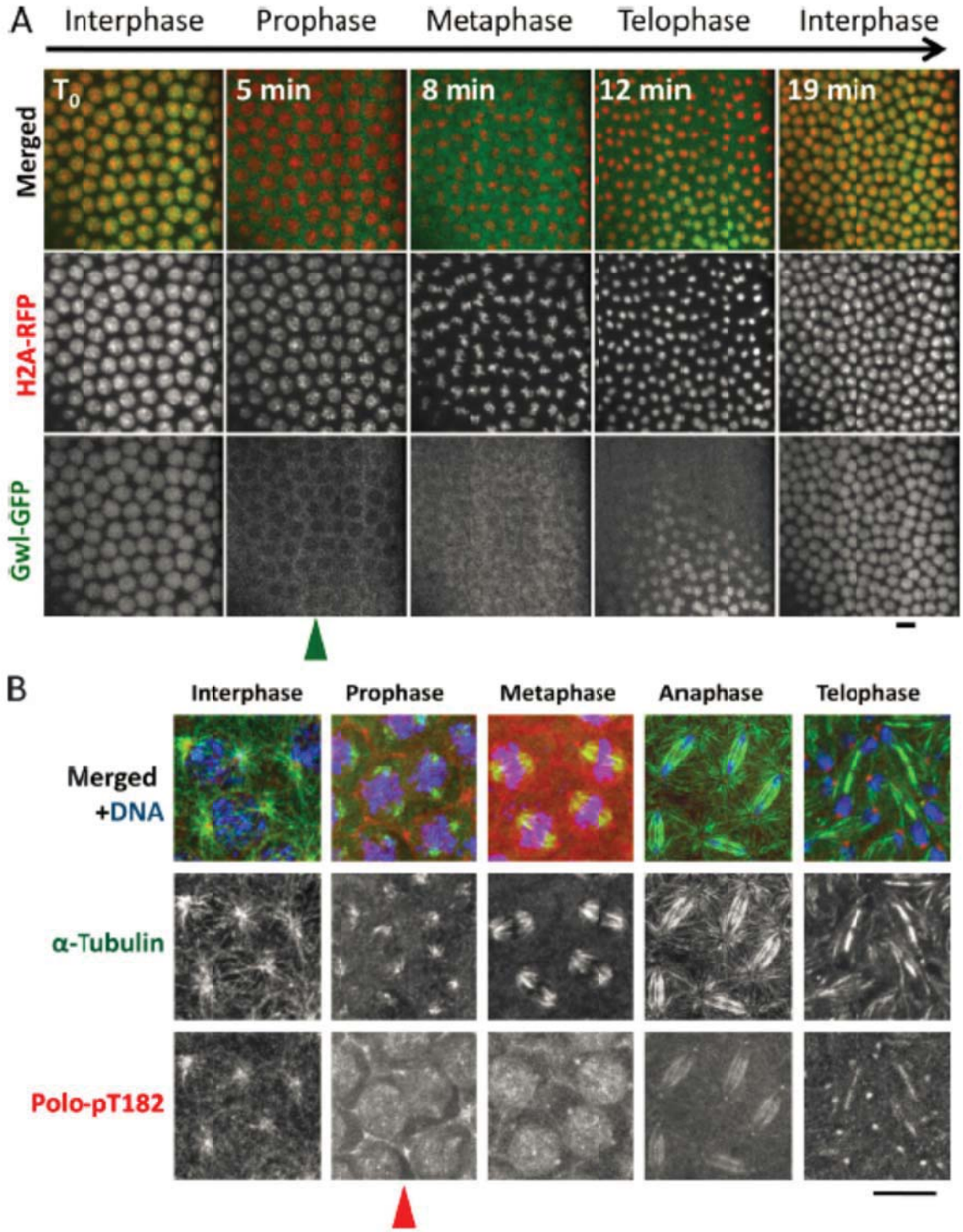
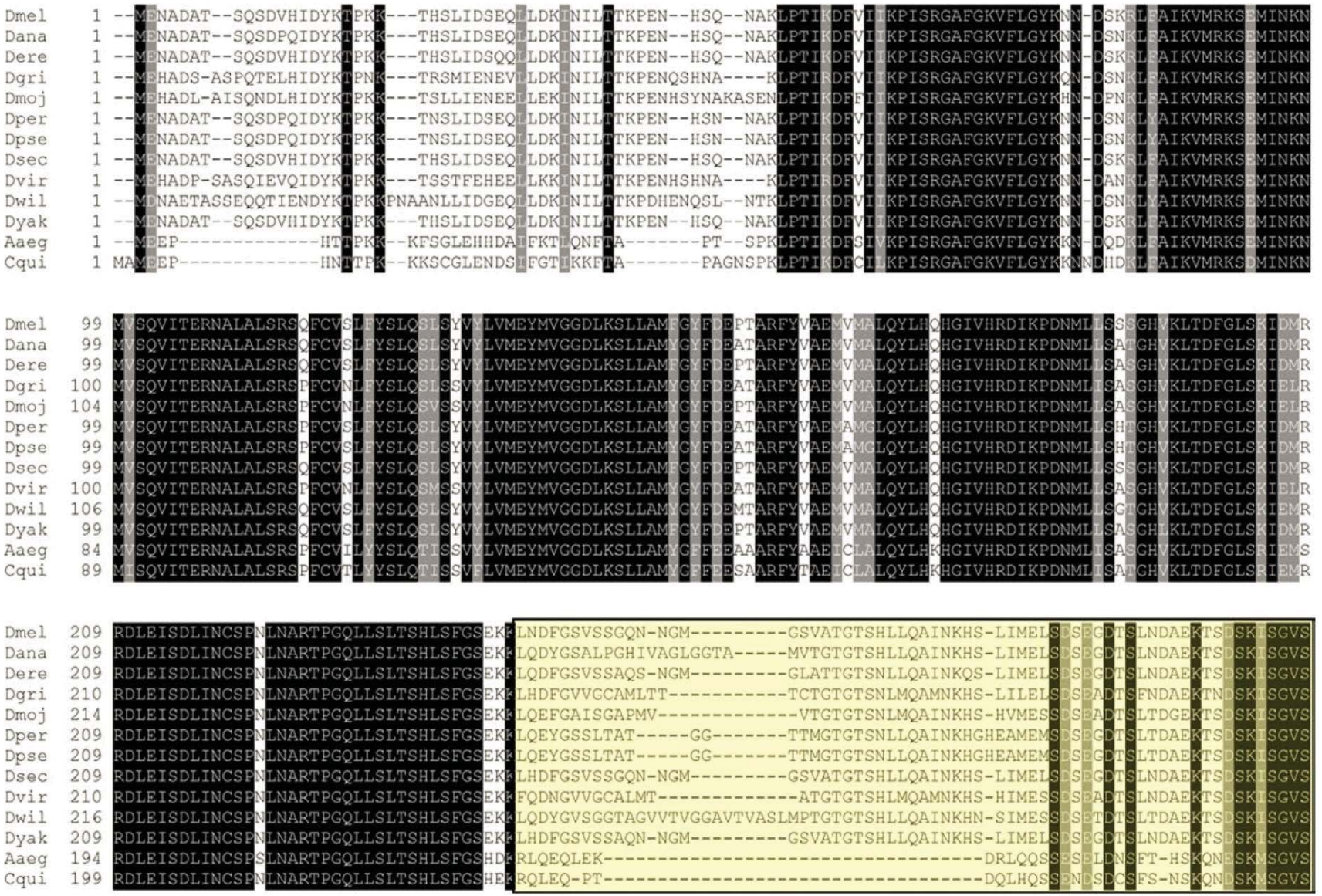


Figure S3.1. The localization of Gwl is cell-cycle regulated (complement to Figure 1). A. Time-lapse imaging of syncytial embryos expressing Gwl-GFP and H2Av-RFP. Note the nuclear exclusion of Gwl-GFP in prophase (green arrowhead). **B.** Active Polo is detected in nuclei in

