

Fluorescent Imaging of Cell Division

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### **Declaration**

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## **List of Publications**

Cell division requires a direct link between microtubule-bound RacGAP and Anillin in the contractile ring.

Research Paper

Stephen L Gregory, **Saman Ebrahimi**, Joanne Milverton, Whitney M. Jones,

Amy Bejsovec, and Robert Saint

Current Biology 18 (2008) 25-29

Polo kinase interacts with RacGAP50C and is required for the localization of the Cytokinesis Initiation Complex.

Research Paper

**Saman Ebrahimi**, Hamilton Fraval, Michael Murray, Robert Saint and Stephen L.

Gregory

Submitted Manuscript

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**Abstract:**

Cytokinesis is the final stage of cell division that divides a cell into two. It requires the coordinated assembly and constriction of an Actin and Myosin based contractile ring in anaphase and telophase. The Rho signalling pathway is known to be a key player in regulating the events that lead to the localisation of components of the contractile ring and activation of its constriction. The immediate upstream regulators of RhoA are: the RhoA activator Pebble/ECT2, the Plus-end directed microtubule associated motor protein Pavarotti Kinesin like Protein (Pav-KLP)/MKLP1 and RacGAP50C/MgcRacGAP. At the onset of Cytokinesis, RacGAP50C and Pav-KLP form a complex, termed the centralspindlin complex, on the microtubules. Pebble binds the centralspindlin complex via RacGAP50C forming a complex, here termed the cytokinesis initiation complex. The complex then travels to the cell equator leading to RhoA activation and the subsequent assembly and constriction of the contractile ring.

Two major aims have been the focus of my PhD studies:

1) To investigate the mechanism behind continuous and stable contractile ring constriction after initiation of cytokinesis:

A stable and continuously constricting contractile ring requires the continuous presence of active RhoA. Both microtubules and the centralspindlin complex have been shown to be essential for this process. However, the mechanism by which they ensure this continual activation of RhoA is yet to be elucidated. In a search for potential candidates that would play a role in this process, Fluorescence Resonance Energy Transfer was adapted to the *Drosophila* system and used to detect direct spatio-temporal interactions between components of the contractile ring and the centralspindlin complex. Subsequently confirmed by Yeast Two-hybrid analysis, a direct interaction was identified between

RacGAP50C and Anillin, a RhoA effector that is attached to the Actin-based cell cortex via direct interactions with Actin and Myosin. Live imaging of neuroblast cells lacking Anillin revealed that the contractile ring destabilizes and falls apart after briefly constricting during anaphase. These results suggest that, during constriction, the centralspindlin complex on the microtubules is continuously linked to the Actin-based ring via an interaction with Anillin leading to the continual presence of Pebble and active RhoA. This explains the long known phenomenon that microtubules are required at the cell equator for a continual and stable contractile ring constriction.

2) To investigate the mechanism behind centralspindlin localisation and the subsequent initiation of cytokinesis:

RhoA activation in anaphase is achieved by the localisation of the cytokinesis initiation complex to the equatorial cortex. More recently, Polo Kinase has been found to play an essential role in this process. FRET and Yeast Two-hybrid Analysis revealed a direct interaction between RacGAP50C and Polo Kinase in anaphase leading to the hypothesis that Polo Kinase may be involved in the localisation of RacGAP50C and initiation of contractile ring assembly. To test this hypothesis, anaphase in *Drosophila polo* mutant cells was examined. Immuno-fluorescence of *polo* mutant cells in anaphase revealed that in the absence of Polo Kinase activity, both RacGAP50C and Pav-KLP stall on the microtubules and do not get to the equator where the contractile ring should assemble. This study revealed an essential role for Polo Kinase in the initial localisation of the RhoA activating complex to the cell equator.



# **CHAPTER I**

## **Introduction**

## **1. Background on cell cycle:**

Cell cycle consists of a series of events that lead to the final division of a parent cell into two daughter cells. The stages of cell cycle can be divided into Gap0 (G0), Gap1 (G1), Synthesis (S phase), Gap2 (G2) and mitosis (M). A cell can remain in G0 for a long period of time until it is stimulated by a signal for transition through to the subsequent stages of cell cycle resulting in division (Hutchison and Glover, 1995). During G1, the cell synthesises the proteins required for DNA synthesis in the subsequent stage of S phase. In S phase the DNA content of the cell doubles in amount and this is followed by G2 during which the cell prepares itself for mitosis by synthesising and or activating the required regulatory molecules. Finally, M phase consists of the three subsequent sub-stages of metaphase, anaphase and telophase that lead to the final partition of all the organelles and the genetic material and division of the cell (Hutchison and Glover, 1995).

Transition through cell cycle is orchestrated by groups of regulatory molecules known as Cyclins, Cyclin dependent kinases (Cdks) which can be inhibited by Cyclin dependent kinase inhibitors (CdkIs) and other genes required during cell-cycle (Li and Brooks, 1999, Hochegger et al., 2008). The action of these regulatory molecules allows progress through the three major transitions in cell cycle; G1 to S, G2 to M and metaphase to anaphase. The G1 to S transition is accompanied by DNA synthesis and coordinated by CyclinE-Cdk2. The G2 to M transition is accompanied by nuclear envelope break-down and controlled by CyclinA-Cdk2/Cdk1 while the metaphase to anaphase transition is marked by separation of the sister chromatids and coordinated by CyclinB-Cdk1 (Figure1) (Hochegger et al, 2008).

Proper transition through cell cycle and proper DNA replication and chromosome segregation is monitored by two major checkpoints, the DNA replication checkpoint and the spindle checkpoint (Russel P, 1998). The function of the DNA replication checkpoint is to ensure that

DNA replication is complete and it can trigger cell cycle arrest at the G2 to M transition stage (Boddy and Russell, 2001). The spindle checkpoint ensures the proper segregation of the sister chromatids and equal distribution of the chromosomes to the two separating daughter cells upon entry into anaphase (Molinari, M 2001). Once triggered, these checkpoints lead to the arrest of the cell during which the integrity of the replication or partition of genetic material is checked and ensured. Alternatively, if the cell cannot achieve the integrity of the genome, it undergoes programmed cell death and is eliminated (Pietenpol **and** Stewart, 2002).

**NOTE:**

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**Figure1: Cyclin control of transition through cell cycle.**

The three transition of Gap1 to Synthesis phase, Gap2 to mitosis and metaphase to anaphase are triggered by CyclinE-Cdk2, CyclinA-Cdk1/Cdk2 and CyclinB-Cdk1.

Source: Adapted from Hochegger et al 2008.

## 1.1 Background on Cytokinesis

Cytokinesis is the stage of the cell cycle by which one cell divides into two. It consists of a series of cellular morphological transitions that start in anaphase and end at the completion of telophase (Figure 1.1). It begins with the assembly of an acto-myosin based contractile ring at the cell cortex at the site of cleavage furrow formation. The ring then constricts leading to the formation of an intracellular bridge between the nuclei of the two separating daughter cells called the midbody. Finally, the bridge is cleaved in a process called abscission and the daughter cells separate (Satterwhite and Pollard, 1992).

**Figure 1.1 Overview of cytokinesis:**

**NOTE:**  
This figure is included on page 8  
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- a) Anaphase A: Sister chromatids move to the opposite poles of the cell.
- b) Anaphase B: A contractile actomyosin ring assembles and constricts, leading to the formation of a cleavage furrow.
- c) Late Telophase: The ring has constricted, resulting in the formation of a midbody.

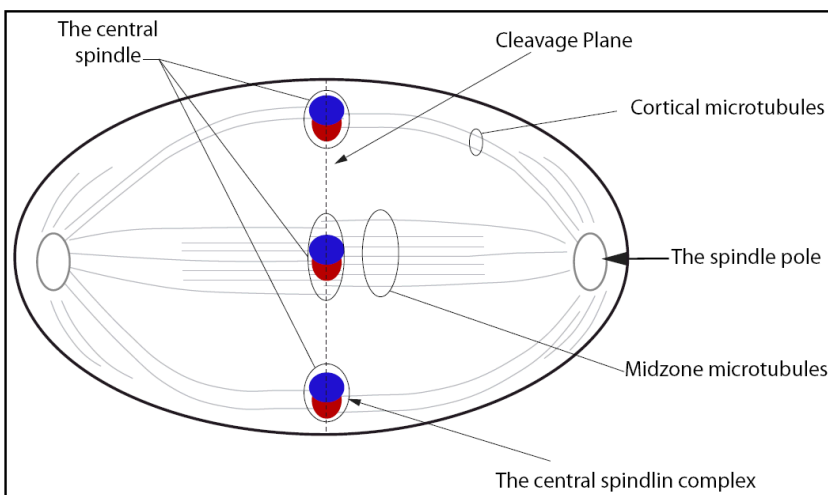
Source: Adapted from Scholey et al 2003.

## 1.2 The events of cytokinesis:

### 1.2.1 Central spindle formation:

The central spindle, which forms during anaphase by reorganization of the mitotic spindle, is a complex of midzone and cortical microtubules that form a dense network of inter-digitating microtubules at the midzone of the cell (Mastrorarde et al., 1993). A microtubule bound protein complex called the centralspindlin complex becomes associated with the inter-digitating plus ends of the central spindle microtubules (Figure 1.2.1) at the time of assembly of the actomyosin contractile ring and condenses as the ring constricts leading to the formation of the midbody (Saxton and McIntosh, 1987). Studies have shown a possible association between the central spindle and the cell cortex leading to the conclusion that the cell-membrane is pulled-in in coordination with central spindle condensation and cytoskeletal rearrangements (Adams et al., 1998, Sisson et al., 2000). In addition, disassembly of the central spindle in late anaphase has been shown to prevent completion of cytokinesis (Wheatley and Wang, 1996). Analysis of a range of *Drosophila* mutants suggested that the proper function of the central spindle is essential for cytokinesis and that the central spindle plays a key role in defining the cleavage plain at the midzone of the cell where the contractile ring assembles (see below) (Gatti et al., 2000).

**Figure 1.2.1 The central spindle**



The central spindle is made up of microtubules that extend between the spindle poles and a protein complex called the central spindlin complex. The cleavage plane is the plane of constriction of the cell at the end of cell division.

### **1.2.2 Cleavage plane specification:**

A range of physical manipulation and genetic approaches have been used to investigate the requirement for different constituents for cytokinesis. Experiments using Echinoderm eggs have shown that physically moving the inter-polar microtubules by poking at the poles so that the position of the midzone of the cell is changed, moves the position of the cleavage furrow, i.e., the furrow disappears upon moving the inter-polar microtubules and reappears where the microtubules reform the midzone (Rappaport, 1978). Consistent with this, Alsop and Zhang (2003) showed that microtubules were essential for cleavage plane specification, while DNA was dispensable (Alsop and Zhang, 2003).

### **1.2.3 Contractile ring assembly and ring constriction:**

The localisation of the central spindle complex to the equator triggers the assembly and constriction of a contractile ring (Somers and Saint, 2003, Zhao and Fang, 2005, Nishimura and Yonemura, 2005, D'Avino et al., 2006). As furrowing progresses, the contractile ring constricts until it reaches the midzone microtubules (figure 1.2.1) at the centre of the cell forming an intracellular bridge (Brill et al., 2000) (Figure 1.1C). It is proposed that the contractile ring is linked to both the membrane and the central spindle resulting in furrow ingression as it constricts drawing the cell membrane in with it (Giansanti et al., 1998).

## **1.3 The Rho small GTPase signalling pathway and Cytokinesis:**

### **1.3.1 Rho small GTPase signalling:**

Rho small GTPase-signalling plays an important role in cellular processes such as cell migration (Raftopoulou and Hall, 2004), adhesion (Malliri and Collard, 2003), proliferation (Debidda et al.,

2005), apoptosis (Dubreuil et al., 2003) and axonal guidance (Yuan et al., 2003). Rho-signalling is also a major player in proper spatial and temporal control of molecular interactions during cytokinesis. Rho signalling has been implicated in the localisation of the contractile ring components and other proteins to the site of cleavage furrow formation, constriction of the ring and invagination of the furrow and abscission (Glotzer, 2001).

Rho proteins act as molecular switches between a GDP (inactive) and GTP-bound (active) form (Bishop and Hall, 2000, Schmidt and Hall, 2002, Moon and Zheng, 2003). They are regulated by guanine nucleotide exchange factors (GEFs), GTPase activating proteins (GAPs) and Guanine nucleotide dissociation inhibitors (GDIs) (Takai et al., 2001). GEFs activate Rho GTPase function by inducing a conformational change that releases GDP, resulting in the uptake of GTP, which is the predominant form of phosphorylated guanosine in the cell. Conversely, GAPs inactivate Rho GTPase by stimulating the intrinsic GTPase activity of Rho-GTPase and converting the bound GTP to GDP. GDIs inhibit activation of Rho GTPases by recruiting RhoGDP from the membrane and keeping it in the cytosol so that it can not be activated by GEFs. However, in the event of an extracellular signal in favour of Rho-signalling, the association between GDIs and Rho GTPases is disrupted, Rho GTPases are recruited to the cell membrane and can then be activated by GEFs resulting in the activation of the Rho-signalling pathway (Figure 1.3.1) (Olofsson, 1999, Antonarakis and Van Aelst, 1998).

### **1.3.2 Rho signalling is required for cytokinesis:**

A role for Rho signalling in cytokinesis was first realized when specific inactivation of RhoA using C3 exo-toxin and injection of a constitutively active dominant negative form of Rho family members led to cytokinetic failure in echinoderm embryos (Mabuchi et al., 1993). Cytokinesis requires the assembly and constriction of an actin and myosin based contractile ring that is made

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**Figure 1.3.1 Regulation of Rho-signalling:**

Guanine nucleotide exchange factors (GEFs) release guanosine diphosphate (GDP) from Rho-GTPases promoting the binding of guanosine triphosphate (GTP) and activation of Rho-GTPases. GDP dissociation inhibitor (GDI) inhibits the dissociation of GDP from Rho-GTPases and thus prevents the association of GDP-GTPases to the cell membrane. GTPase activating proteins (GAPs) stimulate the intrinsic activity of Rho-GTPases and convert the GTP-bound form of Rho-GTPases to the inactive GDP-bound form. In resting cells, Rho-GTPases mostly exist in the GDP-bound form in association with Rho-GDI in the cytosol.

Source: (Antonarakis and Van Aelst, 1998)

up of more than twenty proteins identified to date (Glotzer, 2005). RhoA is known to be first activated by its upstream regulators and then act via its downstream effectors to organise these proteins into a ring structure at the cell division plane that can then stably constrict and divide the cell into two daughter cells (Glotzer, 2001). A variety of upstream regulators and downstream effectors of RhoA have been identified to date to play an important role in this process and they are discussed below (Piekny et al., 2005).

**1.3.3 Activation of RhoA following the metaphase-anaphase transition:**

Components of the contractile ring that act upstream of RhoA are RacGAP50C/MgcRacGAP, Pav-KLP/MKLP1, Pebble/ECT2 and Polo Kinase/PLK1 (Glotzer, 2001, Ohkura et al., 1995,



Petronczki et al., 2007). RacGAP50C and Pav-KLP form a microtubule associated complex, called the centralspindlin complex which has microtubule bundling activity *in vitro* (Mishima et al., 2002). The localisation of the central spindlin complex to the cell cleavage plain is known to be required for localisation of Pebble/ECT2 which activates the function of RhoA in cytokinesis leading to the subsequent assembly and constriction of the contractile ring (Yuce et al., 2005, Somers and Saint, 2003, Zhao and Fang, 2005, Nishimura and Yonemura, 2005, Kamijo et al., 2006). Pav-KLP is a plus-end directed microtubule motor protein known to be essential for cytokinesis (Nislow et al., 1992, Raich et al., 1998, Powers et al., 1998, Adams et al., 1998) and RacGAP50C and Pav-KLP have been shown to be interdependent for their localisation to the equator (Jantsch-Plunger et al., 2000). Various studies have also pointed to an essential role for RacGAP50C early in cytokinesis (Jantsch-Plunger et al., 2000, Hirose et al., 2001, Somers and Saint, 2003, Lee et al., 2004, Canman et al., 2008, Mishima et al., 2002). As well as binding to Pav-KLP, RacGAP50C/MgcRacGAP directly binds the RhoA activator Pebble (Somers and Saint, 2003, Yuce et al., 2005) which is known to bind RhoA and to be essential for RhoA activity in Cytokinesis in both *Drosophila* and mammals (Prokopenko et al., 1999, Tatsumoto et al., 1999).