prophase. Immunofluorescence in syncytial embryos using an antibody specific for the phosphorylated T-loop activation site of Polo (T182) and α-Tubulin. Note the nuclear localization of Polo-pT182 in prophase (red arrowhead). Scale bars: 10 μm.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Dana 1 --MENADAT--SQSDPQIDYKTPKK---THSLIDSEQLLDKINILTTKPEN--HSQ--NAKLPTIKDFVIIKPISRGAFGKVFLGYKNN-DSNKLFAIKVMRKSEMINKN
Dere 1 --MENADAT--SQSDVHIDYKTPKK---THSLIDSQQLLDKINILTTKPEN--HSQ--NAKLPTIKDFVIIKPISRGAFGKVFLGYKNN-DSKRLFAIKVMRKSEMINKN
Dgri 1 --MEHADS-ASPQTELHIDYKTPNK---TRSMIENEVLLDKINILTTKPENQSHNA----KLPTIRDFVIIKPISRGAFGKVFLGYKQN-DSNKLFAIKVMRKSEMINKN
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Dpse 1 --MENADAT--SQSDPQIDYKTPKK---TNSLIDSEQLLDKINILTTKPEN--HSN--NAKLPTIKDFVIIKPISRGAFGKVFLGYKNN-DSNKLYAIKVMRKSEMINKN
Dsec 1 --MENADAT--SQSDVHIDYKTPKK---THSLIDSEQLLDKINILTTKPEN--HSQ--NAKLPTIKDFVIIKPISRGAFGKVFLGYKNN-DSKRLFAIKVMRKSEMINKN
Dvir 1 --MEHADP-SASQIEVQIDYKTPKK---TSSTFEHEELLKKINILTTKPENHSHNA----KLPTIRDFVIIKPISRGAFGKVFLGYKNN-DANKLFAIKVMRKSEMINKN
Dwil 1 --MDNAETASSEQQTIENDYKTPKKPNAANLLIDGEQLLDKINILTTKPDHENQSL--NTKLPTIKDFVIIKPISRGAFGKVFLGYKNN-DSNKLYAIKVMRKSEMINKN
Dyak 1 --MENADAT--SQSDVHIDYKTPKK---THSLIDSEQLLDKINILTTKPEN--HSQ--NAKLPTIKDFVIIKPISRGAFGKVFLGYKNN-DSKRLFAIKVMRKSEMINKN
Aaeg 1 --MEEP-------------HTTPKK--KFSGLEHHDAIFKTLQNFTA-------PT--SPKLPTIKDFSIVKPISRGAFGKVFLGYKKN-DQDKLFAIKVMRKSEMINKN
Cqui 1 MAMEEP-------------HNTTPK--KKSCGLENDSIFGTIKKFTA-------PAGNSPKLPTIKDFCILKPISRGAFGKVFLGYKKNNDHDKLFAIKVMRKSDMINKN
Dmel 99 MVSQVITERNALALSRSQFCVSLFYSLQSLSYVYLVMEYMVGGDLKSLLAMFGYFDEPTARFYVAEMVMALQYLHQHGIVHRDIKPDNMLLSSSGHVKLTDFGLSKIDMR
Dana 99 MVSQVITERNALALSRSQFCVSLFYSLQSLSYVYLVMEYMVGGDLKSLLAMYGYFDEATARFYVAEMVMALQYLHQHGIVHRDIKPDNMLLSATGHVKLTDFGLSKIDMR
Dere 99 MVSQVITERNALALSRSQFCVSLFYSLQSLSYVYLVMEYMVGGDLKSLLAMFGYFDEPTARFYVAEMVMALQYLHQHGIVHRDIKPDNMLLSASGHVKLTDFGLSKIDMR
Dgri 100 MVSQVITERNALALSRSPFCVNLFYSLQSLSSVYLVMEYMVGGDLKSLLAMYGYFDEATARFYVAEMVMALQYLHQHGIVHRDIKPDNMLLSASGHVKLTDFGLSKIEIR
Dmoj 104 MVSQVITERNALALSRSPFCVNLFYSLQSVSSVYLVMEYMVGGDLKSLLAMYGYFDEPTARFYVAEMVMALQYLHQHGIVHRDIKPDNMLLSASGHVKLTDFGLSKIEIR
Dper 99 MVSQVITERNALALSRSPFCVSLFYSLQSLSYVYLVMEYMVGGDLKSLLAMYGYFDEATARFYVAEMAMGLQYLHQHGIVHRDIKPDNMLLSHTGHVKLTDFGLSKIDMR
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Dsec 99 MVSQVITERNALALSRSQFCVSLFYSLQSLSYVYLVMEYMVGGDLKSLLAMFGYFDEPTARFYVAEMVMALQYLHQHGIVHRDIKPDNMLLSSSGHVKLTDFGLSKIDMR
Dvir 100 MVSQVITERNALALSRSPFCVNLTYSLQSMSSVYLVMEYMVGGDLKSLLAMYGYFDEATARFYVAEMVMALQYLHQHGIVHRDIKPDNMLLSASGHVKLTDFGLSKIEIR
Dwil 106 MVSQVITERNALALSRSQFCVSLFYSLQSLSYVYLVMEYMVGGDLKSLLAMYGYFDEMTARFYVAEMVMALQYLHQHGIVHRDIKPDNMLLSGTGHVKLTDFGLSKIEMR
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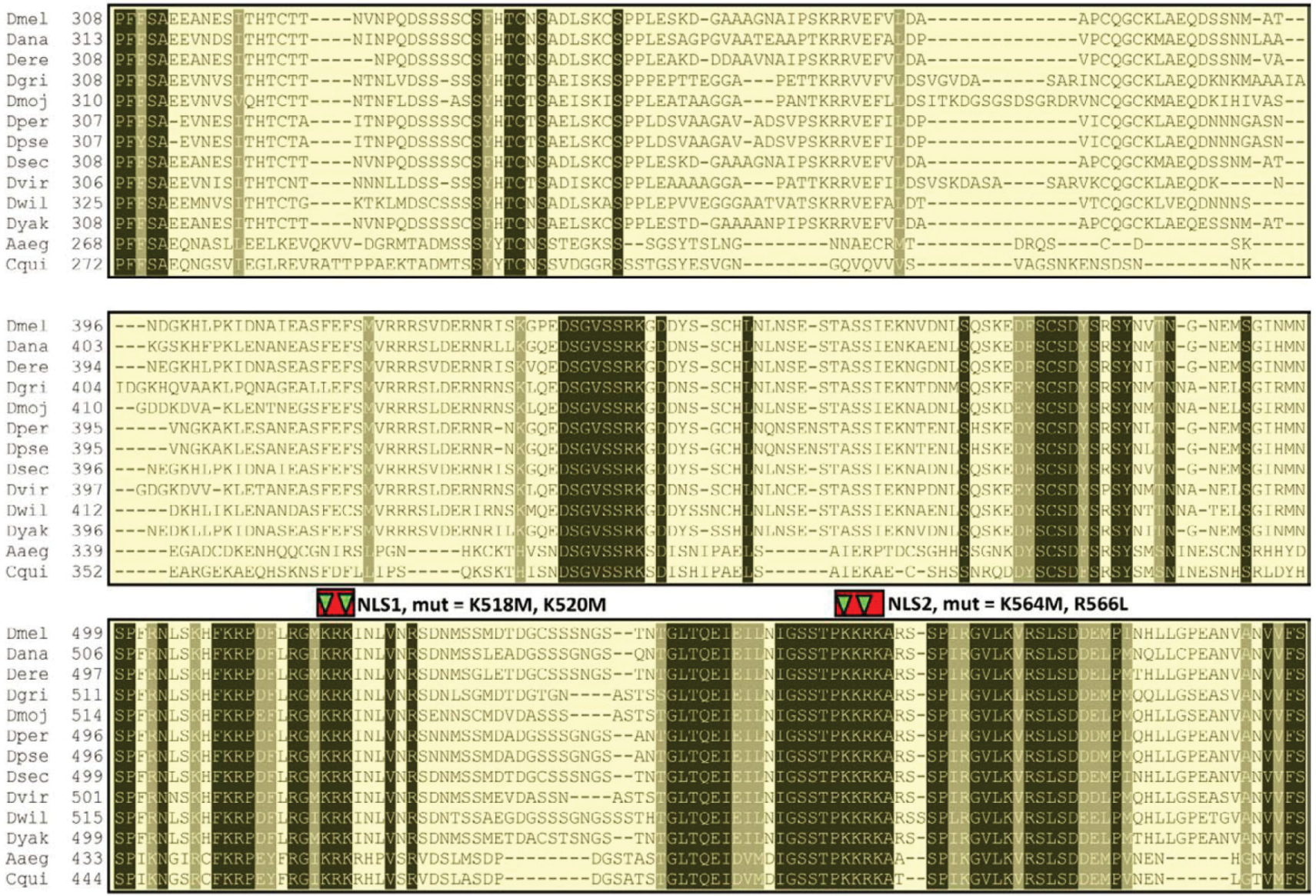
Aaeg 84 MVSQVITERNALALSRSPFCVILYYSLQTISSVYLVMEYMVGGDLKSLLAMYGFFEEAAARFYAAEICLALQYLHKHGIVHRDIKPDNMLISASGHVKLTDFGLSRIEMS
Cqui 89 MISQVITERNALALSRSPFCVTLYYSLQTISSVFLVMEYMVGGDLKSLLAMYGFFEESAARFYTAEICLALQYLHKHGIVHRDIKPDNMLISATGHVKLTDFGLSRIEMR
Dmel 209 RDLEISDLINCSPNLNARTPGQLLSLTSHLSFGSEKKLNDFGSVSSGQN-NGM---------GSVATGTSHLLQAINKHS-LIMELSDSEGDTSLNDAEKTSDSKISGVS
Dana 209 RDLEISDLINCSPNLNARTPGQLLSLTSHLSFGSEKKLQDYGSALPGHIVAGLGGTA-----MVTGTGTSHLLQAINKHS-LIMELSDSEGDTSLNDAEKTSDSKISGVS
Dere 209 RDLEISDLINCSPNLNARTPGQLLSLTSHLSFGSEKKLQDFGSVSSAQS-NGM---------GLATTGTSNLLQAINKQS-LIMELSDSEGDTSLNDAEKTSDSKISGVS
Dgri 210 RDLEISDLINCSPNLNARTPGQLLSLTSHLSFGSEKKLHDFGVVGCAMLTT-----------TCTGTGTSNLMQAMNKHS-LILELSDSEADTSFNDAEKTNDSKISGVS
Dmoj 214 RDLEISDLINCSPNLNARTPGQLLSLTSHLSFGSEKKLQEFGAISGAPMV-------------VTGTGTSNLMQAINKHS-HVMESSDSEADTSLTDGEKTSDSKISGVS
Dper 209 RDLEISDLINCSPNLNARTPGQLLSLTSHLSFGSEKKLQEYGSSLTAT-----GG-------TTMGTGTSNLLQAINKHGHEAMEMSDSEGDTSLTDAEKTSDSKISGVS
Dpse 209 RDLEISDLINCSPNLNARTPGQLLSLTSHLSFGSEKKLQEYGSSLTAT-----GG-------TTMGTGTSNLLQAINKHGHEAMEMSDSEGDTSLTDAEKTSDSKISGVS
Dsec 209 RDLEISDLINCSPNLNARTPGQLLSLTSHLSFGSEKKLHDFGSVSSGQN-NGM---------GSVATGTSHLLQAINKHS-LIMELSDSEGDTSLNDAEKTSDSKISGVS
Dvir 210 RDLEISDLINCSPNLNARTPGQLLSLTSHLSFGSEKKFQDNGVVGCALMT-----------ATGTGTSHLMQAMNKHS-HIMESSDSEADTSLNDAEKTSDSKISGVS
Dwil 216 RDLEISDLINCSPNLNARTPGQLLSLTSHLSFGSEKKLQDYGVSGGTAGVVTVGGAVTVASLMPTGTGTSHLLQAINKHN-SIMESSDSETDTSLTDAEKTSDSKISGVS
Dyak 209 RDLEISDLINCSPNLNARTPGQLLSLTSHLSFGSEKKLHDFGSVSSAQN-NGM---------GSVATGTSHLLQAINKHS-LIMELSDSEGDTSLNDAEKTSDSKISGVS
Aaeg 194 RDLEISDLINCSPSLNARTPGQLLSLTSHLSFGSHDKRLQEQLEK-----------------------------------DRLQQSSESELDNSFT-HSKQNESKMSGVS
Cqui 199 RDLEISDLINCSPNLNARTPGQLLSLTSHLSFGSHEKRQLEQ-PT-----------------------------------DQLHQSSENDSDCSFS-NSKQNDSKMSGVS


NLS1, mut = K518M, K520M
NLS2, mut = K564M, R566L

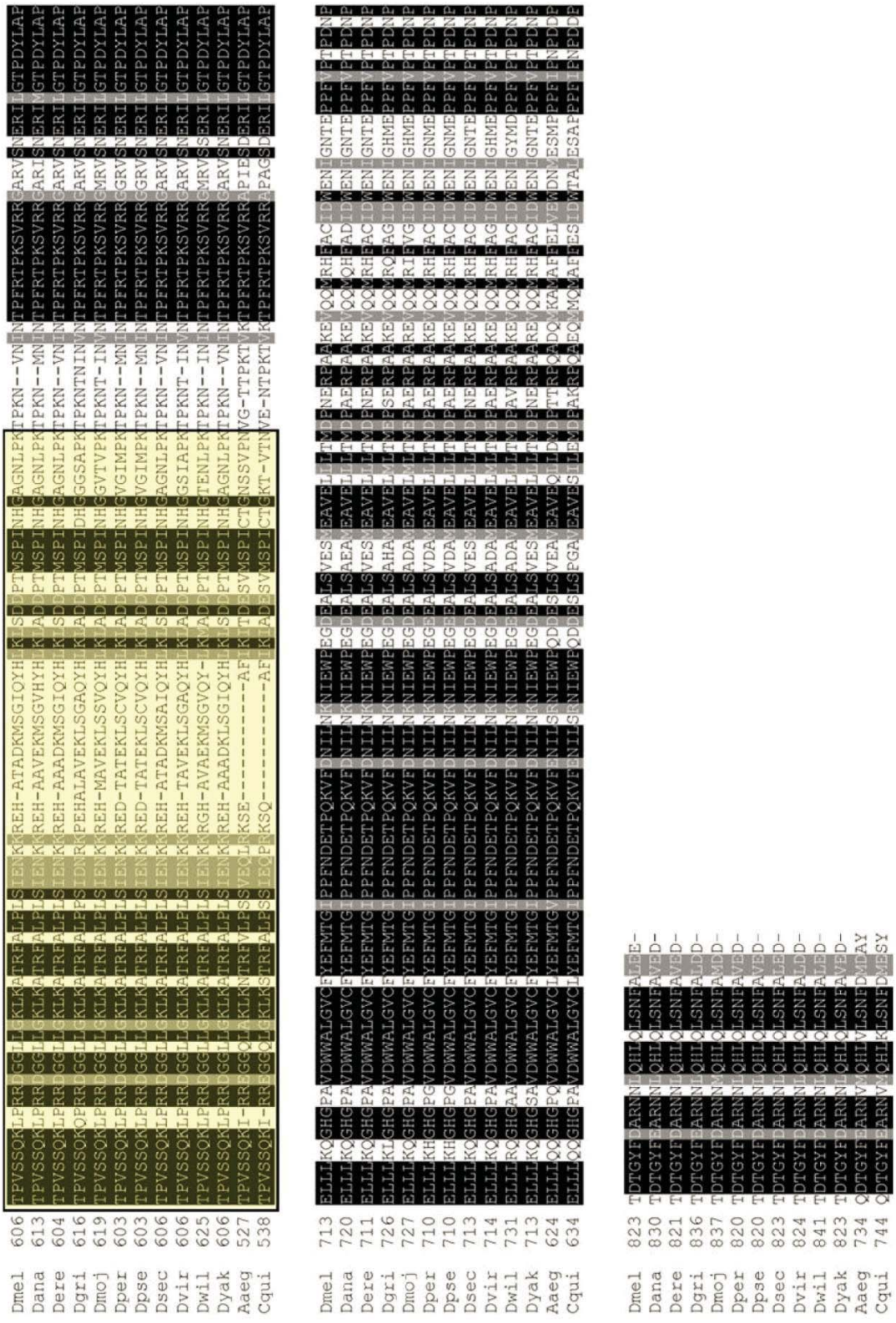


Figure S3.2. Sequence alignment between Gwl orthologs from *Drosophila* and mosquito species. Perfectly conserved residues are in black and residues conserved in charge and

hydrophobicity are in grey. Gwl's central region (in yellow) is poorly conserved, with only 26% sequence identity between these species. However, the part of this region containing the identified NLS1 and NLS2 is better conserved, and the residues making NLS1 and NLS2 (red bars) are perfectly conserved, consistent with their biological function. Residues mutated in this study are marked with green arrowheads. The alignment was done using Clustal Omega and Boxshade 3.21.

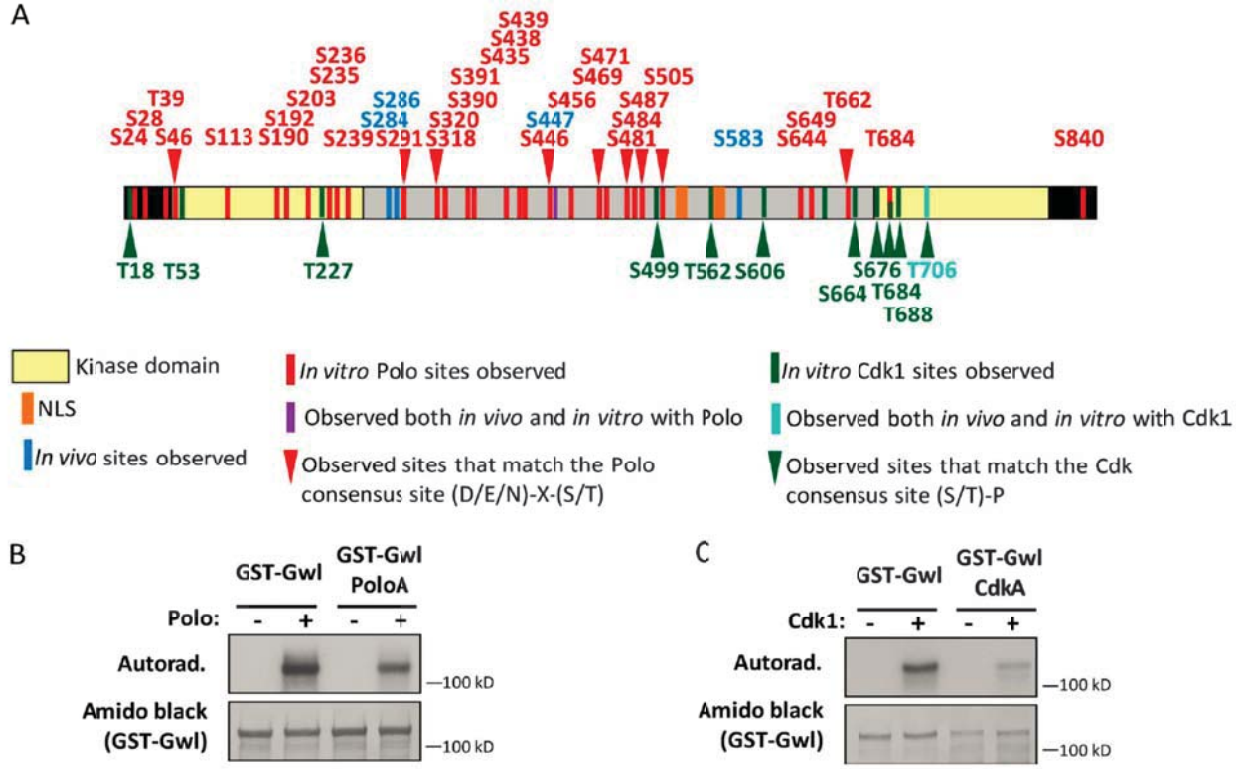


Figure S3.3. Phosphorylation site mapping. *In vitro* Polo and cyclin B-Cdk1 sites were identified following kinase reactions with recombinant Polo or cyclin B-Cdk1 (sites with ≥75% confidence are indicated). *In vivo* sites were identified by mass spectrometry in Gwl-PrA purified from transgenic embryos. Identified phosphoryltion sites that match a Polo or Cdk consensus motif are indicated. Note the high concentration of sites near the NLSs, in the central region of Gwl. **B.** Mutation of all 18 Polo consensus sites (D/E/N)-X-(S/T) between residues 317-661 in Gwl (Gwl-PoloA) reduce its phosphorylation by Polo *in vitro*. **C.** Mutation of all 6 Cdk consensus sites (S/T)-P between residues 349-675 in Gwl (Gwl-CdkA) reduces its phosphorylation by cyclin B-Cdk1 *in vitro*.