RacGAP50C has a C-terminal domain with GAP activity which is shown to be active against RhoA, and two other Rho GTPases Rac1 and Cdc42 *in vitro* (Jantsch-Plunger et al., 2000). A study carried out by Minoshima and colleagues suggests that the GAP activity of MgcRacGAP is specified towards RhoA via phosphorylation of MgcRacGAP by another contractile ring component, AuroraB and that this phosphorylation is required for late but not early stages of cytokinesis (Minoshima et al., 2003). However, this phosphorylation site of MgcRacGAP is not well conserved among the different model systems and although the GAP domain is well conserved, RhoGTPase GAPs vary in this residue (Mishima and Glotzer, 2003). Another study

performed by Yamada and colleagues indicated that impaired cytokinesis by removal of MgcRacGAP can be rescued by the expression of a GAP inactive but not GAP removed mutant form of MgcRacGAP in B-lymphocytes, indicating that the GAP domain but not its activity is essential for cytokinesis (Yamada et al., 2006). In support of the findings of Yamada and colleagues, a study in *Drosophila* showed that disruption of the GAP domain of RacGAP50C leads to defects in cytokinesis while, using genetic studies they found that RacGAP50C and Pebble work synergistically rather than antagonistically (Somers and Saint, 2003, Mishima and Glotzer, 2003). Therefore, although the results of the study carried out by Minoshima and colleagues (Minoshima et al., 2003) clearly points to *in vitro* MgcRacGAP GAP activity towards RhoA, further study is required to address the differences seen in the findings on the GAP activity of RacGAP50C/MgcRacGAP regulation of RhoA in cytokinesis. In addition, there is now strong evidence from a more recent study that the GAP activity is required early in cytokinesis against another member of the RhoGTPase family, Rac (Canman et al., 2008). The study by Canman and colleagues showed that in the presence of a GAP inactive form of MgcRacGAP, furrow ingression was disrupted, but that this was rescued by over-expression of Rac and not RhoA. Their results also showed that ECT2 partial depletion led to the enhancement of the cytokinetic phenotype of GAP inactive RacGAP1 rather than suppression. This is consistent with the results of an earlier study (Somers and Saint, 2003), indicating that RacGAP50C/MgcRacGAP and Pebble/ECT2 synergise rather than antagonize in RhoA functional activation in Cytokinesis in both *Drosophila* and mammals. In addition, co-depletion of WASp and WAVE, two downstream effectors of Rac important for Actin meshwork formation via Arp2/3 (Pollard, 2007), led to significant rescue of the GAP inactive RacGAP1 cytokinetic defects (Canman et al., 2008). Their results indicate that RacGAP50C GAP activity promotes cytokinesis by negatively regulating Rac at the onset of cytokinesis which may otherwise interfere in RhoA mediated actin rearrangement and by positively regulating RhoA

function via Pebble/ECT localisation. Altogether, these results provide further support for RacGAP50C GAP activity early in cytokinesis against Rac while its activity against Rho in cytokinesis is yet to be clarified.

#### **1.3.4 The role of Polo Kinase in initiation of Cytokinesis:**

Polo Kinase was first characterised in *Drosophila* mitotic *polo* mutant cells that displayed abnormal spindle poles suggesting an important role in mitosis (Sunkel and Glover, 1988). The gene was subsequently characterised in mammals (Clay et al., 1993, Hamanaka et al., 1994) and studies on its mammalian homologue, Polo Like Kinase 1 (PLK1), suggested an essential role in various stages of mitosis (Glover et al., 1998) and early in Cytokinesis (Petronczki et al., 2007), specifically in the early process of contractile ring assembly. Using inhibitor analysis, PLK1 has been shown to be required for localisation of the RhoGEF Pebble (ECT2) to the site of cleavage furrow formation and the subsequent activation of RhoA (Petronczki et al., 2007). Different studies have shown that in cells lacking functional PLK1, contractile ring components such as RhoA and Pebble do not localize to the cell equator and Cytokinesis does not initiate (Carmena et al., 1998, Burkard et al., 2007, Brennan et al., 2007, Santamaria et al., 2007). PLK1 has also been shown to localise to the central spindle and the contractile ring during cytokinesis and to co-immunoprecipitate with RhoA suggesting that it is part of the contractile ring (Dai et al., 2007). The polo box domains of PLK1 have been shown to be required for Polo Kinase sub-cellular localisation and its over-expression leads to a failure in cytokinesis (Seong et al., 2002, Elia et al., 2003a, Elia et al., 2003b).

PLK1 also appears to modulate the function of microtubule-associated motor proteins in Cytokinesis. PLK1 appears to bind and Phosphorylate MKLP2 (Neef et al., 2003) and/or

MKLP1 (Liu et al., 2004). There is also evidence for an important role for microtubule-associated motor proteins in the localisation of Polo Kinase in cytokinesis (Adams et al., 1998, Carmena et al., 1998). A recent study using Pav-KLP RNAi, suggests that Pav-KLP may not be required for the localisation of Polo Kinase (D'Avino et al., 2007). This is in sharp contrast to a previous study in *Drosophila* (Adams et al., 1998) and a mammalian study (Liu et al., 2004) which show that Pav-KLP/MKLP1 has a clear role in localising Polo Kinase to the equator. Interpretation of the findings of D'Avino et al., 2007 may be complicated by the presence of residual amounts of Pav-KLP in the Pav-KLP RNAi expressing cells. Therefore, although it is likely that Pav-KLP is required for localisation of Polo Kinase, further study is required to confirm this requirement.

#### **1.3.4.1 The functional domains of Polo Kinase:**

Polo Kinase has two known functional domains; the catalytic N-terminal Kinase domain (Llamazares et al., 1991, Clay et al., 1993, Hamanaka et al., 1994) and the two protein-binding C-terminal Polo Box domains (Elia et al., 2003b, Lowery et al., 2007) (Figure 1.3.4.1). The catalytic domain is encoded by a region that spans amino acids 49 to 310 in mammals and is known to be a serine-threonine kinase domain that phosphorylates substrates at different stages of mitosis (Glover et al., 1998, Donaldson et al., 2001). The Polo Box domains on the other hand, together form one functional protein binding domain that spans amino acids 410 to 592 (Reynolds and Ohkura, 2003, Cheng et al., 2003) and binds to proteins already phosphorylated (phospho-primed) by other protein kinases (Lowery et al., 2007) as well by proteins already phosphorylated by Polo Kinase itself (Neef et al., 2003). These domains are very highly conserved from Yeast to Mammals (Reynolds and Ohkura, 2003, Llamazares et al., 1991) and they are essential for Polo Kinase function in various stages of mitosis (Glover et al., 1998). So far, no particular role has been

reported for the region in between the catalytic and Polo box domains spanning amino acids 311 to 409.

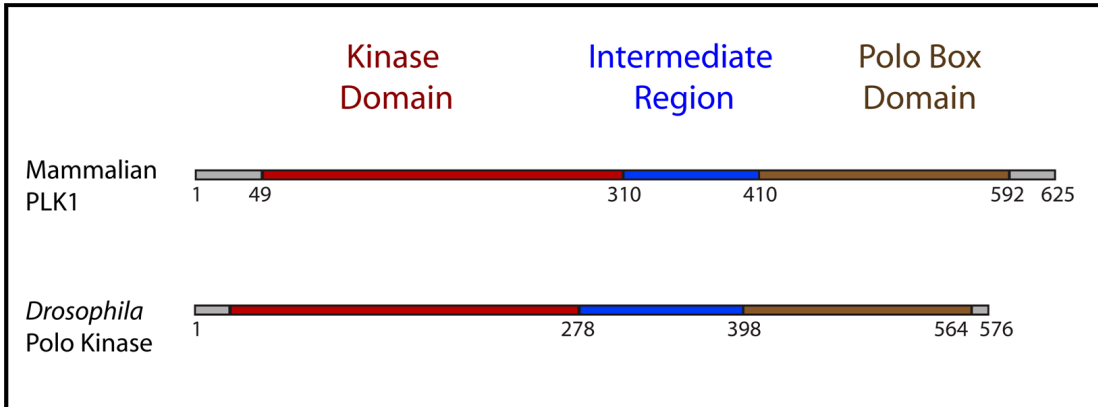


Figure 1.4.3.1 The functional domains of Polo Kinase in *Drosophila* and mammals:

The structure of the mammalian PLK1 and *Drosophila* Polo Kinase. There are two known functional domains; the catalytic Serine-Threonine kinase domain (Red) and the two Polo Box domains that form one Phospho-protein binding domain (Brown). The region in between the two functional domains (Blue) has so far no known function.

### 1.3.4.2 The role of Polo Kinase in the events leading to the onset of Anaphase:

Polo Kinase plays an important role in many mitotic events including those leading to the onset of Anaphase (Nigg, 1998). Entry into Anaphase is achieved by the function of the Anaphase Promoting Complex/Cyclosome (APC/C) at the metaphase to Anaphase transition (Peters, 2002, Harper et al., 2002). The APC/C is a multi-subunit ubiquitin protein ligase enzyme complex that mediates timely ubiquitination and degradation of proteins in mitosis leading to progress in mitotic stages (King et al., 1995, Townsley and Ruderman, 1998). The target proteins of the APC/C are recognised by the existence of sequences called the destruction box (D Box) (Glotzer et al., 1991) and the KEN box (Pfleger and Kirschner, 2000). The specificity of APC/C activity depends on its activating partners Cdc20 and Cadh1 that direct its activity towards D-Box (Glotzer et al., 1991) and Ken Box (Pfleger and Kirschner, 2000) containing proteins respectively. APC/C in

complex with Cdc20 ( $APC^{Cdc20}$ ) functions in the Metaphase to Anaphase transition to promote the onset of Anaphase by degrading Anaphase inhibiting proteins at the right time (Shirayama et al., 1999). Therefore, APC/C plays an important role in the metaphase to Anaphase transition that allows a cell to initiate cytokinesis and carry out the final splitting into two daughter cells.

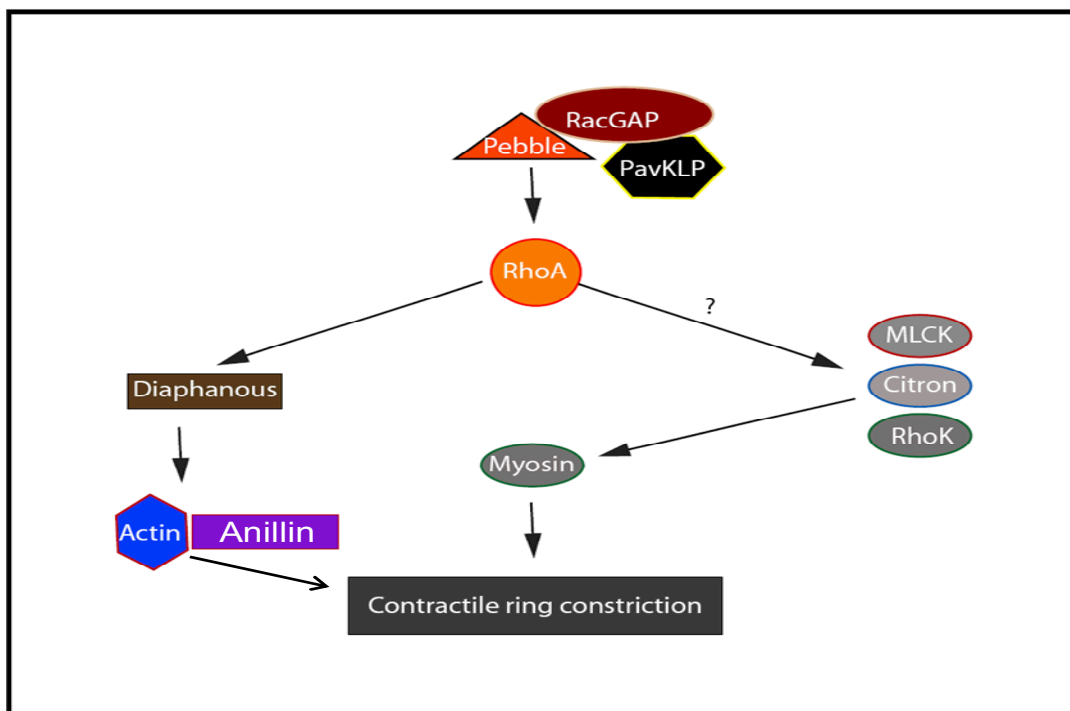
The spindle assembly checkpoint plays an important role in regulating APC/C mediated transition into Anaphase (Yu, 2002). In metaphase, the spindle checkpoint senses chromosome alignment at the metaphase plate, kinetochore attachment to the spindle microtubules and tension at the kinetochore which is required before sister chromatid separation occurs (Li and Nicklas, 1995, Skoufias et al., 2001, Amon, 1999, Burke, 2000). Once these events have taken place, the checkpoint mechanism is inactivated, allowing the release of Cdc20 which had been sequestered away by BubR1 and Mad2, leading to the activation of APC/C (Fang, 2002, Sudakin et al., 2001, Yu, 2002, Shirayama et al., 1999).  $APC^{Cdc20}$  is then able to mediate sister chromatid separation and degrade anaphase inhibiting proteins leading to the transition into Anaphase (Shirayama et al., 1999, Hilioti et al., 2001, Gorbsky and Ricketts, 1993, Nicklas et al., 1995). Sister chromatids are held together by the cohesin protein complex (Nasmyth et al., 2000) and a subunit of the complex, called Scc1 is cleaved at the end of metaphase by separase to facilitate sister chromatid separation (Uhlmann et al., 1999). This function of separase is inhibited by Securin protein (Waizenegger et al., 2002) until the metaphase to anaphase transition when securin is degraded by  $APC/C^{Cdc20}$  (King et al., 1996). The checkpoint is able to inhibit APC/C activity at any time during APC/C function and delay anaphase onset to ensure that the daughter cells get an equal amount of genetic material (Clute and Pines, 1999, Hagting et al., 2002).

Polo Kinase plays an important role in coordinating the metaphase to anaphase transition by the spindle checkpoint and APC/C (Glover et al., 1998, Nigg, 1998, Donaldson et al., 2001). Polo Kinase also plays an important role in chromosome alignment (Matsumura et al., 2007) and kinetochore microtubule attachment through phosphorylation of BubR1, as expression of a form of BubR1 that can not be phosphorylated by Polo kinase leads to a delay in anaphase onset (Elowe et al., 2007). Polo Kinase mediates localisation of spindle checkpoint proteins to the kinetochore where they can sense tension (Wong and Fang, 2007). Polo Kinase also plays a role in the timing of Anaphase onset by phosphorylating Scc1 which is then a substrate for separase cleavage independently of securin degradation (Alexandru et al., 2001). Therefore, Polo Kinase plays many roles in the events leading up to anaphase onset and plays a major role in controlling the timing of anaphase.

### **1.3.5 Targets of activated RhoA during cytokinesis:**

Once activated, RhoA mediates the function of a number of downstream effectors, such as Diaphanous (Dia), Myosin, Actin and Anillin (Glotzer, 2005), that assemble into a contractile ring. Active RhoA reorganises the Actin cytoskeleton via Diaphanous, which leads to the localisation of Actin, Anillin and Myosin (Castrillon and Wasserman, 1994, Watanabe et al., 1997, Miller et al., 1989). RhoA also activates Myosin possibly via Citron kinase (Madaule et al., 1995), Myosin Light Chain Kinase (MLCK) and/or Rho Kinase (Tan et al., 1992, Amano et al., 1996) leading to the constriction of the contractile ring down to an intracellular bridge (Figure 1.3.5) (Trotter and Adelstein, 1979, Tan et al., 1992, Amano et al., 1996). Studies have shown that Myosin depletion prevents constriction of the contractile ring (Straight et al., 2005). Anillin was first identified using F-actin affinity chromatography and has been shown to interact with Actin (Miller et al., 1989) linking it to the cell cortex. Anillin binds to phosphorylated Myosin (Straight et al., 2005, Miller et al., 1989) and RhoA (Piekny and Glotzer, 2008) and may

therefore act as a scaffolding protein in the contractile ring. Anillin and F-actin are shown to co-localize in a RhoA-dependent manner throughout contractile ring constriction (Giansanti et al., 2004). In addition, the equatorial band of Anillin is postulated to be formed through the action of RhoA on its effector, Dia which plays a role in the formation of acto-myosin bundles by acting as a mediator in the microtubule regulation of actin organisation (Gunsalus et al., 1995, Afshar et al., 2000, Giansanti et al., 2004). Consistent with these findings, studies have shown that Anillin is required for maintenance of active myosin at the cleavage furrow and that in the absence of Anillin the ring of active myosin slides to one side of the cell (Straight et al., 2005). Altogether, these studies point at the function of Anillin as a scaffolding protein that may play a role in contractile ring stability.



**Figure 1.3.5 The role of Rho signalling in Cytokinesis:**

At the onset of cytokinesis in Anaphase, RacGAP50C/MgcRacGAP and Pav-KLP/MKLP1 localize the Rho activator Pebble/ECT2 to the site of contractile ring assembly leading to the activation of RhoA function. Active RhoA reorganises the actin cytoskeleton via Diaphanous/mDia leading to the localisation of Anillin and Myosin. RhoA also activates myosin possibly via one of the proteins that activate myosin leading to the constriction of the contractile ring.