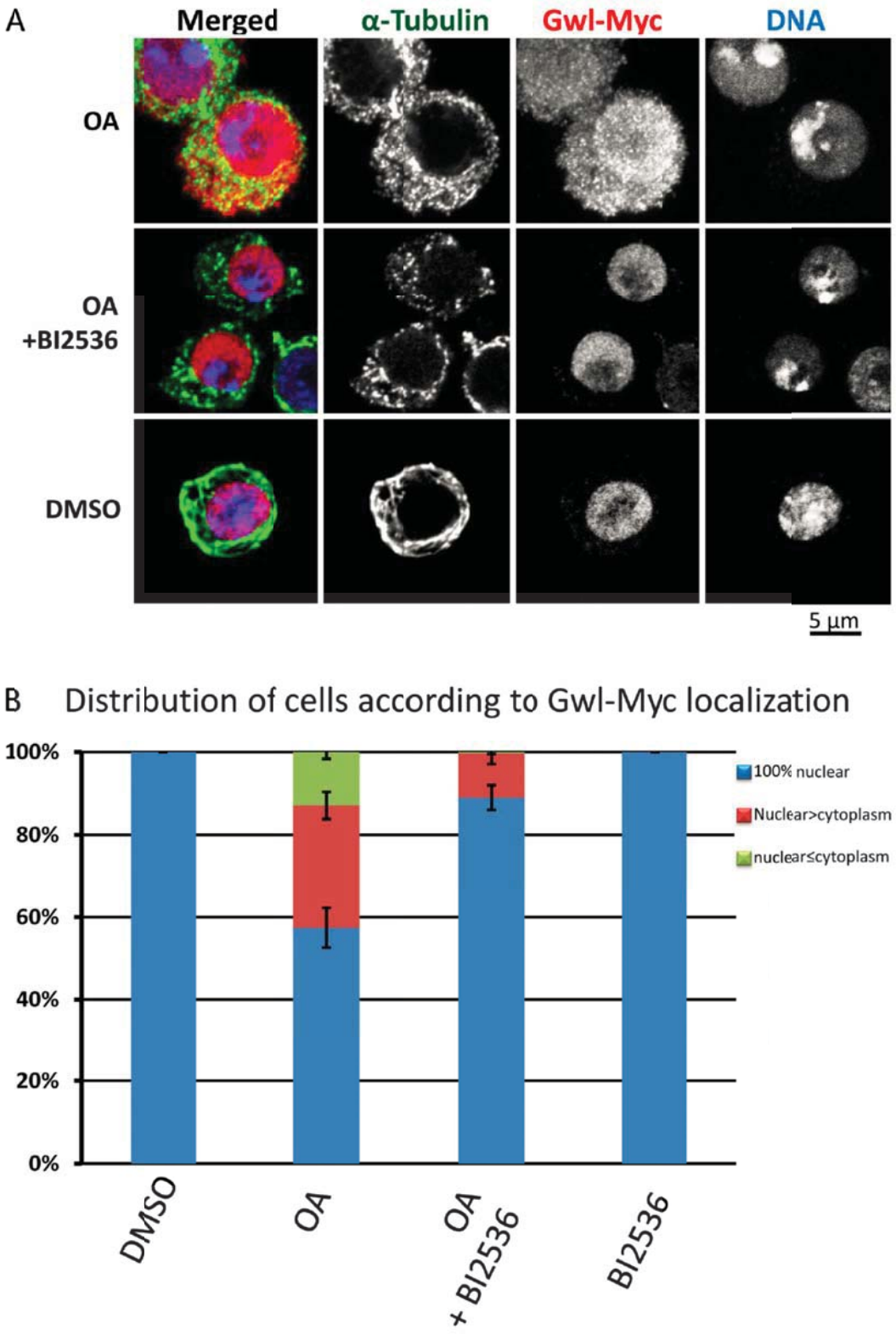


Figure S3.4. Polo phosphorylation of Gwl promotes its cytoplasmic localization (complement to Fig 3.5). A. Cytoplasmic localization of Gwl in response to phosphatase

inhibition depends on Polo activity. Cells were treated with Okadaic acid (100 nM), BI2536 (1 μM) and/or DMSO for 10 min as indicated and examined by immunofluorescence. **B.** Cells were scored according to the localization of Gwl-Myc. Error bars: S.E.M.

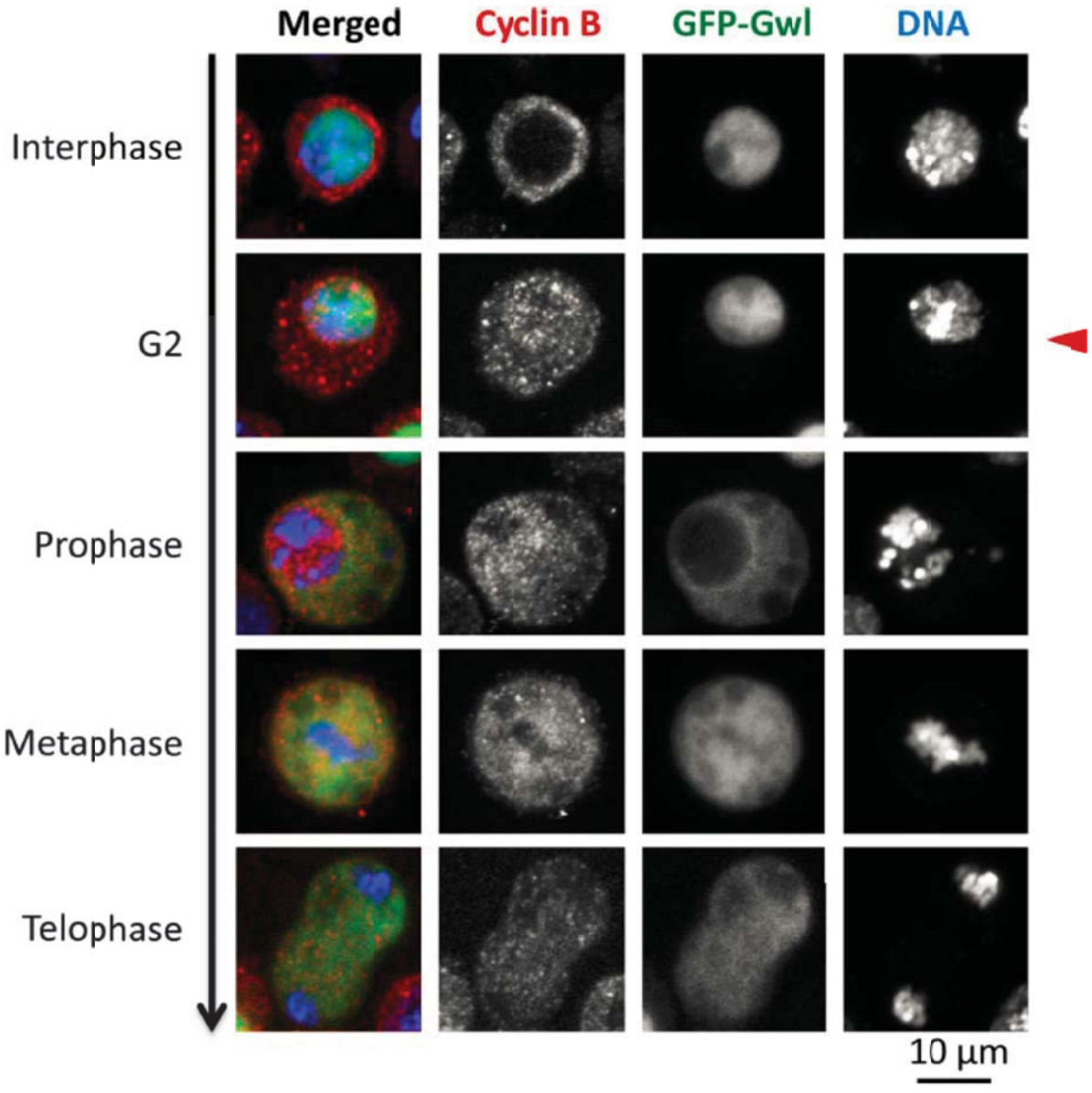


Figure S3.5. Cyclin B is imported in the nucleus while Gwl is still in the nucleus (red arrowhead). Cells were fixed and stained as indicated.

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CHAPTER 4

DISCUSSION AND PERSPECTIVES

4.1 Do PP2A-Tws and Polo function in the same or parallel pathways?

In our laboratory, we have conducted a genetic screen in *Drosophila* embryos to identify genes functioning closely with *polo* and *gwl*. We found that both the catalytic subunit gene (*microtubule star/mts*) of PP2A and its B55 type regulatory subunit gene (*twins/tws*) genetically collaborate with *polo*, but are antagonized by *gwl* [1]. We showed that female flies heterozygous for an amorphic mutant of *polo* ($polo^{11}$) laid embryos where centrosomes detached frequently from nuclei mostly in prophase, whereas females heterozygous for a strong hypomorphic allele of *tws* (tws^{P}) laid embryos where centrosomes detached in telophase [1]. Therefore, we supposed that Polo and PP2A-B55/Tws act in parallel pathways to ensure the cohesion between centrosomes and nuclei. Indeed, although it was demonstrated that both Polo and Tws/B55 are required for centrosome maturation, no common substrate of both proteins at centrosome was reported [2, 3]. Moreover, in *Drosophila* syncytial embryos, centrosome duplication occurs as early as late anaphase and early telophase [4]. Thus, the reduction of PP2A-B55/Tws may impair the earlier centrosome ─ nucleus attachment in ana-telophase, whereas the reduction of Polo may impair this attachment later in prophase. This is also supported by the fact that PP2A-B55/Tws activity is high at mitotic exit and throughout interphase [5], whereas Polo activity increases at G2/M [6]. Therefore, embryos produced by females heterozygous for both $polo^{11}$ and tws^{P} ($+polo^{11}/tws^{P}+$) experienced a dramatical increase in centrosome detachment at all stages of embryonic mitosis probably because the cohesion between centrosome and nucleus has been further weakened during mitotic exit and the next mitotic entry [1]. However, we can't exclude other models. For example, PP2A-B55/Tws could be required for dephosphorylating a certain protein which is important for Polo recruitment at centrosomes. Indeed, such collaboration between Polo and PP2A-B55/Tws has been observed in other contexts. Both Polo and PP2A-B55 target PRC1 (the anti-parallel microtubule-bundling protein), which plays a crucial role in central spindle formation [7-10]. Cyclin B-Cdk1 phosphorylates PRC1 at T481 to prevent both the re-localization of PRC1 to the central spindle and the binding of Plk1 (Polo ortholog in human) to PRC1 in metaphase, whereas PP2A-B55 removes this phosphorylation in anaphase, allowing the recruitment of Plk1 to PRC1 at the central spindle to promote the initiation of cytokinesis [7, 8, 10]. In such case, PP2A-B55 also collaborates with Plk1 and is antagonized by Gwl, where Plk1 appears to be downstream of Gwl and PP2A-B55. However, we later found that Polo promotes

Gwl cytoplasmic retention to mediate PP2A-B55/Tws inhibition before nuclear envelope breakdown (NEBD), suggesting that Polo, Gwl and PP2A-B55/Tws function in the same linear pathway where Polo appears to be the upstream collaborator of Gwl which antagonizes PP2A-B55/Tws [11]. These results collectively indicate that Polo antagonizes PP2A-Tws at mitotic entry, whereas they collaborate with each other at mitotic exit, and there might not be a relationship between these two interplays. This is also supported by the fact that Polo is required for both mitotic entry and mitotic exit [12]. Therefore, the nature of the interplay between Polo and B55/Tws may vary with specific stage and specific event of mitosis. Likewise, the collaboration between Polo and B55/Tws at centrosome ─ nucleus cohesion should differ from their interaction at mitotic entry. However, the collaboration between Polo and Tws/B55 for centrosome attachment appears to be their primary interaction since the centrosome detachment from nucleus is the prominent phenotype observed in embryos laid by +$polo^{11}$/tws^{P}+ females.

In addition to the question we asked above, the other question is how Polo and PP2A-Tws/B55 activities are required for centrosome ─ nucleus cohesion? We pursued the answer by performing both a genetic screen for genes that function with *polo* and *tws* and a proteomic identification of Polo and Tws interacting partners in embryos, and we did obtain some candidates which might regulate the centrosome ─ nucleus attachment. For example, we identified that Polo functions with NudE (unpublished data), a protein which was demonstrated to play an important role in the regulation of dynein, which is required for nuclear attachment of centrosome during mitosis in *Drosophila* [13, 14]. We also found that Spd2, which is essential for centrosome biogenesis and maturation, associates with B55/Tws in the same complex (unpublished data) [15-17]. Further genetic and molecular dissection of these interactions may clarify the precise function of Polo and PP2A-B55/Tws in regulation of centrosome ─ nucleus connection.

4.2 Do Gwl and Polo collaborate with or antagonize each other?

The *Scant* allele of Gwl encodes a hyperactive form of Gwl, and when it is combined with heterozygosity for a null *polo* allele ($polo^{11}$), it can induce complete sterility in *Drosophila* females (+ *Scant*/$polo^{11}$ +) accompanied by a dramatic increase in the level of centrosome detachment from nuclei in syncytial embryos [18]. Interestingly, three mutations, which can suppress the +*Scant*/$polo^{11}$+ phenotype, respectively correspond to *polo* duplications, revertants

of *Scant* to its recessive alleles, and a third-site (later identified as *endos*) [18, 19]. These data suggest that Gwl genetically antagonizes Polo, and this antagonistic interaction might be indirect since the inactivation of one copy of *endos* (*endos*^{00003}), which encodes the sole known substrate of Gwl and the sole known PP2A-B55 specific *in vivo* inhibitor in *Drosophila* [19-21], suppressed the + *Scant*/*polo*^{11} + phenotype. Moreover, no evidence showing that in syncytial embryos, Endos acts on Polo and vice versa has been reported. Thus, I speculated that Gwl could function as an antagonist of Polo by antagonizeing Polo's potent collaborator PP2A-B55 through Endos. In addition, Gwl was observed to antagonize Polo only in mitosis, and Polo was found to collaborate with B55/Tws only in mitosis, whereas Gwl antagonizes B55/Tws in both meiosis and mitosis [1, 18]. Thus, increasing Gwl activity alone may induce some inhibition of PP2A-B55/Tws that is not strong enough to affect cell cycle progression. However, when the increase of Gwl is in combination with the reduction of Polo, which is a potent mitotic-collaborator of B55/Tws, mitotic defects characterized by centrosome detachment would be dramatically enhanced leading to embryonic lethality. Further identification of Polo and PP2A-B55 substrates at centrosome should lead to a better understanding of the antagonism between Gwl and Polo and the collaboration between Polo and PP2A-B55.

The antagonism between Gwl and Polo described above was further perplexed when I found that maternal germline over-expression of Polo in female flies heterozygous for a *gwl* null allele (*gwl*^{6a}) led to a dramatic decrease in embryos hatching, while females over-expressing Polo alone led to only a moderate defect and *gwl*^{6a}/+ females led to no defect on embryo hatching [11]. Thus, the results of this genetic test suggest that Polo somehow antagonizes Gwl, complicating matters. Moreover, *matrimony* (*mtrm*), which was demonstrated to encode an inhibitor of Polo, is a strong enhancer of *endos* [22, 23]. Females heterozygous for a *mtrm* null allele and an *endos* null allele (+ *mtrm*^{126}/*endos*^{00003} +) are sterile because of defects in early embryonic mitosis, and this phenotype can be rescued by the removal of one copy of *polo* [23]. This result further suggests that elevated Polo activity antagonizes the Gwl-Endos pathway. I argued that elevated Polo activity might lead to premature cytoplasmic retention of Gwl even before Gwl is activated by Cdk1 in the nucleus. Indeed, I found that in embryos expressing half the normal dose of Gwl and over-expressing Polo, there was a significant fraction of nuclei that failed to enter into mitosis when most of nuclei were already in prometa-/metaphase [11]. Such phenotype might be due to the lack of sufficient activity of Gwl. I later found that removal of

half of B55/Tws in females over-expressing Polo and expressing half the normal dose of Gwl efficiently rescued embryo hatching (unpublished data). This result suggests that the antagonist relationship between Gwl/Endos and Polo relies on the PP2A-Tws/B55 activity. Moreover, through modification of Polo, Gwl/Endos and PP2A-Tws/B55 expression levels, it was demonstrated in various ways that the balance between these proteins is crucial for both meiotic and mitotic progression in *Drosophila* embryos. As most of these genetic tests were reviewed by Dr. David M. Glover [24], I won't show them here in detail. Thus far, several genetic results suggest that Polo and Gwl antagonize each other, but these antagonistic relationships were observed only when we artificially increased one kinase activity and simultaneously decreased the other kinase activity in *Drosophila* syncytial embryos. These genetic tests did greatly help us to uncover new interacting partners and understand better interacting genes functions; however they may not exactly reflect their physiological relationships.