## **1.4 Areas studied further in this thesis:**

### **1.4.1 Molecular requirements for a continual constriction of the contractile ring:**

As mentioned earlier, activation of RhoA function by the RhoGEF Pebble/ECT2 is essential for the assembly and constriction of the contractile ring (Prokopenko et al., 1999, Tatsumoto et al., 1999). Various studies suggest that the localisation of Pebble/ECT2 to the equator where it can activate RhoA is dependent on the microtubule-bound central spindlin complex (Somers and Saint, 2003, Zhao and Fang, 2005, Nishimura and Yonemura, 2005, Zavortink et al., 2005, Yuce et al., 2005). In addition, microtubules have been shown to be required for a continuous constriction of the contractile ring during cytokinesis independently of other contents of the cell (Rappaport, 1978, Rappaport, 1997). This leads to the hypothesis that the central spindlin complex somehow governs the continual activation of RhoA at the cleavage plane throughout anaphase and telophase. However, it is not yet clear what other interactions at the cleavage plane lead to the maintenance of the contractile ring once the process is initiated. Therefore, it is important to uncover the molecular interactions within the contractile ring that persist after the initiation of constriction.

### **Aim and Hypothesis 1:**

**Aim1:** To investigate the mechanism behind continuous and stable contractile ring constriction after initiation of cytokinesis:

**Hypothesis:** Direct interactions between some of the components of the contractile ring at the cleavage plane ensure the continuous activation of RhoA function and stable contractile ring constriction throughout cytokinesis.

### **1.4.2 Molecular requirements for initiation of Cytokinesis**

As mentioned earlier, Polo Kinase has been shown to be essential for the localisation of the RhoA activator Pebble to the site of contractile ring assembly as well as the localisation of all the downstream effectors of RhoA tested so far. However, the mechanism by which Polo Kinase performs coordinates this early contractile ring assembly and activation of RhoA is yet to be elucidated. In order to further understand the function of Polo Kinase early in cytokinesis, there was a need to look for potential binding partners of Polo Kinase in anaphase to clarify the mechanism by which Polo Kinase mediates initiation of cytokinesis.

#### **Aim and Hypothesis2**

Aim2: To investigate the mechanism behind the initiation of cytokinesis.

Hypothesis2: Polo Kinase is involved in initiation of Cytokinesis by interacting with components of the cytokinetic machinery upstream of RhoA signalling.

### **1.5 Experimental Approach**

#### **1.5.1 Fluorescence Resonance Energy Transfer:**

In order to test for the mechanism behind continual and stable contractile ring constriction, there was a need to test for a direct interaction between components of the contractile ring at the cleavage plane. Fluorescence Resonance Energy Transfer (FRET) has emerged as a method of choice for testing direct spatio-temporal interactions inside the tissue of interest (Kenworthy, 2001, Periasamy, 2001). FRET is the transfer of energy from an excited-state donor fluorophore to an acceptor fluorophore mediated by dipole-dipole coupling (Förster, 1948). In order for this transfer of energy to occur, the fluorophores used to label the two proteins of interest need to be within an extremely close distance (5nm) (You et al., 2006). The transfer of energy is also dependent on the type of fluorophores used to label the two proteins of interest; that is, the

emission of one of the fluorophores (Donor fluorophore) needs to overlap the excitation of the other fluorophore (acceptor fluorophore) (Figure 1.5.1 A). This, in turn causes the excitation of the acceptor fluorophore by the energy transferred from the donor fluorophore (Periasamy, 2001). Even though, in strict terms, FRET detects the distance between two proteins, large scale FRET experiments have shown that positive FRET signals highly statistically correlate with a direct interaction, making it possible to detect protein-protein associations in time and space within the cell (You et al., 2006, Chen et al., 2003). FRET would therefore be an ideal method of choice to detect the timing and location of protein-protein interactions at the cleavage plane of a dividing cell.

One of the methods used for detecting FRET interaction between two fluorophores is by photo-bleaching of the acceptor fluorophore (Kenworthy, 2001). In any FRET interaction between two fluorophores, energy is transferred from the donor fluorophore (eg. Cy3) to the acceptor fluorophore (e.g Cy5). However, if the acceptor fluorophore, Cy5 is strongly excited for a period of 1 to 5 minutes, it can no longer accept any energy transferred onto it from Cy3 (Konig et al., 2006). This means that Cy3 regains its full emission and its fluorescence increases. Therefore, by comparing Cy3 fluorescence before and after photo-bleaching Cy5, any FRET interaction between Cy3 and Cy5 can be calculated. Using wide-field Microscopy, this method can be automated and the same parameters can be used to test a large number of cells both for the sample and the negative controls (Kenworthy, 2001, Periasamy, 2001).

In order to adapt this technique to the *Drosophila* system, a positive control was established by using the anti-phospho-histone 3 antibody to stain DNA in dividing cells. This antibody gives strong fluorescence and labelling it with both Cy3 and Cy5 provides an opportunity to test for the right conditions for FRET to occur. Labelling the same protein with both the donor and the

acceptor dyes ensures that they are within the distance required for energy transfer to occur. After adjusting for different parameters in the immuno-staining protocol, an increase in the

**NOTE:**  
This figure is included on page 24 of the print copy of the thesis held in the University of Adelaide Library.

Figure 1.5.1A: Fluorescence Resonance Energy transfer:

Cy3 Excitation (Blue) leads to emission of Cy3 (Red). Emission of Cy3 overlaps with the excitation of Cy5 (Green filled colour) annotated as Spectrum Overlap and leads to the emission of Cy5 (Red filled colour) annotated as emission. This overlap only allows energy transfer when the two fluorophores are within 5nm.

fluorescence of the Cy3 label was seen after photo-bleaching the Cy5 label indicating FRET interaction. The same conditions were then used to test FRET on the contractile ring component, Anillin labelled with both Cy3 (used as the donor ) and Cy5 (used as the acceptor) and a positive FRET signal was seen (Figure 1.5.1 B). The technique was therefore ready to be used in analysis of protein-protein interactions between components of the contractile ring at the cleavage plane.

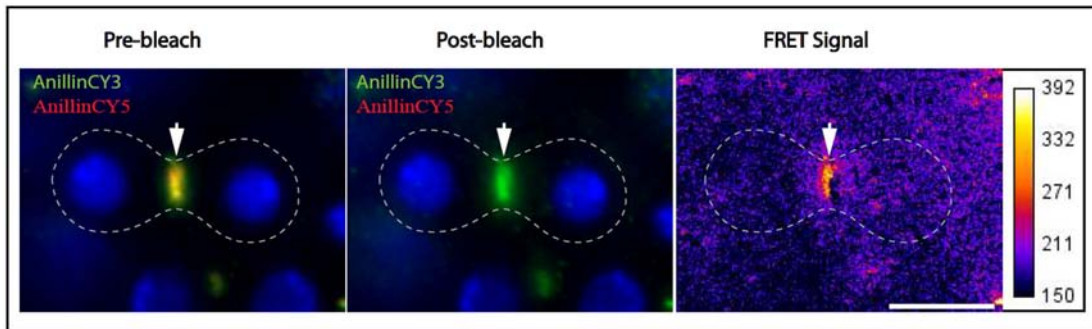


Figure 1.5.1B Positive control for FRET analysis:

A third instar larval *Drosophila* neuroblast in early telophase at a stage in which cytokinetic furrowing has initiated. Anillin is tagged with a mixture of Cy3 (green) and Cy5 (red) secondary antibodies, revealing the expected contractile ring localization (arrow) between the Telophase nuclei stained with DAPI (Blue). Upon photo-bleaching the Cy5 label (Post-bleach) there is no more Cy5 signal (red) and the FRET signal shows increase in Cy3 fluorescence at the contractile ring (arrow in FRET signal) as calculated by subtracting Post-bleach Cy3 fluorescence to that of the Pre-bleach Cy3 fluorescence. The bar next to the FRET signal indicates pixel intensity and the scale bar indicates 10um.

### 1.5.2 Using *Drosophila* genetics and cell biology to clarify the function of Polo Kinase in contractile ring assembly and initiation of Cytokinesis:

In order to study the role of Polo Kinase specifically in cytokinesis, first I decided to test for early cytokinetic genes that interact with Polo Kinase using FRET. For this purpose, RacGAP50C and Pav-KLP would be suitable candidates since they are both upstream of RhoA activation and contractile ring assembly as they localise the RhoA activator Pebble (Mishima et al., 2002, Somers and Saint, 2003, Nishimura and Yonemura, 2005, Zhao and Fang, 2005). Next, in order to dissect the functional importance of Polo Kinase early in cytokinesis, it is necessary to generate *Drosophila* cells in Anaphase that lack Polo Kinase activity. These cells could then be used for phenotypic analysis of contractile ring assembly and constriction in the absence of Polo kinase. However, Polo Kinase is essential for metaphase events and cells lacking functional Polo Kinase activate the spindle checkpoint and arrest at the end of metaphase making it difficult to study the functional importance of

Polo in cytokinesis (Donaldson et al., 2001). The spindle checkpoint component, Mad2 has been shown to be specific to regulating the checkpoint, and *mad2* mutant *Drosophila* have been shown to be checkpoint deficient, viable and fertile with no reported abnormalities in cell division (Buffin et al., 2007). Therefore, creating *polo mad2* double mutant flies should allow the study of Polo kinase function specifically in cytokinesis.

## CHAPTER II

### Research Paper I

#### **Cell division requires a direct link between microtubule-bound RacGAP and Anillin in the contractile ring.**

Stephen L. Gregory<sup>1</sup>, Saman Ebrahimi<sup>1</sup>, Joanne Milverton<sup>1</sup>, Whitney M. Jones<sup>2</sup>, Amy Bejsovec<sup>2</sup>, and Robert Saint<sup>3\*</sup>,

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**Current Biology, 2008, Vol 18, 25-29**

Stephen L. Gregory

Designed the experimental approach, supervised the progress of experimental work, established FRET analysis, performed Yeast Two-hybrid analysis and live imaging of Anillin and RacGAP50C and wrote the paper.

Certification that the statement of authorship is accurate and permission is given for the inclusion of the paper in the thesis

Signed

**Saman Ebrahimi**

Co-established and performed FRET analysis of Anillin and RacGAP50C and Actin, performed live imaging of Anillin and RacGAP50C, generated transgenic *Drosophila*, and commented on the manuscript.

Certification that the statement of authorship is accurate and permission is given for the inclusion of the paper in the thesis

Signed



Joanne Milverton

Generated transgenic flies, carried out injection of fluorescent- tagged wilde-type and mutant proteins into embryos for analysis and commented on the manuscript.

Certification that the statement of authorship is accurate and permission is given for the inclusion of the paper in the thesis

Signed

Amy Bejsovec

Supervised the original yeast two-hybrid screen that indicated an interaction between Anillin and RacGAP50C.

Certification that the statement of authorship is accurate and permission is given for the inclusion of the paper in the thesis

Signed

Whitney M Jones

Performed the original yeast two-hybrid screen that indicated an interaction between Anillin and RacGAP50C.

Certification that the statement of authorship is accurate and permission is given for the inclusion of the paper in the thesis

Signed

Robert Saint

Supervised and Directed the Project and revised the manuscript.

Certification that the statement of authorship is accurate and permission is given for  
the inclusion of the paper in the thesis

Signed

# Cell Division Requires a Direct Link between Microtubule-Bound RacGAP and Anillin in the Contractile Ring

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Joanne Milverton,<sup>1</sup> Whitney M. Jones,<sup>2</sup>  
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## CHAPTER III

### Research Paper II

# **Polo kinase interacts with RacGAP50C and is required for the localization of the Cytokinesis Initiation Complex.**

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**Submitted Manuscript (Journal of Cell Science)**

**Saman Ebrahimi**

Performed localisation, Yeast Two-Hybrid and FRET analysis of Polo Kinase, RacGAP50C and Pav-KLP, generated transgenic *Drosophila*, performed immunostaining of Polo Kinase mutant cells for RacGAP50C and Pav-KLP and wrote the Paper.

Certification that the statement of authorship is accurate and permission is given for the inclusion of the paper in the thesis

Signed

**Hamilton Fraval**

Performed mutagenesis of RacGAP50C to be tested in Yeast Two-Hybrid Analysis and commented on the manuscript.

Certification that the statement of authorship is accurate and permission is given for the inclusion of the paper in the thesis

Signed

Michael Murray

Performed live imaging of kinase-inactive Polo Kinase transgenic *Drosophila* and commented on the manuscript.

Certification that the statement of authorship is accurate and permission is given for the inclusion of the paper in the thesis

Signed

Robert Saint

Supervised and Directed the Project and revised the manuscript.

Certification that the statement of authorship is accurate and permission is given for the inclusion of the paper in the thesis

Signed

Stephen L. Gregory

Designed and co-directed the experimental approach and supervised the progress of experimental work, performed mutagenesis of Polo Kinase, generated *Drosophila* transgenics and revised the manuscript.

Certification that the statement of authorship is accurate and permission is given for the inclusion of the paper in the thesis

Signed



**Polo kinase interacts with RacGAP50C and is required for the  
localization of the Cytokinesis Initiation Complex.**

Saman Ebrahimi<sup>1</sup>, Hamilton Fraval<sup>2</sup>, Michael Murray<sup>2</sup>, Robert Saint<sup>2</sup> and Stephen L.  
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Running Title: Polo kinase localises centralspindlin

Ebrahimi, S., Fraval, H., Murray, M., Saint, R. & Gregory, S.L. (2010) Polo kinase interacts with  
RacGAP50C and is required to localize the cytokinesis initiation complex.  
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## ***Summary***

The proper assembly of the actomyosin-based contractile ring in anaphase is essential for cytokinesis. The initial assembly and the subsequent initiation of ring constriction is dependent on activation of RhoA at the equatorial cortex by a complex, here termed the cytokinesis initiation complex, between a microtubule-associated kinesin-like protein (KLP), a member of the RacGAP family and the RhoGEF Pebble. Recently, the activity of the mammalian Polo kinase ortholog Plk1 has been implicated in the formation of this complex. We show here that Polo kinase is, in fact, a component of the cytokinesis initiation complex via an association with RacGAP50C. We find that a new domain of Polo kinase, termed the intermediate domain, interacts directly with RacGAP50C and that Polo kinase is essential for localization of the KLP-RacGAP centralspindlin complex to the cell equator and spindle midzone. In the absence of Polo kinase, RacGAP50C and Pav-KLP fail to localise normally, instead decorating microtubules along their length. Our results indicate that Polo kinase is a component of the conserved cytokinesis initiation complex and is required to trigger centralspindlin localization as a first step in cytokinesis.

## ***Introduction***

Cytokinesis is the final stage of cell division that splits a cell into two. The process initiates in anaphase with the localisation of a microtubule associated protein complex to the cell equator and the subsequent assembly and constriction of an acto-myosin-based contractile ring coordinated by the Rho signalling pathway (Glotzer, 2005). In anaphase, RhoA activation is achieved by the localisation of the RhoGEF Pebble/ECT2 to the sites of cleavage furrow formation (Prokopenko et al., 1999). This localisation is mediated by RacGAP50C/RacGAP1 (Prokopenko et al., 1999; Somers and Saint, 2003; Tatsumoto et al., 1999) which is in a complex with the microtubule associated motor protein Pav-KLP/MKLP1 called the centralspindlin complex (Mishima et al., 2002). This leads to the assembly and constriction of the contractile ring and finally, division of the cell (Nishimura and Yonemura, 2006). Evidence suggests that Pebble and RacGAP50C bind directly and that this binding is essential for the localization of Pebble to the equator and the subsequent activation of Rho signalling in anaphase (Somers and Saint, 2003). RacGAP50C and Pav-KLP have been shown to be interdependent in their localisation to the cell equator suggesting that a functional centralspindlin complex is required for Rho activation (Jantsch-Plunger et al., 2000). Recent studies in mammals suggest an important role for Polo-like kinase 1 (PLK1) for the interaction between RacGAP50C and Pebble, as inhibiting the function of PLK1 in anaphase prevents the localisation of Pebble/ECT2 to the equator and the subsequent ring assembly, although the centralspindlin complex still localises (Brennan et al., 2007; Petronczki et al., 2007).