In contrast to genetically antagonist interplay between Gwl and Polo discovered in flies, several data support that they collaborate for mitotic progression. First, Gwl contributes to the activation of Cdc25 through inhibition of PP2A-B55 [25, 26], while Polo activates Cdc25 by direct phosphorylation [27-29]. Therefore, they collaborate to promote MPF auto-amplification loop by activating Cdc25. Moreover, Polo associates with and phosphorylates Gwl *in vitro* both in *Drosophila* and in *Xenopus*, whereas Gwl does not phosphorylate Polo *in vitro* [11, 30, 31]. Moreover, phosphorylation of Gwl by Polo has no significant effect on Gwl activity [30, 32]. However, as described above, we found that Polo phosphorylates Gwl likely to promote the cytoplasmic retention of Gwl, which subsequently mediates the inhibition of PP2A-B55 [11]. These results collectively indicate that Polo and Gwl function together to promote mitotic progression. In addition, interdependency between Polo and Gwl has been delineated during recovery from G2/M DNA damage checkpoint arrest [31]. During checkpoint recovery, Polo pre-phosphorylated Gwl was observed to promote the dephosphorylation of Smc1 (structural maintenance of chromosomes), which is a component of the cohesin complex, thus promoting sister chromatid separation [31, 33]. Gwl did not phosphorylate Polo during checkpoint recovery, but promotes Polo activation by Aurora A kinase [31]. Therefore, Gwl and Polo depend on each other for recovery from DNA damage checkpoint arrest. In yeast, Polo kinase (Cdc5), Gwl kinase (Rim15) and Zds1 were identified as suppressors of growth defects of *kog1-105* (a mutant of KOG1 which is an essential component of TORC1 (Tor complex 1)) at non-permissive

temperature [34]. Interestingly, *kog1-105* prevents the mitotic entry at non-permissive temperature, whereas overexpression of either Polo or Rim15 or Zds1 rescued mitotic entry, suggesting a collaborating relationship between yeast Polo and Gwl at mitotic entry [34]. Moreover, both Rim15 and Zds1 were demonstrated to be important regulators of spatial localization and activity of yeast PP2A-B55 (PP2A-Cdc55) [35-38]. Thus, there might also be an interaction between Polo kinase and PP2A-B55 in yeast. Indeed, unlike animal PP2A-B55, yeast PP2A-Cdc55 does play a positive role at mitotic entry [39]. It would be interesting to further investigate the possible interaction between Polo and PP2A-B55 in yeast. Altogether, these results indicate that Polo and Gwl collaborate with each other to promote mitotic progression across different species.

4.3 How important is Gwl for mitotic progression?

First studies conducted in *Xenopus* egg extracts demonstrated that the Gwl depleted cycling extracts never enter mitosis [25, 30]. Later, in starfish oocyte and *Xenopus* oocyte, Gwl as well as cyclin B-Cdk1 were proposed to be two essential constituents of MPF (M phase-promoting-factor) due to the fact that when immature oocyte lacking of Gwl was injected with fully activated cyclin B-Cdk1 alone, the ooctye was unable to enter M phase, whereas the injection of both fully activated cyclin B-Cdk1 and Gwl into immature oocyte induced M phase entry [40]. In addition, several independent works demonstrated that human Gwl (Mastl: microtubule associated serine/threonine kinase like) is required for faithful mitotic progression, suggesting that Gwl may be a highly relevant target for cancer therapy [41-43]. Indeed, the group of Dr. Marcos Malumbres recently obtained promising results in tumor regression by targeting Gwl in mouse (scientific communication in CSH Cell Cycle Meeting, 2014). However, without cyclin B-Cdk1, Gwl alone can never induce mitosis, whereas Gwl depletion can be rescued by either constitutively active cyclin B-Cdk1 or by inhibiting PP2A-B55 [5, 25, 26, 30]. Therefore, the control of mitotic entry and maintenance is indeed a war between cyclin B-Cdk1 and PP2A-B55, while Gwl is rather an assistant of cyclin B-Cdk1 that functions to inhibit PP2A-B55.

As an assistant of cyclin B-Cdk1, Gwl is not always essential in all tissues or all organisms. In *Drosophila*, several combinations of *gwl* loss-of-function mutant alleles caused the lethality at late larvae or pupae stage. Nonetheless, when larvae carrying any one of these combinations of *gwl* loss-of-function mutant alleles are simultaneously heterozygous for a loss-

of-function allele *tws*^{P} (the single B55 in flies), the viability was completely rescued [44]. In other words, removal of only half of Tws/B55 can compensate for the complete loss of Gwl, suggesting that Gwl is dispensable if the ratio of cyclin B-Cdk1 activity relative to PP2A-B55/Tws activity is high enough. This is further supported by the observation that larvae completely lacking of *endos*, but heterozygous for *tws*^{P} also developed into normal adults [44].

Compared to *Drosophila,* surprisingly, no obvious orthologs of Gwl in nematode *C.elegans* were identified, thus raising the question of how worms can progress successfully through mitosis without Gwl [30, 44]. In section 1.5, I described that cyclin B-Cdk1 might be able to prevent PP2A-B55 holoenzyme assembly by directly phosphorylating B55 [45]. Moreover, PP2A-B55 holoenzyme assembly is also regulated by cell cycle dependent methylation of the catalytic subunit [46, 47]. These mechanisms might provide an additional level of regulation of PP2A-B55 activity without the requirement for Gwl. Additionally, PP2A-B55 level in *C.elegans* cells is so low that its Cdk1-counteracting effect was assumed to be less important [44]. Indeed, RNAi of Endos did not induce any clear effect in somatic and germline cell divisions in *C.elegans* [44].

Unlike in *C.elegans*, Gwl does exist in starfish oocyte. However, cyclin B-Cdk1 can bypass Gwl and directly phosphorylate Arpp19 (the single homolog of Endos/Arpp19 in starfish) [48]. Cylin B-Cdk1 phosphorylates Endos at serine 69 to mediate the initial PP2A-B55 inhibition, leading to cyclin B-Cdk1 autoregulatory activation at mitotic entry, whereas Gwl phosphorylates Arpp19 at another conserved site to ensure downstream mitotic progression [48]. However, this Cdk1 site was not found to be conserved in *Drosophila* Endos (the single homolog of Endos/Arpp19 in flies) [48]. Although it was suggested that this Cdk1 site on *Drosophila* Endos corresponds to a phosphomimetic mutation of serine/threonine on aspartic acid [48], the simultaneous depletion of both Endos and Gwl or the depletion of either protein alone in *Drosophila* tissue culture cells, induced similar chromosome condensation and nuclear envelope breakdown defects [19]. This result indicated that at least in *Drosophila* tissue culture cells, cyclin B-Cdk1 is unlikely to bypass Gwl and to acts directly on Endos to induce mitotic entry.

In addition to the importance of Gwl function in organisms described above, Gwl doesn't appear to be required for mitotic entry in mammal cells, even though it is needed for faithful mitotic progression [41, 42, 49]. Moreover, despite the fact that yeast Gwl (Rim15) ─ Endos

(Igo1/Igo2) pathway also contributes to mitotic progression by regulating PP2A-Cdc55(B55 in yeast) [37], their contribution is minor compared to the Zds1/Zds2 pathway [50].

However, in contrast to organisms described above, why Gwl depletion prevents mitotic entry in *Xenopus* cycling extracts? One reason could be that in *Xenopus* egg extracts, Gwl is required for triggering the initial step in phosphorylation and activation of Cdc25 in a manner independent of MPF (M-phase promoting factor), whereas only later M phase phosphorylation of Cdc25 depends on MPF [25]. Nevertheless, thus far, there is no evidence showing that such Gwl-dependent and MPF-independent early Cdc25 activation is conserved in other organisms. Moreover, in contrast to organisms that don't need Gwl for mitotic entry, in immature *Xenopus* oocyte, only a small fraction of Gwl localizes in the nucleus, raising the possibility that Gwl-dependent Cdc25 activation is relevant to such particular Gwl distribution [40]. Thus, it will be interesting to study the function of *Xenopus* Gwl cytoplasmic localization by forcing it to localize completely in nucleus.

In conclusion, although Gwl is demonstrated to play an essential role in protecting the Cdk1 phosphorylated mitotic substrates by inhibiting PP2A-B55, the importance of Gwl is variable in different tissues and organisms.

4.4 Spatiotemporal regulation of kinases and phosphatases is required for mitotic progression.

Cell cycle progression requires the regulation of both kinases and phosphatases. CyclinB-Cdk1 is a principal kinase phosphorylating a large number of substrates to trigger mitotic entry. In metazoans, it is counterbalanced by a major regulator, the protein phosphatase 2A (PP2A)-B55 [5, 26]. Greatwall (Gwl) kinase activation at mitotic entry leads to the inactivation of PP2A-B55 to promote the phosphorylation of cyclin B-Cdk1 substrates [5, 26, 43]. The activation Gwl depends on its phosphorylation by cyclin B-Cdk1 and on its auto-phosphorylation [32], whereas its inactivation at mitotic exit depends on PP2A-B55 [51, 52]. However, at mitotic entry, how Gwl itself escapes from PP2A-B55 activity to be activated by cyclin B-Cdk1 and then protects other cyclin B-Cdk1 substrates is still less understood? I therefore investigated the spatiotemporal localization of these regulators. First, in accordance with previous studies, I found that Tws, which is the sole member of B55 regulatory subunit family in *Drosophila*,

localizes predominantly in cytoplasm until NEBD (unpublished data) [53, 54]. Then, we demonstrated that the sub-cellular localization of Gwl changes dramatically during the cell cycle. Gwl is nuclear in interphase, and delocalizes rapidly to the cytoplasm in prophase, a few minutes only before nuclear envelope breakdown (NEBD) [11]. Interestingly, I found that cyclin B enters the nucleus before that Gwl delocalizes into cytoplasm [11]. Indeed, upon mitotic entry, initial low cyclin B-Cdk1 activity is sufficient to trigger its own nuclear import and rapid nuclear accumulation [55, 56]. Therefore, I proposed that the nuclear envelope may serve as an intrinsic barrier to separate Gwl from PP2A-B55, thus promoting Gwl activation by cyclin B-Cdk1 in the nucleus. Although one recent work suggests that an unknown phosphatase other than PP2A-B55 dephosphorylates and inactivates Gwl [57], Gwl nuclear localization could still facilitate its activation by cyclin B-Cdk1 or might also segregate Gwl from its unidentified inactivating phosphatase in the cytoplasm. In accordance with my supposition, it was recently shown that active Gwl was first detected in the nucleus [52]. Moreover, Gwl NLS mutant, which localizes constantly in the cytoplasm, appears to be less phosphorylated than wild type Gwl in *Drosophila* ovaries [11]. Most importantly, our results showed that Gwl nuclear localization is necessary for its function [11].