As well as the mammalian data, a number of studies in *Drosophila* and other models point to a role for Polo kinase in cytokinesis. Polo was first identified in *Drosophila*

(Sunkel and Glover, 1988), and an early study showed that the activity of Polo kinase peaks in anaphase and telophase suggesting a role in cytokinesis (Fenton and Glover, 1993). A subsequent study examined a hypomorphic form of Polo kinase in *Drosophila* spermatocytes and showed early cytokinetic defects such as the formation of an abnormal spindle midzone and actin ring and the eventual failure of cytokinesis as indicated by the formation of multinucleate cells (Carmena et al., 1998). The fission yeast homologue of Polo kinase, *plp1*, has been shown to be essential for the formation of the actin ring during septum formation indicating that Plo1 may play a role in cytokinesis (Ohkura et al., 1995). Consistent with these studies, a role for Polo kinase in regulating the centralspindlin complex in cytokinesis was also suggested by the data of Herrmann and colleagues as they showed that *polo* mutant cells had a reduced central spindle (Herrmann et al., 1998). Altogether, these studies along with the mammalian data (Brennan et al., 2007; Burkard et al., 2007; Petronczki et al., 2007; Santamaria et al., 2008) suggest an important role for Polo kinase early in cytokinesis. However, the mechanism by which Polo regulates the centralspindlin complex consisting of RacGAP50C/RacGAP1 and Pav-KLP/MKLP1 is so far not clear. In this study we provide evidence for a novel role for Polo kinase in cytokinesis. We show that Polo kinase directly interacts with the linker protein RacGAP50C and that it is required for localisation of both RacGAP50C and Pav-KLP to the equator.

## ***Results***

### ***Polo kinase colocalises with the central spindlin complex in cytokinesis***

To investigate how Polo kinase may play its regulatory role at the onset of cytokinesis (Petronczki et al., 2007) we followed the localisation of Polo kinase and the central spindlin component RacGAP50C, using immuno-fluorescence of *Drosophila* larval brain cells. In metaphase, Polo kinase localises to the spindle poles (Figure 1A-1D) and to tight bands on the DNA while RacGAP50C is diffusely cytoplasmic (Figure 1A-1D). However, during anaphase, at the onset of cytokinesis, both proteins relocate to the cell equator (Figure 1E-1H) while Polo kinase also maintains its presence at the poles. The equatorial co-localisation continues as cleavage furrow localisation to the end of telophase (Figure 1I-1L, S1).

### ***Polo kinase interacts directly with RacGAP50C and forms part of the cytokinesis initiation complex***

To test for a direct interaction between Polo kinase and RacGAP50C during cytokinesis, we carried out Fluorescence Resonance Energy Transfer (FRET) by acceptor photo-bleaching (Kenworthy, 2001). We have previously shown that this technique can be used to detect molecular interactions *in situ* in *Drosophila* brain tissue (Gregory et al., 2008). FRET has been previously shown to identify proteins that are in extremely close proximity (<5nm), highly correlated with a direct molecular interaction (You et al., 2006). *Drosophila* brain tissues were incubated with antibodies directed against Polo kinase and RacGAP50C or Pav-KLP and stained with secondary antibodies labelled with the Cy3 and Cy5 fluorophores respectively. Upon photo-bleaching of Cy5 (compare Figure 2A and 2B), we saw a significant increase in the Cy3 signal (Figure 2C) indicating FRET between the RacGAP50C and

Polo kinase associated fluorophores. This interaction was apparent in cells at all stages of cytokinesis, but not at metaphase (data not shown). We tested whether this FRET signal was due to cross-reactivity of the secondary antibodies by staining with the same secondary antibodies that were used to mark Polo kinase and RacGAP50C but in the absence of the primary antibody against RacGAP50C. These samples showed no significant FRET signal (Figure 2D-2F). These results indicate that the positive FRET signal in Figure 2C is not due to cross-reactivity.

We then tested for a FRET signal between Polo kinase and Pav-KLP, which has previously been shown to bind and co-localize with RacGAP50C (Somers and Saint, 2003; Zavortink et al., 2005). As expected, Pav-KLP co-localises with Polo kinase (Figure 2G), but no significant FRET was observed between the Polo kinase and Pav-KLP associated fluorophores (Figure 2G-2I). This illustrates the specificity of our assay, in detecting the interaction between Polo kinase and RacGAP50C but not the Pav-KLP component of centralspindlin, and shows that the FRET signal is not due to random juxtaposition of any two proteins in the central spindle. Quantification of the FRET results showed that the interaction between Polo kinase and RacGAP50C is highly statistically significant ( $p < 0.001$ ) (Figure 2J).

To confirm that these FRET results indicated a direct physical interaction between Polo kinase and RacGAP50C, we carried out yeast two-hybrid analysis. We found that a new region of Polo kinase, between the kinase domain and the Polo Box domain, here named the intermediate domain, can physically bind a region of RacGAP50C between amino acids 103 to 173 of RacGAP50C (Figure 3). This region of RacGAP50C is adjacent to its Pebble and Pav-KLP binding regions (Somers and

Saint, 2003) and deletion of those binding regions did not disrupt the interaction with Polo kinase (Figure 3). Targeted mutagenesis of conserved residues in the Polo binding region resulted in loss of Polo binding (Figure S2). These mutations also reduced or removed binding to Pav-KLP, suggesting that the Polo binding region is necessary for overall protein structure. Polo kinase binding to its targets is usually mediated via its Polo Box domain, in a phospho-priming dependent manner (Elia et al., 2003b). Binding between Polo kinase and RacGAP50C occurred via the intermediate region and not via the Polo Box domain. The intermediate region is quite divergent across taxa, but mutation of two conserved amino acids removes RacGAP binding, as well as preventing proper localization of Polo throughout mitosis (Figure S2).

***Polo kinase is required for the localisation of the centralspindlin complex to the equator***

To elucidate the role Polo kinase plays in the *Drosophila* cytokinesis initiation complex, we examined the behaviour of RacGAP50C in cells deficient for Polo kinase. Previous work has shown that *polo* mutant cells arrest in metaphase due to the activation of the spindle checkpoint, preventing analysis of cytokinesis (Donaldson et al., 2001). To bypass the metaphase arrest and look at the role of Polo kinase specifically in cytokinesis, we generated *polo mad2* double mutant flies. Mad2 is essential for spindle checkpoint activation (Li and Murray, 1991) but Mad2 deficient *Drosophila* have been shown to be viable and fertile, with cells dividing correctly with no apparent abnormalities (Buffin et al., 2007). Removal of Mad2 therefore does not normally disrupt cell division apart from removing the checkpoint block. To confirm that our *polo mad2* mutant *Drosophila* tissue did in fact bypass the metaphase

arrest, we compared the mitotic index of *polo mad2* mutant cells with that of *polo* mutants and wild-type cells (Figure 4A). We saw a strong rescue of the mitotic arrest of *polo* mutants by the *mad2* mutation. To determine whether the reduction of mitotic cells was due to increased apoptosis or mitosis exit; we carried out TUNEL staining for apoptotic cells (Figure 4B). We found that in a *mad2* mutant background, the rate of apoptosis in *polo* mutants was reduced close to that of wild type cells, and progression through mitosis was not drastically arrested (Figure S3). Taken together these results indicate that the *polo mad2* double mutant cells are released from metaphase arrest, allowing us to examine the requirement for Polo in cytokinesis.

To investigate the role of Polo kinase in localisation of the Pav-KLP-RacGAP50C centralspindlin complex, we stained *polo mad2* double mutant larval brains for RacGAP50C or Pav-KLP (Figure 5). We saw a complete lack of RacGAP50C and Pav-KLP at the cell equator and no specific localization at the spindle midzone in all mutant cells (Figures 5D, 5F, 5J and 5L; n=12 for RacGAP50C, n=15 for Pav-KLP) compared to the control cells (Figure 5C and 5I; n=35 for RacGAP50C, n=33 for Pav-KLP). However, unlike the situation in wild type cells or *mad2* mutants (Figure S4), in *polo mad2* double mutants both components were found throughout the length of the mitotic spindle (Figures 5D, 5F, 5J and 5L arrowheads). To further examine the localisation of RacGAP50C, we carried out live imaging of *polo mad2* brains expressing Venus-tagged RacGAP50C. Consistent with the fixed tissue and in contrast to wild type controls (Movie S1), we saw RacGAP50C decorating interpolar filaments in dividing cells with no association with the equatorial region or specific localization to the midzone (Movie S2).



## ***Discussion***

### ***Polo kinase initiates centralspindlin-driven cell cleavage***

In the absence of Polo kinase, we have found that the centralspindlin complex remains associated with the microtubule spindle, but cannot translocate to the plus ends of the microtubules at the equator and spindle midzone. This lack of normal centralspindlin localisation contrasts with previous work suggesting that the mammalian homolog of *Drosophila* Polo kinase, Polo-like kinase 1 (PLK1), is not required for the localisation of centralspindlin to the equator (Brennan et al., 2007). In these experiments, however, PLK1 was chemically inhibited after the onset of anaphase at a time when PLK1 may have already carried out some of its anaphase functions. By bypassing the metaphase arrest in Polo kinase deficient cells, we have been able to demonstrate the need for Polo kinase in the localisation of RacGAP50C and Pav-KLP to the cell equator. This is highly significant because it reveals a well-characterized mitotic kinase as a regulator of the midzone localization of centralspindlin. (Mishima et al., 2002; Nishimura and Yonemura, 2006; Somers and Saint, 2003; Zhao and Fang, 2005) placing Polo at the top of the regulatory hierarchy that drives cell cleavage. It will be of considerable interest to determine the molecular mechanism by which Polo facilitates the movement of centralspindlin. From our data it appears that in mutant cells even when there are no gross defects in the anaphase spindle (Figure S5), centralspindlin does not accumulate at the ends of microtubules (Figure 5). We found that this could be rescued by normal, but not kinase inactive Polo (data not shown). We hypothesize, therefore, that the failure is in direct molecular regulation of the complex rather than as a result of an indirect structural defect. The phenotype we have observed may be related to that of inactivated Pav-KLP, which also remains on the spindle without localizing to the plus ends (Minestrini et al, 2004). It is possible

that Polo is required for the recruitment or function of other centralspindlin regulators such as Cdks or phosphatases (Mishima 2004), though to date none of these has shown a similar phenotype.

***A new domain of Polo kinase mediates the initial formation of the cytokinesis initiation complex***

Our studies provide evidence for a direct interaction between RacGAP50C and Polo kinase. RacGAP50C has previously been shown to bind a number of other proteins in cytokinesis such as Anillin (Gregory et al., 2008), Pebble and Pavarotti (Somers and Saint, 2003) indicating an important role for RacGAP50C in localisation and anchoring of proteins at the contractile ring early in cytokinesis. In addition, our yeast two-hybrid interaction assay in conjunction with our FRET data indicates not only that Polo kinase and RacGAP50C bind directly, but also that they interact in anaphase at a time when cytokinesis initiates. This interaction occurs via a new domain of Polo kinase with no previously known function. It will therefore be interesting to investigate whether this domain is also required for events prior to cytokinesis or if it confers cytokinesis-specific functions on Polo – our mutagenesis of the region suggests the former (Fig S2). Previous studies have focussed on Polo binding via the Polo Box domains and have identified several metaphase targets such as Vimentin, INCENP and Cdc25 phospho-primed by the mitotic kinase Cdk1/Cyclin B (Elia et al., 2003a; Elia et al., 2003b; Goto et al., 2006; Yamaguchi et al., 2005). Because the Polo RacGAP interaction is not via the Polo Box regions, the possibility exists that it is not phospho-dependent, which might be better suited to maintaining interaction through anaphase, when the kinase Cdk1/Cyclin B has been degraded by the APC/C (Clute and Pines, 1999; Peters, 2002).

In summary, the evidence presented here shows that Polo kinase binds RacGAP50C, a component of the cytokinesis initiation complex and that it functions upstream of Pav-KLP and RacGAP50C localisation, an event that must occur immediately after the onset of anaphase as a critical step in the cascade of regulatory events leading to cytokinesis.

## **Materials and Methods**

### **Immuno-histochemistry**

Brains were dissected from 3rd instar larvae in ice-cold PBS with 4% paraformaldehyde using forceps in two-well microscope slide. Frozen ice-packs were put under the dissecting microscope stage to keep it cold. The dissected brains were then moved to a clean well on the same slide and shredded with fine needles. Four brains were done at a time and the whole dissecting and fixing procedure was carried out within ten minutes. The fix was then removed from the well without removing the brain pieces and replaced with PBS plus 0.2% Triton X-100. The brains were transferred into a microcentrifuge tube and washed with PBS plus 0.2% Triton X-100 for 2 hours at room temperature and blocked in PBS plus 0.5% Triton X-100 and 10% Goat Serum for another two hours at room temperature. Primary antibodies were added in the same blocking solution and incubated overnight at 4°C, followed by a two hour wash in PBS plus 0.2% Triton X-100. Secondary antibodies were diluted in the blocking solution and added for 4 hours at room temperature followed by a two hour wash in PBS plus 0.2% Triton X-100. Hoechst 33258 (1ug/ml) staining was carried out for ten minutes before another thirty minute wash at room temperature. Brain pieces were then mounted in 80% glycerol/PBS. Samples were viewed on a

Zeiss Axioplan2 upright microscope with Semrock 'Brightline' filter sets using a 63x PlanApo (n.a. 1.4) objective. Images were acquired using an AxioCam MRm CCD camera and if necessary deconvolved using Axiovision software (Carl Zeiss).

Antibodies used were: Mouse anti-Polo kinase from Claudio E Sunkel (1:50), rat anti-RacGAP (1:300), rabbit anti-RacGAP (1:50) and Rabbit anti-Pav-KLP (1:500).

Secondary antibodies (1:200) were all "highly cross-absorbed" from Rockland or Jackson Immunochemicals. TUNEL staining to visualize degrading DNA in dying cells was carried out using a TMR-Red In situ cell death detection kit (Roche) on fixed brain squashes following a three minute wash in 0.1M Citrate/ 0.1% Triton X-100 at room temperature and a brief wash in PBS. TUNEL labelling was carried out for one hour at 37C, followed by a PBS wash and Hoechst 33342 labelling (0.5ug/ml 10 minutes).

### **FRET analysis**

Fixed third instar larval brain cells were first stained for Polo kinase and RacGAP50C using secondary antibodies tagged with Cy3 (donor) and Cy5 (acceptor) fluorophores respectively, as described above. Images of first the acceptor, then the donor fluorophore were acquired using a Zeiss Axioplan2 upright microscope with Semrock 'Brightline' GFP filter set using a 63x PlanApo (n.a. 1.4) objective. These images are referred to in figure 2 as pre-bleach. Then the acceptor was photobleached at maximum light intensity for 30-180 seconds, followed by imaging of the donor, then acceptor again and these images were referred to as post-bleach. This sequence was automated and identical imaging conditions were used pre- and post bleaching. Effectiveness of photobleaching was confirmed by loss of signal in the acceptor fluorescence post-bleaching. Donor fluorophore images were imported into ImageJ

(Abramoff et al., 2004.) and aligned to correct any lateral displacement using the StackReg plugin (Thévenaz et al., 1998). The FRET signal was displayed using the “fire” lookup table. We did not show the results as FRET efficiency ( $E = 1 - \text{pre/post}$ ) as this method has been shown to give less effective discrimination from background (Konig et al., 2006). This procedure, with the same conditions was repeated for the cross-reactivity (secondary only) and concentration (Polo kinase and Pav-KLP) controls.

### **Fly stocks and constructs**

The fly stocks used were, daughterless-Gal4 (Bloomington 8641), *polo*<sup>10</sup> (Szeged No.2123), Deficiency (Df(3L)rgdC-co2, Bloomington No.2052) and *mad2* (*mad2*<sup>E1Y1687</sup>, Bloomington 22495). *polo*<sup>10</sup> *mad2* and *Deficiency mad2* double mutants were generated by recombining the two alleles onto the same chromosome. For immuno-fluorescence analysis of localisation of RacGAP50C and Pav-KLP in the absence of Polo kinase *polo*<sup>10</sup> *mad2* /*Deficiency mad2* flies were compared to *polo*<sup>10</sup> *mad2* /TM6 or *Deficiency mad2* / TM6 controls. For live analysis of localisation of RacGAP50C, UAS-Venus-tagged RacGAP ; *polo*<sup>10</sup> *mad2* /*Deficiency mad2 da-Gal4* flies were compared to UAS-Venus-tagged RacGAP ;*Deficiency mad2 da-Gal4* / TM6.

### **Yeast two-hybrid analysis**

The Yeast L40 strain (*MATa his3Δ200 trp1-901 leu2-3112 ade2 LYS::(lexAop)<sub>4</sub> – HIS3 URA3::(lexAop)<sub>8</sub>-lacZ GAL4 gal80*) and vectors pVP16 and pNLX (Hollenberg et al., 1995. ) were used to detect protein-protein interactions as previously described (Somers and Saint, 2003). Interactions were tested by lacZ filter assay according to

the Breeden lab protocol ([http://www.fhrc.org/science/labs/breeden/Methods/b-GAL\\_filterassay.html](http://www.fhrc.org/science/labs/breeden/Methods/b-GAL_filterassay.html)). Polo kinase was used as the bait using pNLX and RacGAP50C constructs were used as the prey using pVP16. A construct of RacGAP50C containing amino acids 363 to 625 and the empty vector were used as negative controls. All regions of RacGAP50C tested are indicated in Figure 3.

### **Live imaging**

Third instar larval brains were dissected in a 1 to 1 ratio of Halocarbon oil 700 and Halocarbon oil 27 (SIGMA) and spread on cover-slips using needles as described (Savoian and Rieder, 2002). Venus-tagged RacGAP50C was imaged in *Drosophila* neuroblast cells double mutant for *polo mad2* every 30sec on a Zeiss Axioplan2 upright microscope with Semrock 'Brightline' GFP filter set using a 63x PlanApo (n.a. 1.4) objective.

### **Supplementary Information**

The movies described in the text (S1 and S2) are provided as supplementary materials.

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## References

- Abramoff, M. D., Magelhaes, P. J. and Ram, S. J.** (2004.). Image Processing with ImageJ. *Biophotonics Intl* **11**, 36-42.
- Archambault, V., D'Avino, P. P., Deery, M. J., Lilley, K. S. and Glover, D. M.** (2008). Sequestration of Polo kinase to microtubules by phosphoprime-independent binding to Map205 is relieved by phosphorylation at a CDK site in mitosis. *Genes & Dev.* **22**, 2707 - 2720.
- Brennan, I. M., Peters, U., Kapoor, T. M. and Straight, A. F.** (2007). Polo-Like Kinase Controls Vertebrate Spindle Elongation and Cytokinesis. *PLoS ONE* **2**, e409.
- Buffin, E., Emre, D. and Karess, R. E.** (2007). Flies without a spindle checkpoint. *Nat Cell Biol* **9**, 565-572.
- Burkard, M. E., Randall, C. L., Larochelle, S. p., Zhang, C., Shokat, K. M., Fisher, R. P. and Jallepalli, P. V.** (2007). Chemical genetics reveals the requirement for Polo-like kinase 1 activity in positioning RhoA and triggering cytokinesis in human cells. *Proc Natl Acad Sci* **104**, 4383-4388.
- Carmena, M., Riparbelli, M. G., Minestrini, G., Tavares, A. M., Adams, R., Callaini, G. and Glover, D. M.** (1998). Drosophila Polo Kinase Is Required for Cytokinesis. *J. Cell Biol.* **143**, 659-671.
- Clute, P. and Pines, J.** (1999). Temporal and spatial control of cyclin B1 destruction in metaphase. *Nat Cell Biol* **1**, 82-87.
- Donaldson, M. M., Tavares, A. A. M., Hagan, I. M., Nigg, E. A. and Glover, D. M.** (2001). The mitotic roles of Polo-like kinase. *J Cell Sci* **114**, 2357-2358.
- Elia, A. E. H., Cantley, L. C. and Yaffe, M. B.** (2003a). Proteomic Screen Finds pSer/pThr-Binding Domain Localizing Plk1 to Mitotic Substrates. *Science* **299**, 1228-1231.
- Elia, A. E. H., Rellos, P., Haire, L. F., Chao, J. W., Ivins, F. J., Hoepker, K., Mohammad, D., Cantley, L. C., Smerdon, S. J. and Yaffe, M. B.** (2003b). The Molecular Basis for Phosphodependent Substrate Targeting and Regulation of Plks by the Polo-Box Domain. *Cell* **115**, 83-95.
- Fenton, B. and Glover, D. M.** (1993). A conserved mitotic kinase active at late anaphase-telophase in syncytial Drosophila embryos. *Nature* **17**, 637-40.
- Glotzer, M.** (2005). The Molecular Requirements for Cytokinesis. *Science* **307**, 1735-1739.
- Goto, H., Kiyono, T., Tomono, Y., Kawajiri, A., Urano, T., Furukawa, K., Nigg, E. and Inagaki, M.** (2006). Complex formation of Plk1 and INCENP required for metaphase-anaphase transition. *Nat Cell Biol* **8**, 180-7.
- Gregory, S. L., Ebrahimi, S., Milverton, J., Jones, W. M., Bejsovec, A. and Saint, R.** (2008). Cell Division Requires a Direct Link between Microtubule-Bound RacGAP and Anillin in the Contractile Ring. *Curr Biol* **18**, 25-29.
- Herrmann, S., Amorim, I. and Sunkel, C. E.** (1998). The POLO kinase is required at multiple stages during spermatogenesis in Drosophila melanogaster. *Chromosoma* **107**, 440-51.
- Hollenberg, S. M., Sternglanz, R., Cheng, P. F. and Weintraub, H.** (1995. ). Identification of a new family of tissue-specific basic helix-loop-helix proteins with a two-hybrid system. *Mol. Cell. Biol.* **15**, 3813-3822.
- Jantsch-Plunger, V., Gonczy, P., Romano, A., Schnabel, H., Hamill, D., Schnabel, R., Hyman, A. A. and Glotzer, M.** (2000). CYK-4: A Rho Family GTPase

- Activating Protein (GAP) Required for Central Spindle Formation and Cytokinesis. *J. Cell Biol.* **149**, 1391-1404.
- Kenworthy, A. K.** (2001). Imaging Protein-Protein Interactions Using Fluorescence Resonance Energy Transfer Microscopy. *Methods* **24**, 289-296.
- Konig, P., Krasteva, G., Tag, C., Konig, I. R., Arens, C. and Kummer, W.** (2006). FRET-CLSM and double-labeling indirect immunofluorescence to detect close association of proteins in tissue sections. *Lab Invest* **86**, 853-864.
- Li, R. and Murray, A. W.** (1991). Feedback control of mitosis in budding yeast. *Cell* **66**, 519-31.
- Minestrini, G., Harley, A.S., and Glover, D. M.** (2003) Localization of Pavarotti-KLP in living *Drosophila* embryos suggests roles in reorganizing the cortical cytoskeleton during the mitotic cycle. *Mol Biol Cell* **14**, 4028-4038
- Mishima, M., Kaitna, S. and Glotzer, M.** (2002). Central Spindle Assembly and Cytokinesis Require a Kinesin-like Protein/RhoGAP Complex with Microtubule Bundling Activity. *Dev Cell* **2**, 41-54.
- Mishima, M., Pavicic, V., Gruneberg, U., Nigg, E. A., and Glotzer, M.** (2004). Cell cycle regulation of central spindle assembly. *Nature* **430**, 908-913.
- Nishimura, Y. and Yonemura, S.** (2006). Centralspindlin regulates ECT2 and RhoA accumulation at the equatorial cortex during cytokinesis. *J Cell Sci* **119**, 104-14.
- Ohkura, H., Hagan, I. M. and Glover, D. M.** (1995). The conserved *Schizosaccharomyces pombe* kinase *pl*1, required to form a bipolar spindle, the actin ring, and septum, can drive septum formation in G1 and G2 cells. *Genes Dev* **9**, 1059-1073.
- Peters, J.-M.** (2002). The Anaphase-Promoting Complex: Proteolysis in Mitosis and Beyond. *Mol Cell* **9**, 931-943.
- Petronczki, M., Glotzer, M., Kraut, N. and Peters, J.-M.** (2007). Polo-like Kinase 1 Triggers the Initiation of Cytokinesis in Human Cells by Promoting Recruitment of the RhoGEF Ect2 to the Central Spindle. *Dev Cell* **12**, 713-725.
- Prokopenko, S. N., Brumby, A., O'Keefe, L., Prior, L., He, Y., Saint, R. and Bellen, H. J.** (1999). A putative exchange factor for Rho1 GTPase is required for initiation of cytokinesis in *Drosophila*. *Genes Dev.* **13**, 2301-2314.
- Santamaria, A., Neef, R., Eberspacher, U., Eis, K., Husemann, M., Mumberg, D., Prectl, S., Schulze, V., Siemeister, G., Wortmann, L., et al.** (2007). Use of the novel Plk1 inhibitor ZK-thiazolidinone to elucidate functions of Plk1 in early and late stages of mitosis. *Mol Biol Cell* **18**, 4024-4036.
- Savoian, M. S. and Rieder, C. L.** (2002). Mitosis in primary cultures of *Drosophila melanogaster* larval neuroblasts. *J Cell Sci* **115**, 3061-3072.
- Somers, W. G. and Saint, R.** (2003). A RhoGEF and Rho Family GTPase-Activating Protein Complex Links the Contractile Ring to Cortical Microtubules at the Onset of Cytokinesis. *Dev Cell* **4**, 29-39.
- Sunkel, C. E. and Glover, D. M.** (1988). Polo, a mitotic mutant of *Drosophila* displaying abnormal spindle poles. *J Cell Sci* **89**, 25-38.
- Tatsumoto, T., Xie, X., Blumenthal, R., Okamoto, I. and Miki, T.** (1999). Human ECT2 Is an Exchange Factor for Rho GTPases, Phosphorylated in G2/M Phases, and Involved in Cytokinesis. *J. Cell Biol.* **147**, 921-928.
- Thévenaz, P., Ruttimann, U. E. and Unser, M.** (1998). A Pyramid Approach to Subpixel Registration Based on Intensity. *IEEE Trans. Image Process.* **7** 27-41.



- Yamaguchi, T., Goto, H., Yokoyama, T., Sillje, H., Hanisch, A., Uldschmid, A., Takai, Y., Oguri, T., Nigg, E. A. and Inagaki, M.** (2005). Phosphorylation by Cdk1 induces Plk1-mediated vimentin phosphorylation during mitosis. *J Cell Sci* **171**, 431-436.
- You, X., Nguyen, A. W., Jabaiah, A., Sheff, M. A., Thorn, K. S. and Daugherty, P. S.** (2006). Intracellular protein interaction mapping with FRET hybrids. *Proc Natl Acad Sci* **103**, 18458-18463.
- Zavortink, M., Contreras, N., Addy, T., Bejsovec, A. and Saint, R.** (2005). Tum/RacGAP50C provides a critical link between anaphase microtubules and the assembly of the contractile ring in *Drosophila melanogaster*. *J Cell Sci* **118**, 5381-5392.
- Zhao, W.-m. and Fang, G.** (2005). MgcRacGAP controls the assembly of the contractile ring and the initiation of cytokinesis. *Proc Natl Acad Sci U S A* **102**, 13158-13163.

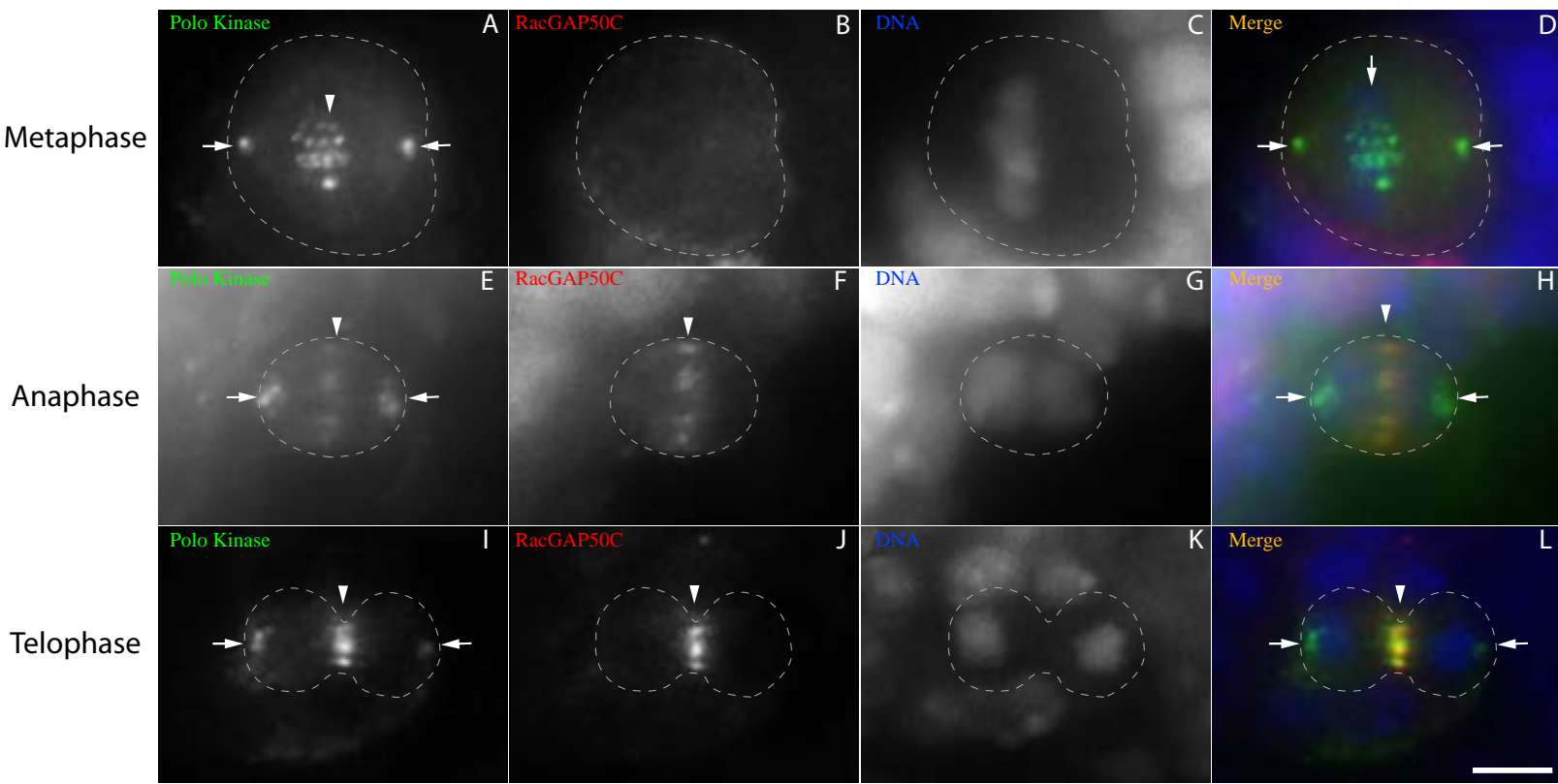


Figure 1. RacGAP50C and Polo kinase co-localise during early cytokinesis.

Third instar larval brain cells were imaged at the plane of the spindle poles for Polo kinase (A,E,I), RacGAP50C (B,F,J) and DNA (C,G,K). Merged images (D,H,L) show Polo kinase in green, RacGAP50C in red and DNA in blue. (A-D) A metaphase cell showing Polo kinase localised to the spindle poles (arrows) and to the centromeres at the metaphase plate (arrowhead) while RacGAP is diffusely cytoplasmic. (E-H) An anaphase cell, showing Polo kinase and RacGAP50C localised to the contractile ring (arrowheads, and see Fig SX) while Polo kinase is also present at the poles. (I-L) A telophase cell showing localisation of both Polo kinase and RacGAP50C to the contracted central spindle (arrowheads) while Polo kinase is also remains at the poles (arrows). Scale bar 5 $\mu$ m (L).