In addition to Gwl, in *Drosophila*, the nuclear envelope also separates other cyclin B-Cdk1 nuclear substrates from PP2A-B55. Thus, this spatial segregation may also allow cyclin B-Cdk1 to efficiently phosphorylate its nuclear substrates without PP2A-B55 disturbance until NEBD. This might explain why Gwl depletion did not prevent mitotic entry in *Drosophila* cultured cells as well as in many other organisms (e.g. human cells) [44, 49]. However, this spatial segregation can't explain why Gwl depleted *Drosophila* cultured cells and human cells displayed chromosome condensation and NEBD defects [44, 58]. Recently, I found that PP2A-B55 contains a functional and conserved NLS (nuclear localization signal), despite that PP2A-B55 is predominantly cytoplasmic and is never concentrated in nucleus during unperturbed cell cycle progression (unpublished data). This suggests that PP2A-B55 may be a nucleocytoplamic shuttling protein. Therefore, in Gwl depletion cells, instantaneous nuclear localization of PP2A-B55 might be sufficient for disturbing cyclin B-Cdk1 activity and thus delaying chromosome condensation and NEBD. Moreover, it was demonstrated that cyclin B-Cdk1 nuclear localization promotes cyclin B phosphorylation and further cyclin B-Cdk1 nuclear localization [59]. PP2A-B55 was supposed to be the phosphatase which dephosphorylates cyclin B, thus preventing

cyclin B nuclear localization [59]. Therefore, in Gwl depleted cells, PP2A-B55 may slow down cyclin B nuclear accumulation leading to delayed chromosome condensation and NEBD. Interestingly, in Gwl depleted mouse cells, there were less Cdk1 phosphorylated substrates in the nucleus compared with that in wild-type mouse cells [49], which might be due to the decrease in the nuclear fraction of cyclin B-Cdk1. Thus, it will be interesting to investigate the nuclear accumulation velocity of cyclin B in Gwl depleted cells.

If Gwl nuclear localization is required for its activation in the nucleus, then why is Gwl sent into cytoplasm before NEBD [11, 49]? Cyclin B-Cdk1 is first activated in the cytoplasm, however, the earliest Cdk1 cytoplasmic substrates, which are required for cytoskeleton reorganization and cell rounding, are thought to be totally exposed to fully active PP2A-B55 [55, 56]. Therefore, I speculated that Gwl is promptly sent into cytoplasm before NEBD in order to protect Cdk1 cytoplasmic substrates against PP2A-B55 activity, thus synchronizing phosphorylation events in both nucleus and cytoplasm. This nuclear export of Gwl before NEBD was indeed demonstrated to be important for its function and mitotic progression [11, 49]. However, there are still some concerns about the localization of the protein Endos. Endos is a small protein of about 20 kilodaltons [20], thus we expect that Endos is capable of diffusing freely through nuclear pores. Indeed, Endos-GFP was demonstrated to localize in both cytoplasm and nucleus in *Drosophila* syncytial embryos and cultured cells [19]. If Gwl phosphorylated Endos can diffuse freely through nuclear pores into cytoplasm to inhibit PP2A-B55, Gwl cytoplasmic translocation might not be important. I have speculated that the passive nucleocytoplasmic transport of Endos might be less efficient than the facilitated nuclear export of Gwl. Immunofluorescence of Gwl phosphorylated Endos with phosphospecific antibody may uncover its nucleocytoplasmic dynamic. In contrast to Endos localization reported in flies, it was recently delineated that in immature starfish oocyte, Endos is predominantly cytoplasmic [48]. I did find that different tags had different effects on Endos localization. For example, Endos tagged with GFP or RFP localized in both cytoplasm and nucleus, whereas Endos tagged with PrA, Myc or Flag principally localized in the cytoplasm (unpublished data). Therefore, previously reported Endos-GFP localization might have not exactly depicted the localization of endogenous Endos, and it will be important to further examine its true localization. If Endos is cytoplasmic, it may explain why Gwl should be exported into cytoplasm before NEBD; however, it will be interesting to investigate what prevent its nuclear diffusion. Recently, Maxime Cormier,

a PhD student in the laboratory, found that Endos can constitutively associate with B55/Tws. As B55/Tws localizes in the cytoplasm, Maxime will continue to investigate if it is B55/Tws which retains Endos in the cytoplasm.

In addition, our studies had uncovered some regulatory mechanisms of Gwl spatial localization. We identified two nuclear localization signals (NLS1 and NLS2, respectively) in Gwl uncharacterized long insert region [11]. Phosphorylation by Polo and Cdk1 in this region of Gwl promotes its cytoplasmic localization in prophase before NEBD [11]. Phosphorylation by Polo likely promotes the association of Gwl with 14-3-3ε leading to its cytoplasmic retention, whereas Cdk1 regulates Gwl localization through an unknown mechanism [11]. However, human Gwl nuclear export in prophase has been shown to depend on its own kinase activity and its activation by cyclinB-Cdk1 [49]. Moreover, the existence of nuclear export signals (NES) in both *Drosophila* and human Gwl were suggested but yet to be further characterized [11, 49]. During my thesis redaction, I further investigated the role of Cdk1 and PP2A-B55/Tws in regulation of Gwl spatial localization. However, as some essential results are still preliminary and some important experiments should be repeated with higher resolution imaging, I decided to just describe them briefly. I found that Cdk1 can phosphorylate Gwl at threonine 562 leading to the NLS2 inactivation. I further demonstrated that B55/Tws can associate with Gwl which in the cytoplasm during cytokinesis. Moreover, Gwl is more phosphorylated and less nuclear in interphase in embryos laid by female heterozygous for a null allele of *tws* (tws^{P}), suggesting that PP2A-B55/Tws may dephosphorylate Gwl to allow its relocalization into the nucleus in late cytokinesis. However, the possibility that PP2A-B55/Tws also targets the Cdk1 site threonine 562 on Gwl should be further investigated.

In conclusion, mitotic entry and progression depends on not only the essential kinase and its counteracting phosphatase activity ratio but also on their spatiotemporal localizations. Almost all mitotic kinases and phosphatases (e.g. Polo kinase, Aurora kinases, Cdc25 phosphatase, Wee1 phosphatase, cyclin B-Cdk1 kinase, Gwl kinase, and PP2A-B55 phosphatase) described in this thesis are spatially regulated. Study of individual enzyme localization may help to better understand its functions; however, the elucidation of whole regulatory mechanism of mitotic entry and progression greatly relies on the understanding of chronological and subcellular localization of all enzymes involved in this network.

4.5 The regulation of Gwl, Endos/Arpp19, and PP2A-B55 at mitotic exit

It is still controversial which phosphatases are essential for the dephosphorylation of the bulk of Cdk1 mitotic substrates at mitotic exit. It could be protein phosphatase PP1 [5, 60, 61], or PP2A [26, 41, 45, 62-64], both [8, 43, 57], or neither [52]. There are also many discrepancies between the proposed models for Gwl and Endos inactivation and PP2A-B55 reactivation at mitotic exit. Here I will not argue which phosphatase is essential for mitotic exit or which proposed regulatory model of Gwl, Endos/Arpp19 and PP2A-B55 at mitotic exit is correct; I will only discuss my presumption based on previous works performed by different laboratories. In Gwl depleted *Xenopus* CSF extracts, it has been demonstrated that addition of Gwl pre-phosphorylated Endos/Arpp19 can't rescue the mitotic state, as Endos/Arpp19 are rapidly dephosphorylated [20]. However, addition of active Gwl or Gwl thio-phosphorylated Endos/Arpp19 was able to rescue the mitotic state [20, 21, 30]. Moreover, the depletion of Arpp19 from CSF extracts led to a rapid dephosphorylation of Gwl and mitotic exit due to the reactivation of PP2A [20]. In addition, it was demonstrated that phosphorylation of Gwl-specific site on Endos decreases simultaneously with dephosphorylation of Gwl at mitotic exit [20]. These results collectively suggest several characteristics of phosphatases that dephosphorylate Gwl/Endos at mitotic exit. First, an anti-Endos phosphatase competes with Gwl, but the phosphorylation of Endos by Gwl is more rapid than the dephosphorylation of Endos by the anti-Endos phosphatase, and this phosphatase does not inactivate Gwl. Second, the putative anti-Gwl phosphatase follows the activity of PP2A-B55. Third, the anti-Gwl phosphatase and anti-Endos phosphatase are not the same, but function at the same time at mitotic exit. Indeed, two recent works demonstrated that either PP2A-B55 dephosphorylates Gwl and Fcp1 dephosphorylates Endos [52] or PP2A-B55 dephosphorylates Endos and an unknown phosphastase dephosphorylates Gwl [57]. Previously, some evidences did support that PP2A-B55 dephosphorylates Gwl. First, in *Xenopus* egg extracts, both $PP2A_D$ and B55 are able to associate with Gwl in the same complex [51, 64]. Moreover, the PP2A catalytic subunit dephosphorylates and inactivates Gwl *in vitro* [51]. However, if PP2A-B55 dephosphorylates Gwl, then how could this be initiated when PP2A-B55 is being inhibited by active Gwl/Endos? I speculated that Gwl phosphorylated Endos might induce an incomplete inhibiton of PP2A-B55, thus after cyclin B-Cdk1 inactivation, partially active PP2A-B55 removes Gwl phosphorylation which is unable to be replaced by Cdk1. However, besides the fact that complete suppression of PP2A-B55 activity