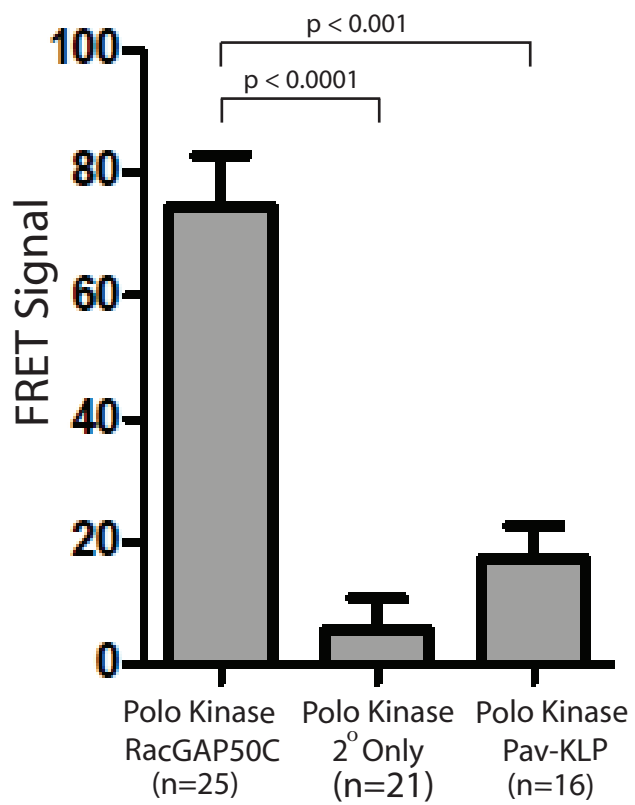
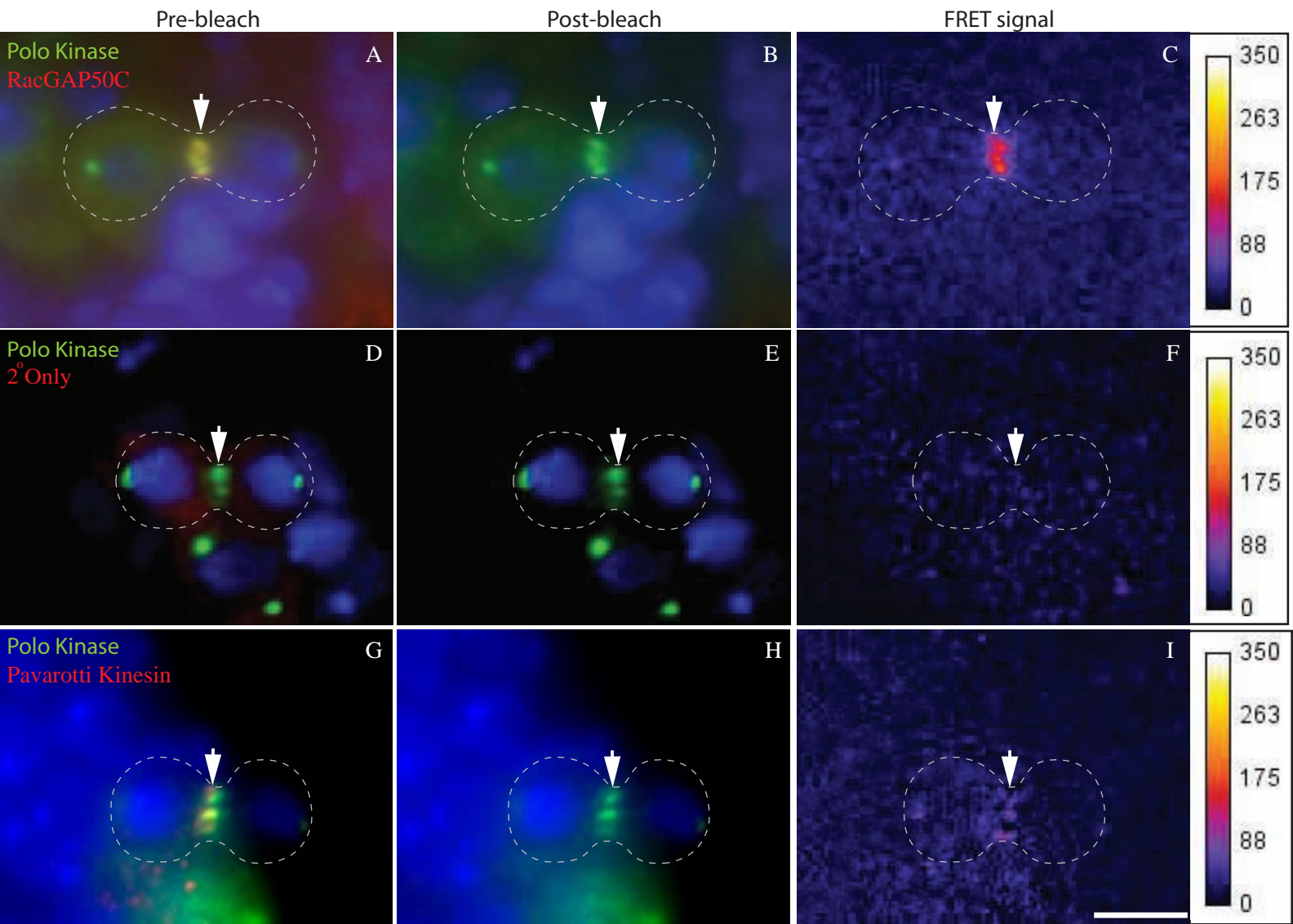
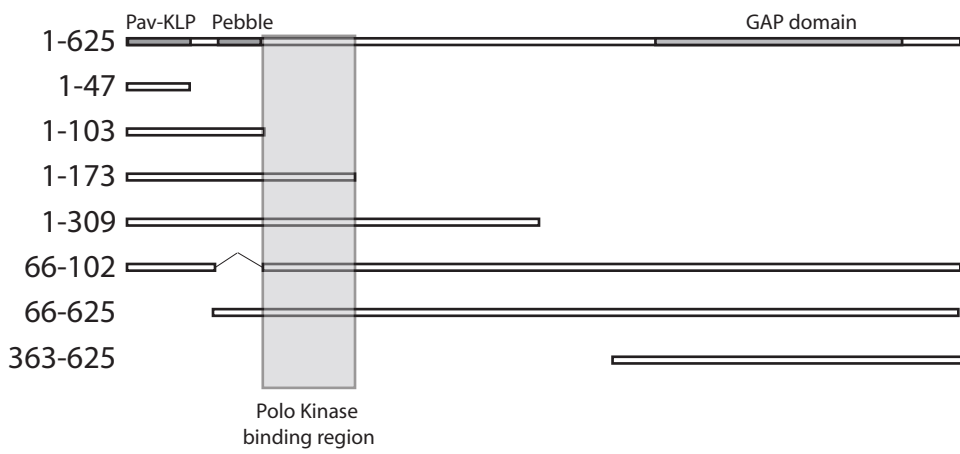


Figure 2. FRET Analysis of Polo kinase, RacGAP50C and Pav-KLP using Cy3 donor and Cy5 acceptor fluorophores.

Third instar larval brains were stained with anti-Polo kinase and a Cy3-labelled secondary antibody (green A, B, D, E, G, H), anti-RacGAP50C (A, B) or anti-Pav-KLP (G, H) and a Cy5-labelled secondary antibody (red) and Hoechst 33258 to visualize DNA (blue). (A) Cy3-labelled Polo kinase and Cy5-labelled RacGAP50C are localized to the cell equator (arrow) before bleaching. (B) After bleaching of the acceptor tag Cy5, only the Cy3 signal remains (arrow). (C) A FRET signal is observed at the cell equator. (D-F) Staining as for (A-B), except that the primary anti-RacGAP50C antibody is not included. (D) Cy3-labelled Polo kinase is present at the cell equator, with background Cy5 staining, (E) Bleaching has eliminated the Cy5 signal. (F) No FRET signal is observed. (G-I) Pav-KLP and Polo kinase show no FRET. (G) Pav-KLP and Polo kinase both localize to the cell equator. (H) Only the Polo kinase-Cy3 signal remains after bleaching of the Cy5-labelled Pav-KLP. (I) No FRET signal is observed. Scale bar 5 $\mu$ m. (J) Mean FRET signal for rings from the sample (n=25), cross-reactivity control (n=21) and concentration control (n=16) is shown as means with 95% CI obtained using the non-parametric Mann-Whitney test (unpaired, two tailed) (J).

# RacGAP50C

# Interaction with Polo Kinase



Kinase Domain	Intermediate Domain	Polo Box Domain
1	278	397
		576

Construct	Interaction
1-625	++
1-47	-
1-103	-
1-173	++
1-309	++
66-102	+
66-625	+
363-625	-

Figure 3. Yeast two-hybrid analysis of the RacGAP50C and Polo kinase interaction. RacGAP50C constructs in pVP16 vector (prey) were tested against the intermediate region (amino acids 276 to 391) of Polo kinase (bait). RacGAP50C constructs containing amino acids 1-47, 1-103 and 363-625 do not show any interaction with Polo kinase, while RacGAP50C constructs including amino acids 103-173 show strong interaction. The interaction is not disrupted by removal of the Pav-KLP (11-47) or Pebble (66-102) binding domains in RacGAP50C.

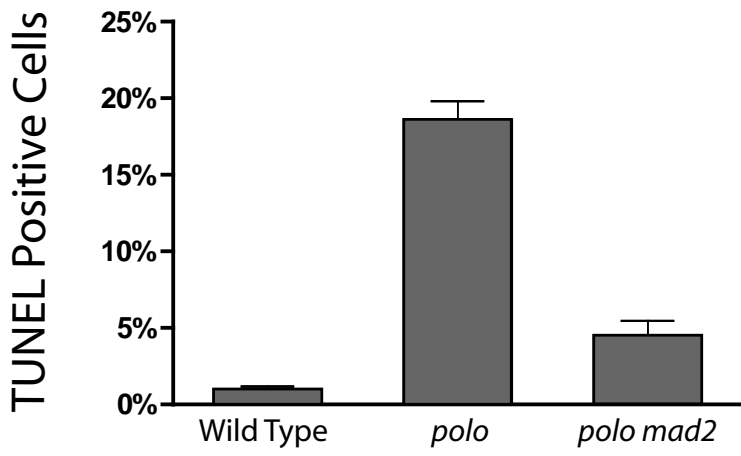
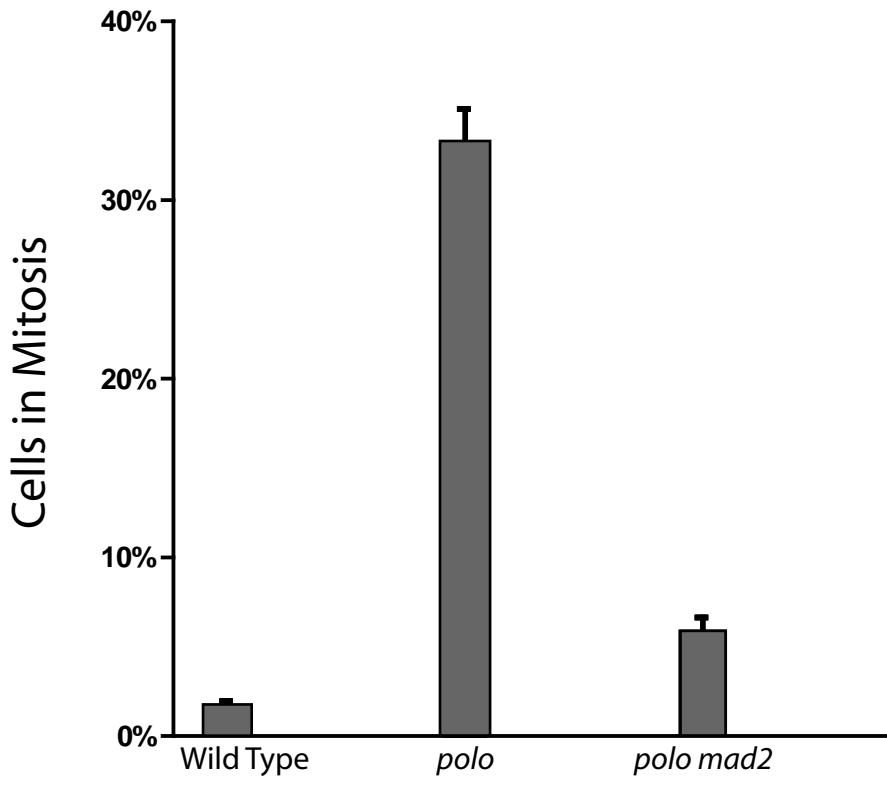




Figure 4. *polo mad2* double mutant cells exit mitosis.

(A) Third instar larval brains were stained with Hoechst and the percentage of cells in mitosis was calculated for *polo* mutants (33%, n=1777), *polo mad2* double mutants (5.8%, n=3082) and wild type cells (1%, n=6575). Note that in every case the *polo* mutants and double mutants used were trans-heterozygous for *polo*<sup>10</sup> over a Deficiency (see supplementary materials).

(B) Third instar larval brains were stained with TUNEL and the percentage of cells in apoptosis was calculated for *polo* mutants (18.5%, n=3415), *polo/mad2* double mutants (4.4%, n=1469) and wild types (0.9%, n=4267). The number of mitotic and TUNEL positive cells is shown as means with 95% CI obtained using the non-parametric Mann-Whitney test (unpaired, two tailed).

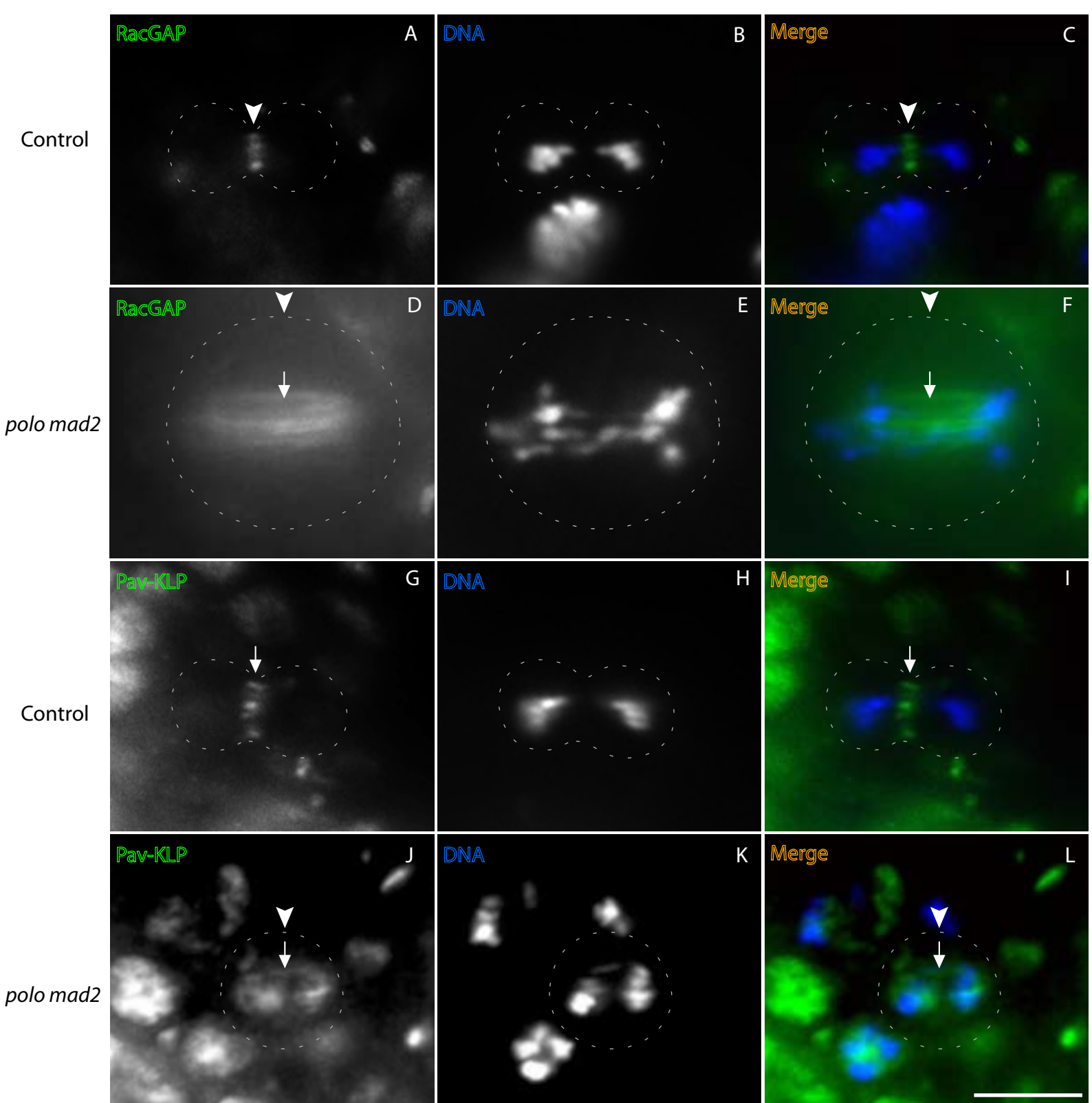


Figure 5. The centralspindlin complex does not localise to the equatorial cortex in the absence of Polo kinase.

Third instar larval brains were stained with anti-RacGAP50C (A, D and green in C, F) or anti-Pav-KLP (G, J and green in I, L) antibodies and Hoechst 33258 to detect DNA (B, E, H, K and blue in C, F, I, L). (A-C) A wild type anaphase cell showing RacGAP50C localisation to the cell equator (arrowheads). (D-F) A *polo mad2* mutant anaphase cell showing RacGAP50C localisation to microtubules connecting the two poles (arrows) but no equatorial or specific localization to the spindle midzone (arrowheads). (G-I) A wild type anaphase cell showing Pav-KLP localisation at the cell equator (arrowheads). (J-L) A *polo mad2* mutant anaphase cell showing Pav-KLP localisation to the microtubules connecting the two poles (arrows) but not at the equator or the spindle midzone (arrowheads).GAP50C.

## **CHAPTER IV**

### **Future directions**

#### **4. Significance of this study and future directions:**

The coordination of cytokinetic events requires a series of complex molecular interactions between the components of the Rho signalling pathway and the cytoskeleton. The Rho-signalling pathway is the main player in directing the cellular morphological transitions that take place during cytokinesis. Some of the interactions that need to take place between the components of the Rho signalling pathway for progression and completion of this final stage of cell division have been revealed. The outcomes of this PhD project have now added useful insights into how the acto-myosin based contractile ring is stabilised and how continuous RhoA activation is achieved for a successful cell division.