appears to be not necessary for mitotic progression in flies [44], there is no other evidence supporting my hypothesis. I do not think that Endos is inactivated first since Gwl appears more potent than the anti-Endos phosphatase. I can't argue if the anti-Endos phosphatase is Fcp1, because its regulation in mitosis is not known at all. The only known information about Fcp1 is that it is a nuclear phosphatase, playing a role in the initiation of transcription by targeting the C-terminal domain of the largest subunit of RNA polymerase II (Pol II) [65]. It was suggested that Fcp1 mediates Cdk1 inactivation and promotes the action of other phosphatase at mitotic exit in human cells [66]. Thus, I speculate that Fcp1 should be a strong antagonist of Gwl since it inactivates both Gwl activator and Gwl substrate. It might be interesting to investigate further the potential inhibition of Gwl by Fcp1. However, the regulation of Gwl/Endos by PP2A-B55 and Fcp1 is strongly opposed by another study, which demonstrated that Endos regulates PP2A-B55 through an unfair competition mechanism [57]. It indicates that Gwl-phosphorylated Endos binds tightly PP2A-B55 and blocks the PP2A active site, while PP2A-B55 has a constitutive anti-Endos activity, which is however less efficient compared with its anti-Cdk1 activity [57]. Therefore, during M phase, Gwl-phosphorylated Endos hinders completely PP2A-B55 activity from dephosphorylating Cdk1 substrates, whereas at mitotic exit, when Gwl is inactivated, PP2A-B55 dephosphorylated Endos can't be replaced, thus PP2A-B55 can efficiently dephosphorylate Cdk1 substrates [57]. However, this work suggests that Gwl should be inactivated before Endos by an unknown phosphatase other than PP2A, a fact that challenges several previous works [20, 51, 52, 57]. Altogether, it is largely accepted that both Gwl and Endos should be inactivated leading to the reactivation of PP2A-B55, and such regulation has been repeatedly shown crucial for mitotic exit. However, the actual regulatory modes of Gwl, Endos and PP2A-B55 at mitotic exit remain ambiguous and controversial. Additional studies are required for a better elucidation of their regulatory mechanisms at mitotic exit. Finally, according to all the data described above, I proposed a model for the spatiotemporal regulation of the Gwl ─ PP2A-B55/Tws axis in the control of mitotic entry and exit (figure 4.1).

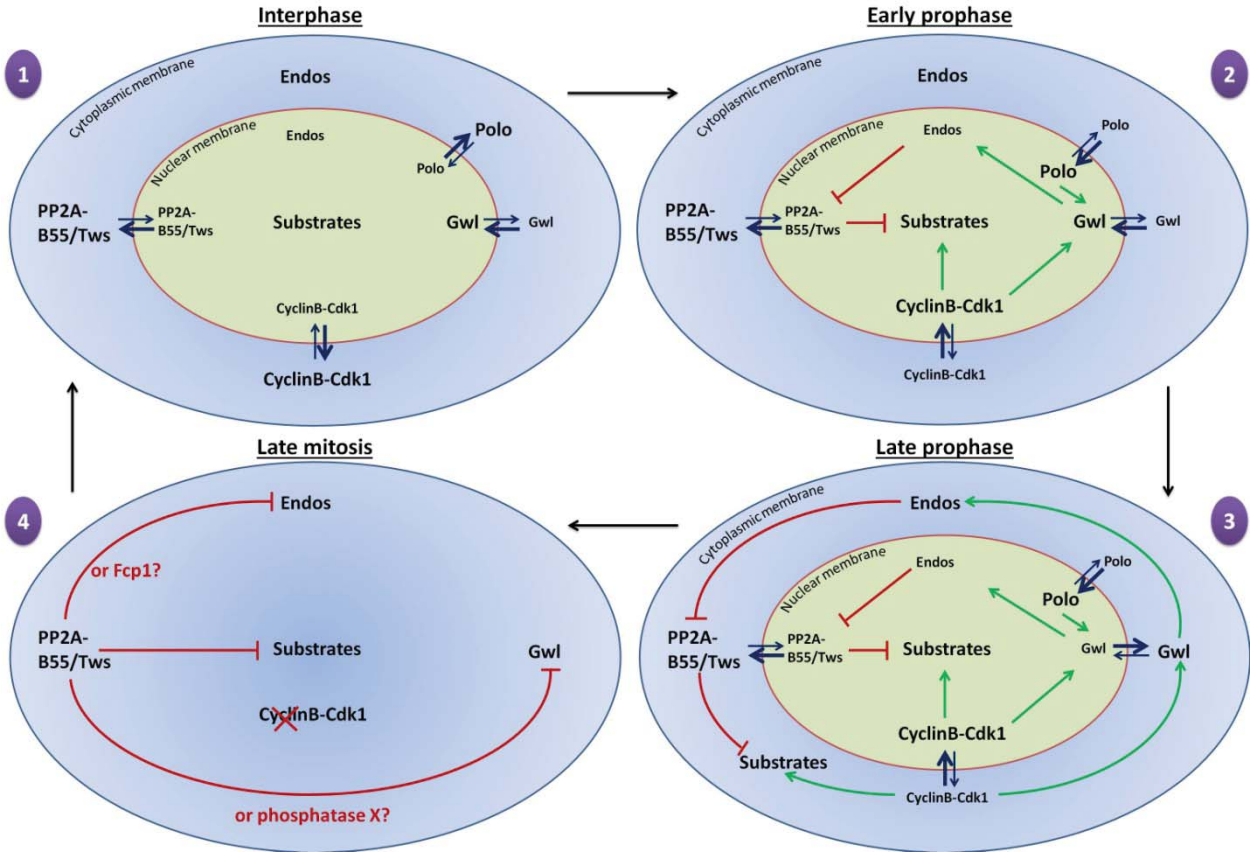

Figure 4.1. A model for spatiotemporal regulation of the Gwl-PP2A axis in the control of mitotic entry and exit. The relative rate of nuclear import and export of each protein are represented by arrows of varying thickness. In interphase, cyclin B-Cdk1 is inactive, while PP2A-B55/Tws is active. Cyclin B-Cdk1, Endos, Polo, and PP2A-B55 are mostly cytoplasmic, while Gwl is predominantly nuclear (1). In early prophase, cyclin B-Cdk1 begins to accumulate in the nucleus and becomes active through the auto-amplification loop (not shown). In the nucleus, cyclin B-Cdk1 activates Gwl which mediates the inhibiton of nuclear PP2A-B55/Tws by phosphorylating Endos (2). Phosphorylation of Gwl by both Polo and cyclin B-Cdk1 promotes Gwl cytoplasmic relocalization before NEBD leading to cytoplasmic PP2A-B55/Tws inhibition (3). Thus, the activity of cyclin B-Cdk1/PP2A-B55 ratio is high throughout the cell before nuclear envelope breakdown (NEBD). After cyclin B degradation and Gwl inactivation, PP2A-B55/Tws inactivates Endos and then dephosphorylates several Cdk1 substrates leading to mitotic exit (4). Dephosphorylation of Gwl by PP2A-B55/Tws may contribute to Gwl nuclear relocalizaiton in late mitosis, whereas the inactivation of Gwl may depend on an unindentified phosphatase 'X' other than PP2A-B55.

4.6 Perspectives

As described above, I found that cyclin B-Cdk1 and PP2A-B55 may have opposite effects on Gwl localization. I demonstrated that cyclin B-Cdk1 can phosphorylate Gwl at threonine 562 leading to the NLS2 (nuclear localization signal) inactivation. However, the fact that PP2A-B55/Tws might also target Cdk1 site ─ threonine 562 on Gwl should be further investigated. I demonstrated that the suppression of B55/Tws by RNAi in *Drosophila* cultured D-MEL cells leads to a significant delay of Gwl nuclear relocalization at mitotic exit. I supposed that this delay is partially due to NLS2 reactivation failure, as that the phosphorylated T562 residue of Gwl can't be dephosphorylated in B55/Tws depleted cells. Thus the Gwl T562A mutation, which is supposed to induce a constitutively active NLS2, should significantly rescue the delay of nuclear relocalization of Gwl at mitotic exit in B55/Tws depleted cells. We are currently optimising time-lapse microscopy conditions for this experiment.

Moreover, as described above, I found that B55/Tws can abnormally accumulate in the nucleus. I will check the significance of the localization of B55/Tws by forcing it nuclear localization. I will test which types of stimulus can trigger B55/Tws nuclear accumulation. For this test, I am preparing different assays such as DNA damage, laser radiation, and B55/Tws interacting viral protein E4orf4 transfection. If I identify stimuli that trigger B55/Tws nuclear localization, I will continue to work on the signaling pathway involved in the regulation of B55/Tws localization and the function of this change of localization.

4.7 References

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