The first component of this PhD project provided strong evidence for the molecular mechanism behind continual RhoA activation and stable contractile ring constriction after initiation of cytokinesis (Figure 4). The results suggest that the contractile ring is connected to the microtubules via a direct and persistent interaction between Anillin and RacGAP50C (Figure 4). Anillin is a large protein that binds both actin and myosin while RacGAP50C binds microtubules, Pav-KLP, Pebble and Polo Kinase. The Anillin-bound portion of the contractile ring is then connected to the RacGAP50C-bound portion leading, I propose, to the continual availability of Pebble/ECT2 to activate RhoA function and connecting the contractile ring in a fixed and stable position to the microtubule-based central spindle complex. This in turn provides the requirements for stable and continual contractile ring constriction throughout anaphase and telophase. In the absence of Anillin, RacGAP50C is lost from the equatorial cortex and furrow ingression fails. These results provide a molecular link to account for the long unexplained phenomenon that upon moving the microtubules physically, the contractile ring disassembles and a new one assembles at the new site where microtubules meet (Rappaport, 1978) and that microtubules are continually

required for cytokinesis (Wheatley and Wang, 1996, Alsop and Zhang, 2003). They are also consistent with the observation that in Anillin RNAi expressing cells, the ring of active myosin slides to one side of the cell (Straight et al., 2005, Zhao and Fang, 2005). While this study provides further evidence for a persistent connection between the contractile ring and microtubules at the equatorial cortex, another connection vital to cytokinesis remains to be explained. When the ring constricts, the cell membrane at the site of furrow formation follows the contractile ring down to the intra-cellular bridge (Adams et al., 1998, Sisson et al., 2000). However, the link between the cell-membrane and the contractile ring is yet to be discovered. One possibility is that there is a link between the Anillin PH domain and the phospholipids that the domain binds to, or the link could be via Actin or other contractile ring components and components of the cell membrane. Membrane and actin dynamics at the furrow require the function of septins (Kinshita and Noda, 2001) and the Phosphatidylinositol 4-kinase *four wheel drive* (*fwd*) (Brill, et al 2000). It is possible that *fwd* mediates the interaction between Actin and Anillin with the cell membrane either directly or by regulating membrane components. All of these would be good candidates to test in FRET and genetic interaction assays to see if such linkages exist. It is plausible that a large protein such as Anillin may in fact act as a scaffolding protein that not only connects the contractile ring to the microtubule-based central spindle complex, but also provides a link between the cell membrane and the contractile ring via its binding to Actin, RacGAP50C and the cell membrane. Experiments such as testing for direct and genetic interactions between Anillin and other contractile ring components on the one hand and components of the cell membrane on the other hand may clarify the mechanism by which the cell membrane is connected to the contractile ring. This could then be verified by the removal of Anillin or any component that tests positive for an interaction to see if the

contractile ring and cell membrane connection is disrupted, although the multi-functionality of these proteins means that such tests will have to be carefully constructed.

The important finding from the second aim of this PhD project concerns the early role of Polo Kinase in cytokinesis. Due to the difficulty of looking at the role of Polo Kinase very early in cytokinesis because of its role in earlier cell cycle events, its essential role in localising the central spindlin complex to the equator has gone unnoticed (Figure 4). It is clear from this study that there is a direct interaction between Polo Kinase and RacGAP50C making Polo Kinase part of the Cytokinesis Initiation Complex. This interaction is via a previously un-identified domain of Polo Kinase and may not be dependent on prior phosphorylation events since an interaction involving the Polo Box domain was not detected. This therefore, adds a new region into the structure of Polo Kinase. It will be interesting to investigate whether this new region of Polo Kinase plays any role in events other than cytokinesis. This could be done by looking for interactions between this region and proteins in other stages of mitosis and by removing the domain and testing its ability to carry out the Polo kinase mitotic functions. To date, studies in mammals have involved inhibition of the kinase function of PLK1 after the onset of Anaphase at a time when PLK1 may have already initiated the localisation of those components to the equator. Other studies in which PLK1 function was inhibited at the end of Metaphase and cells were pushed through to Anaphase by inhibiting components of the spindle checkpoint machinery (Lénárt et al., 2007) showed no evidence of furrow formation, but did not investigate the early localisation of the central spindlin complex to the equator. In this study, bypassing the metaphase arrest in Polo Kinase mutant cells revealed that in *polo* mutant cells, RacGAP50C and Pav-KLP decorate the inter-polar microtubules but do not get to the equator. This provides strong support for the suggestion

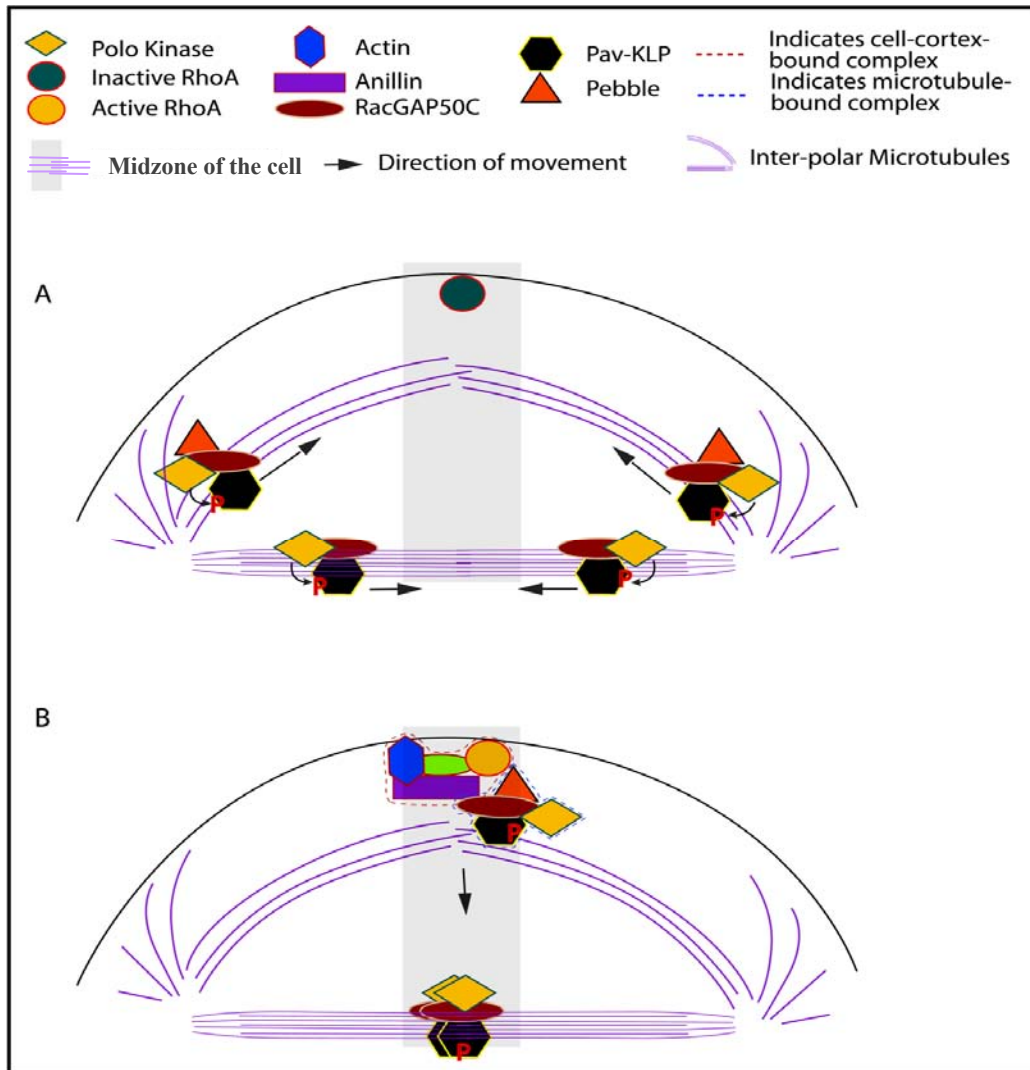
that Polo Kinase is required for the localisation of the Cytokinesis Initiation Complex to the site of contractile ring assembly at the cell equator. The absence of Pav-KLP localisation to equator mimics that of inactive (motor-dead) Pav-KLP (Minestrini et al., 2003). It will be interesting to see whether Pav-KLP motor-dead cells give the same RacGAP50C localisation phenotype and if Polo Kinase directly activates Pav-KLP motor activity, as studies suggest that PLK1 can phosphorylate Pav-KLP/MKLP1 in mammals (Liu et al., 2004). This can be done by first identifying the residues on Pav-KLP that would be phosphorylated by Polo Kinase using *in vitro* kinase assays and then removing that phosphorylation site to see if Pav-KLP still localises to the equator. It is already shown in mammals that PLK1 can phosphorylate hsCyk4 (RacGAP50C) and that this phosphorylation is required the binding of hsCyk4 and the Rho activator ECT2 (Pebble) and hence the subsequent activation of RhoA (Burkard et al., 2009). The results of this study together with the established roles of Polo Kinase in checkpoint control and in APC/C regulation raise the possibility that Polo Kinase is a key coordinator of the sequence of events that includes correct timing of anaphase onset and the subsequent initiation of cytokinesis.

In order to further characterize the possible role of Polo Kinase in the timing of entry into anaphase, it will also be of considerable interest to investigate whether Polo kinase regulates other known proteins at the onset of anaphase. This can be done by looking at functional activation and/or inhibition of proteins at the onset of anaphase and investigate whether Polo kinase is involved. Polo kinase phosphorylation of proteins prior to and throughout cytokinesis may be a means by which the timing and progress of cytokinesis is coordinated with the timing of the end of mitosis. For instance, it is possible that polo kinase phosphorylation of Pav-KLP (Liu et al., 2004) is required for Pav-KLP and RacGAP50C



interaction (Somers and Saint, 2003) to facilitate their localisation to the furrow, followed by Polo kinase phosphorylation of RacGAP50C (Burkard et al., 2009) mediating RacGAP50C and pebble interaction (Somers and Saint, 2003, Yuce et al., 2005) and the subsequent activation of RhoA. It will also be interesting to investigate if Polo Kinase phosphorylation of RacGAP50C is required for RacGAP50C deactivation of Rac to permit entry into anaphase (Canman et al., 2008) . Finally, there could be other Polo Kinase targets at the onset of anaphase. A combination of kinase assays and genetic interaction studies may provide valuable insight into these possible roles of Polo Kinase and would clarify the existence of a cross-talk between Polo kinase and RacGAP50C in the metaphase to anaphase transition and initiation of cytokinesis.

Finally, these findings along with previous studies suggest a model for the role of RacGAP50C in cytokinesis as a gene that performs three different consecutive functions: 1) Inhibition of Rac at the onset of anaphase which is required for cytokinesis (Canman et al., 2008) 2) Initiation of Cytokinesis by interacting with Polo Kinase and taking Pebble/ECT2 to activate RhoA and 3) ensuring a continued presence of RhoA signal and stable contractile ring constriction by interacting with Anillin.



**Figure 4 An updated model for initiation of cytokinesis and contractile ring stability.**

A) The cytokinesis initiation complex forms in anaphase by an interaction between Polo, RacGAP50C, Pav-KLP and Pebble. The Polo Kinase and RacGAP50C interaction brings Polo Kinase close to Pav-KLP. Polo Kinase then phosphorylates Pav-KLP (indicated by P) leading to localisation of the cytokinesis initiation complex to the equator.

B) At the equator, Pebble activates RhoA leading to contractile ring assembly and initiation of cytokinesis. Here, Anillin binds to Actin, Myosin and RhoA forming a complex bound to the cell cortex while RacGAP50C is bound to Pav-KLP, Polo Kinase and Pebble forming a complex that is associated with the inter-polar microtubules. An interaction between Anillin and RacGAP50C then anchors the ring to both the cell cortex and the cortical microtubules stabilising the contractile ring.

## References:

- Adams, R. R., Tavares, A. A. M., Salzberg, A., Bellen, H. J. & Glover, D. M. (1998) Pavarotti encodes a kinesin-like protein required to organize the central spindle and contractile ring for cytokinesis. *Genes Dev*, 12, 1483-1494.
- Afshar, K., Stuart, B. & Wasserman, S. (2000) Functional analysis of the *Drosophila* diaphanous FH protein in early embryonic development. *Development*, 127, 1887-1897.
- Alexandru, G., Uhlmann, F., Mechtler, K., Poupard, M.-A. & Nasmyth, K. (2001) Phosphorylation of the Cohesin Subunit Scc1 by Polo/Cdc5 Kinase Regulates Sister Chromatid Separation in Yeast. *Cell*, 105, 459-472.
- Alsop, G. B. & Zhang, D. (2003) Microtubules are the only structural constituent of the spindle apparatus required for induction of cell cleavage. *J. Cell Biol.*, 162, 383-390.
- Amano, M., Ito, M., Kimura, K., Fukata, Y., Chihara, K., Nakano, T., Matsuura, Y. & Kaibuchi, K. (1996) Phosphorylation and Activation of Myosin by Rho-associated Kinase (Rho-kinase). *J. Biol. Chem.*, 271, 20246-20249.
- Amon, A. (1999) The spindle checkpoint. *Curr Opin Genet Dev*, 9, 69-75.
- Antonarakis, S. & Van Aelst, L. (1998) Mind the GAP, Rho, Rab and GDI. *Nat Genet*, 19, 106-108.
- Bishop, A. L. & Hall, A. (2000) Rho GTPases and their effector proteins. *Biochem. J.*, 348, 241-255.
- Brennan, I. M., Peters, U., Kapoor, T. M. & Straight, A. F. (2007) Polo-Like Kinase Controls Vertebrate Spindle Elongation and Cytokinesis. *PLoS ONE*, 2, e409.
- Brill, J.A., Hime, G., Scharer-Schuksz, M. & Fuller, M. (2000) A phospholipid kinase regulates actin organization and intercellular bridge formation during germline cytokinesis. *Development*, 127, 3855-3864.
- Boddy M.N., and Russell, P., (2001) DNA replication checkpoint. *Current Biology*, 11, R953-R956
- Buffin, E., Emre, D. & Karess, R. E. (2007) Flies without a spindle checkpoint. *Nat Cell Biol*, 9, 565-572.
- Burkard, M. E., Randall, C. L., Laroche, S. P., Zhang, C., Shokat, K. M., Fisher, R. P. & Jallepalli, P. V. (2007) Chemical genetics reveals the requirement for Polo-like kinase 1 activity in positioning RhoA and triggering cytokinesis in human cells. *Proc Natl Acad Sci*, 104, 4383-4388.
- Burkard, M. E., Maciejowski, J., Rodriguez-Bravo, V., Repka, M., Lowery, D.M., Clauser, K.R., Zhang, C., Shokat, K.M., Carr, S.A., Yaffe, M.B. & Jallepalli, P.V. (2009) Plk1 Self-Organization and Priming Phosphorylation of HsCYK-4 at the Spindle Midzone Regulate the Onset of Division in Human Cells. *PLoS Biol*, 7, e100110.
- Burke, D. J. (2000) Complexity in the spindle checkpoint. *Curr Opin Genet Dev*, 10, 26-31.
- Canman, J. C., Lewellyn, L., Laband, K., Smerdon, S. J., Desai, A., Bowerman, B. & Oegema, K. (2008) Inhibition of Rac by the GAP Activity of Centralspindlin Is Essential for Cytokinesis. *Science*, 322, 1543-1546.
- Carmena, M., Riparbelli, M. G., Minestrini, G., Tavares, A. M., Adams, R., Callaini, G. & Glover, D. M. (1998) *Drosophila* Polo Kinase Is Required for Cytokinesis. *J. Cell Biol.*, 143, 659-671.
- Castrillon, D. H. & Wasserman, S. A. (1994) Diaphanous is required for cytokinesis in *Drosophila* and shares domains of similarity with the products of the limb deformity gene. *Development*, 120, 3367-3377.

- Chen, Y., Mills, J. D. & Periasamy, A. (2003) Protein localization in living cells and tissues using FRET and FLIM. *Differentiation*, 71, 528-541.
- Cheng, K.-Y., Lowe, E. D., Sinclair, J., Nigg, E. A. & Johnson, L. N. (2003) The crystal structure of the human polo-like kinase-1 polo box domain and its phospho-peptide complex. *EMBO J*, 22, 5757-5768.
- Clay, F. J., McEwen, S. J., Bertoncillo, I., Wilks, A. F. & Dunn, A. R. (1993) Identification and cloning of a protein kinase-encoding mouse gene, Plk, related to the polo gene of *Drosophila*. *Proc Natl Acad Sci U S A*, 90, 4882-4886.
- Clute, P. & Pines, J. (1999) Temporal and spatial control of cyclin B1 destruction in metaphase. *Nat Cell Biol*, 1, 82-87.
- D'Avino, P. P., Archambault, V., Przewloka, M. R., Zhang, W., Lilley, K. S., Laue, E. & Glover, D. M. (2007) Recruitment of Polo Kinase to the Spindle Midzone during Cytokinesis Requires the Feo/Klp3A Complex. *PLoS ONE*, 2, e572.
- D'Avino, P. P., Savoian, M. S., Capalbo, L. & Glover, D. M. (2006) RacGAP50C is sufficient to signal cleavage furrow formation during cytokinesis. *J Cell Sci*, 119, 4402-4408.
- Dai, B. N., Yang, Y., Chau, Z. & Jhanwar-Uniyal, M. (2007) Polo-like kinase 1 regulates RhoA during cytokinesis exit in human cells. *Cell Prolif*, 40, 550-557.
- Debidda, M., Wang, L., Zang, H., Poli, V. & Zheng, Y. (2005) A Role of STAT3 in Rho GTPase-regulated Cell Migration and Proliferation. *J. Biol. Chem.*, 280, 17275-17285.
- Donaldson, M. M., Tavares, A. A. M., Hagan, I. M., Nigg, E. A. & Glover, D. M. (2001) The mitotic roles of Polo-like kinase. *J Cell Sci*, 114, 2357-2358.
- Dubreuil, C. I., Winton, M. J. & Mckerracher, L. (2003) Rho activation patterns after spinal cord injury and the role of activated Rho in apoptosis in the central nervous system. *J. Cell Biol.*, 162, 233-243.
- Elia, A. E. H., Cantley, L. C. & Yaffe, M. B. (2003a) Proteomic Screen Finds pSer/pThr-Binding Domain Localizing Plk1 to Mitotic Substrates. *Science*, 299, 1228-1231.
- Elia, A. E. H., Rellos, P., Haire, L. F., Chao, J. W., Ivins, F. J., Hoepker, K., Mohammad, D., Cantley, L. C., Smerdon, S. J. & Yaffe, M. B. (2003b) The Molecular Basis for Phosphodependent Substrate Targeting and Regulation of Plks by the Polo-Box Domain. *Cell*, 115, 83-95.
- Elowe, S., Hammer, S., Uldschmid, A., Li, X. & Nigg, E. A. (2007) Tension-sensitive Plk1 phosphorylation on BubR1 regulates the stability of kinetochore-microtubule interactions. *Genes Dev*, 21, 2205-2219.
- Fang, G. (2002) Checkpoint Protein BubR1 Acts Synergistically with Mad2 to Inhibit Anaphase-promoting Complex. *Mol. Biol. Cell*, 13, 755-766.
- Förster, T. (1948) Intermolecular energy migration and fluorescence. *Ann Phys*, 6, 55-75.
- Gatti, M., Giansanti, M. G. & Bonaccorsi, S. (2000) Relationships between the central spindle and the contractile ring during cytokinesis in animal cells. *Micros Res Tech*, 49, 202-208.
- Giansanti, M. G., Bonaccorsi, S., Williams, B., Williams, E. V., Santolamazza, C., Goldberg, M. L. & Gatti, M. (1998) Cooperative interactions between the central spindle and the contractile ring during *Drosophila* cytokinesis. *Genes Dev.*, 12, 396-410.
- Giansanti, M. G., Farkas, R. M., Bonaccorsi, S., Lindsley, D. L., Wakimoto, B. T., Fuller, M. T. & Gatti, M. (2004) Genetic Dissection of Meiotic Cytokinesis in *Drosophila* Males. *Mol. Biol. Cell*, 15, 2509-2522.
- Glotzer, M. (2001) Animal Cell Cytokinesis. *Annu Rev Cell Dev Biol*, 17, 351-386.

- Glotzer, M. (2005) The Molecular Requirements for Cytokinesis. *Science*, 307, 1735-1739.
- Glotzer, M., Murray, A. W. & Kirschner, M. W. (1991) Cyclin is degraded by the ubiquitin pathway. *Nature*, 349, 132-138.
- Glover, D. M., Hagan, I. M., Tavares, A. & Lvaro, A. M. (1998) Polo-like kinases: a team that plays throughout mitosis. *Genes Dev*, 12, 3777-3787.
- Gorbsky, G. J. & Ricketts, W. A. (1993) Differential expression of a phosphoepitope at the kinetochores of moving chromosomes. *J. Cell Biol.*, 122, 1311-1321.
- Gunsalus, K., Bonaccorsi, S., Williams, E., Verni, F., Gatti, M. & Goldberg, M. (1995) Mutations in twinstar, a Drosophila gene encoding a cofilin/ADF homologue, result in defects in centrosome migration and cytokinesis. *J. Cell Biol.*, 131, 1243-1259.
- Hagting, A., Den Elzen, N., Vodermaier, H. C., Waizenegger, I. C., Peters, J.-M. & Pines, J. (2002) Human securin proteolysis is controlled by the spindle checkpoint and reveals when the APC/C switches from activation by Cdc20 to Cdh1. *J. Cell Biol.*, 157, 1125-1137.
- Hamanaka, R., Maloid, S., Smith, M. R., O'connell, C. D., Longo, D. L. & Ferris, D. K. (1994) Cloning and characterization of human and murine homologues of the Drosophila polo serine-threonine kinase. *Cell Growth Differ*, 5, 249-257.
- Harper, J. W., Burton, J. L. & Solomon, M. J. (2002) The anaphase-promoting complex: it's not just for mitosis any more. *Genes Dev*, 16, 2179-2206.
- Hilioti, Z., Chung, Y.-S., Mochizuki, Y., Hardy, C. F. J. & Cohen-Fix, O. (2001) The anaphase inhibitor Pds1 binds to the APC/C-associated protein Cdc20 in a destruction box-dependent manner. *Curr Biol*, 11, 1347-1352.
- Hirose, K., Kawashima, T., Iwamoto, I., Nosaka, T. & Kitamura, T. (2001) MgcRacGAP Is Involved in Cytokinesis through Associating with Mitotic Spindle and Midbody. *J. Biol. Chem.*, 276, 5821-5828.
- Hochegger, H., Takeda, S., and Hunt, T (2008) Cyclin-dependent kinases and cell-cycle transitions: does one fit all? *Nat Rev Mol Cell Biol*, 9, 910-916.
- Hutchison, C., Glover D.M., eds. (1995) Cell cycle control. New York: Oxford University Press.
- Jantsch-Plunger, V., Gonczy, P., Romano, A., Schnabel, H., Hamill, D., Schnabel, R., Hyman, A. A. & Glotzer, M. (2000) CYK-4: A Rho Family GTPase Activating Protein (GAP) Required for Central Spindle Formation and Cytokinesis. *J. Cell Biol.*, 149, 1391-1404.
- Kamijo, K., Ohara, N., Abe, M., Uchimura, T., Hosoya, H., Lee, J.-S. & Miki, T. (2006) Dissecting the Role of Rho-mediated Signaling in Contractile Ring Formation. *Mol Biol Cell*, 17, 43-55.
- Kenworthy, A. K. (2001) Imaging Protein-Protein Interactions Using Fluorescence Resonance Energy Transfer Microscopy. *Methods*, 24, 289-296.
- King, R. W., Deshaies, R. J., Peters, J.-M. & Kirschner, M. W. (1996) How Proteolysis Drives the Cell Cycle. *Science*, 274, 1652-1659.
- King, R. W., Peters, J.-M., Tugendreich, S., Rolfe, M., Hieter, P. & Kirschner, M. W. (1995) A 20s complex containing CDC27 and CDC16 catalyzes the mitosis-specific conjugation of ubiquitin to cyclin B. *Cell*, 81, 279-288.
- Kinoshita, M., & Noda, m. (2001) Roles of Septins in the Mammalian Cytokinesis Machinery. *Cell Struct Funct*, 26, 667-670
- Konig, P., Krasteva, G., Tag, C., Konig, I. R., Arens, C. & Kummer, W. (2006) FRET-CLSM and double-labeling indirect immunofluorescence to detect close association of proteins in tissue sections. *Lab Invest*, 86, 853-864.
- Lee, J.-S., Kamijo, K., Ohara, N., Kitamura, T. & Miki, T. (2004) MgcRacGAP regulates cortical activity through RhoA during cytokinesis. *Exp Cell Res*, 293, 275-282.

- Lénárt, P., Petronczki, M., Steegmaier, M., Di Fiore, B., Lipp, J. J., Hoffmann, M., Rettig, W. J., Kraut, N. & Peters, J.-M. (2007) The Small-Molecule Inhibitor BI 2536 Reveals Novel Insights into Mitotic Roles of Polo-like Kinase 1. *Curr Biol*, 17, 304-315.
- Li J.M., and Brooks, G. (1999) Cell cycle regulatory molecules (cyclins, cyclin-dependent kinases and cyclin-dependent kinase inhibitors) and the cardiovascular system Potential targets for therapy? *Eur Heart J*, 20, 406–420.
- Li, X. & Nicklas, R. B. (1995) Mitotic forces control a cell-cycle checkpoint. *Nature*, 373, 630-632.
- Liu, X., Zhou, T., Kuriyama, R. & Erikson, R. L. (2004) Molecular interactions of Polo-like-kinase 1 with the mitotic kinesin-like protein CHO1/MKLP-1. *J Cell Sci*, 117, 3233-3246.
- Llamazares, S., Moreira, A., Tavares, A., Girdham, C., Spruce, B. A., Gonzalez, C., Karess, R. E., Glover, D. M. & Sunkel, C. E. (1991) polo encodes a protein kinase homolog required for mitosis in Drosophila. *Genes Dev*, 5, 2153-2165.
- Lowery, D. M., Clauser, K. R., Hjerrild, M., Lim, D., Alexander, J., Kishi, K., Ong, S., Gammeltoft, S., Carr, S. A. & Yaffe, M. B. (2007) Proteomic screen defines the Polo-box domain interactome and identifies Rock2 as a Plk1 substrate. *EMBO J*, 26, 2262–2273.
- Mabuchi, I., Hamaguchi, Y., Fujimoto, H., Morii, N., Mishima, M. & Narumiya, S. (1993) A rho-like protein is involved in the organisation of the contractile ring in dividing sand dollar eggs. *Zygote*, 1, 325-331.
- Madaule, P., Furuyashiki, T., Reid, T., Ishizaki, T., Watanabe, G., Morii, N. & Narumiya, S. (1995) A novel partner for the GTP-bound forms of rho and rac. *FEBS Lett*, 377, 243-248.
- Malliri, A. & Collard, J. G. (2003) Role of Rho-family proteins in cell adhesion and cancer. *Curr Opin Cell Biol*, 15, 583-589.
- Mastronarde, D., McDonald, K., Ding, R. & McIntosh, J. (1993) Interpolar spindle microtubules in PTK cells. *J. Cell Biol.*, 123, 1475-1489.
- Matsumura, S., Toyoshima, F. & Nishida, E. (2007) Polo-like Kinase 1 Facilitates Chromosome Alignment during Prometaphase through BubR1. *J Biol Chem.*, 282, 15217-15227.
- Miller, K. G., Field, C. M. & Alberts, B. M. (1989) Actin-binding proteins from Drosophila embryos: a complex network of interacting proteins detected by F-actin affinity chromatography. *J. Cell Biol.*, 109, 2963-2975.
- Minestrini, G., Harley, A. S. & Glover, D. M. (2003) Localization of Pavarotti-KLP in Living Drosophila Embryos Suggests Roles in Reorganizing the Cortical Cytoskeleton during the Mitotic Cycle. *Mol Biol Cell*, 14, 4028–4038.
- Minoshima, Y., Kawashima, T., Hirose, K., Tonzuka, Y., Kawajiri, A., Bao, Y. C., Deng, X., Tatsuka, M., Narumiya, S., May Jr, W. S., Nosaka, T., Semba, K., Inoue, T., Satoh, T., Inagaki, M. & Kitamura, T. (2003) Phosphorylation by Aurora B Converts MgcRacGAP to a RhoGAP during Cytokinesis. *Dev Cell*, 4, 549-560.
- Mishima, M. & Glotzer, M. (2003) Cytokinesis: a logical GAP. *Curr Biol*, 13, R589-R591.
- Mishima, M., Kaitna, S. & Glotzer, M. (2002) Central Spindle Assembly and Cytokinesis Require a Kinesin-like Protein/RhoGAP Complex with Microtubule Bundling Activity. *Dev Cell*, 2, 41-54.
- Molinari, M., (2001) Cell cycle checkpoints and their inactivation in human cancer. *Cell Prolif*, 33, 261-274.
- Moon, S. Y. & Zheng, Y. (2003) Rho GTPase-activating proteins in cell regulation. *Trends Cell Biol*, 13, 13-22.

- Nasmyth, K., Peters, J.-M. & Uhlmann, F. (2000) Splitting the Chromosome: Cutting the Ties That Bind Sister Chromatids. *Science*, 288, 1379-1384.
- Neef, R., Preisinger, C., Sutcliffe, J., Kopajtich, R., Nigg, E. A., Mayer, T. U. & Barr, F. A. (2003) Phosphorylation of mitotic kinesin-like protein 2 by polo-like kinase 1 is required for cytokinesis. *J. Cell Biol.*, 162, 863-876.
- Nicklas, R. B., Ward, S. C. & Gorbsky, G. J. (1995) Kinetochore chemistry is sensitive to tension and may link mitotic forces to a cell cycle checkpoint. *J. Cell Biol.*, 130, 929-939.
- Nigg, E. A. (1998) Polo-like kinases: positive regulators of cell division from start to finish. *Curr Opin Cell Biol*, 10, 776-783.
- Nishimura, Y. & Yonemura, S. (2005) Centralspindlin regulates ECT2 and RhoA accumulation at the equatorial cortex during cytokinesis. *J Cell Sci*, 119, 104-114.
- Nislow, C., Lombillo, V. A., Kuriyama, R. & McIntosh, J. R. (1992) A plus-end-directed motor enzyme that moves antiparallel microtubules in vitro localizes to the interzone of mitotic spindles. *Nature*, 359, 543-547.
- Ohkura, H., Hagan, I. M. & Glover, D. M. (1995) The conserved *Schizosaccharomyces pombe* kinase *plp1*, required to form a bipolar spindle, the actin ring, and septum, can drive septum formation in G1 and G2 cells. *Genes Dev*, 9, 1059-1073.
- Olofsson, B. (1999) Rho Guanine Dissociation Inhibitors: Pivotal Molecules in Cellular Signalling. *Cell Signal*, 11, 545-554.
- Periasamy, A. (2001) Fluorescence resonance energy transfer microscopy: a mini review. *J Biomed Opt*, 6, 287-291.
- Peters, J.-M. (2002) The Anaphase-Promoting Complex: Proteolysis in Mitosis and Beyond. *Mol Cell*, 9, 931-943.
- Petronczki, M., Glotzer, M., Kraut, N. & Peters, J.-M. (2007) Polo-like Kinase 1 Triggers the Initiation of Cytokinesis in Human Cells by Promoting Recruitment of the RhoGEF Ect2 to the Central Spindle. *Dev Cell*, 12, 713-725.
- Pfleger, C. M. & Kirschner, M. W. (2000) The KEN box: an APC recognition signal distinct from the D box targeted by Cdh1. *Genes Dev*, 14, 655-665.
- Piekny, A., Werner, M. & Glotzer, M. (2005) Cytokinesis: welcome to the Rho zone. *Trends Cell Biol*, 15, 651-658.
- Piekny, A. J. & Glotzer, M. (2008) Anillin Is a Scaffold Protein That Links RhoA, Actin, and Myosin during Cytokinesis. *Curr Biol*, 18, 30-36.
- Pietenpol, J.A., and Stewart, Z.A., (2002) Cell cycle checkpoint signalling: cell cycle arrest versus apoptosis. *Toxicology*, 181-182:475-81.
- Pollard, T. D. (2007) Regulation of Actin Filament Assembly by Arp2/3 Complex and Formins. *Annu Rev Biophys Biomol Struct*, 36, 451-477.
- Powers, J., Bossinger, O., Rose, D., Strome, S. & Saxton, W. (1998) A nematode kinesin required for cleavage furrow advancement. *Curr Biol*, 8, 1133-1136.
- Prokopenko, S. N., Brumby, A., O'keefe, L., Prior, L., He, Y., Saint, R. & Bellen, H. J. (1999) A putative exchange factor for Rho1 GTPase is required for initiation of cytokinesis in *Drosophila*. *Genes Dev.*, 13, 2301-2314.
- Raftopoulou, M. & Hall, A. (2004) Cell migration: Rho GTPases lead the way. *Dev Biol*, 265, 23-32.
- Raich, W. B., Moran, A. N., Rothman, J. H. & Hardin, J. (1998) Cytokinesis and Midzone Microtubule Organization in *Caenorhabditis elegans* Require the Kinesin-like Protein ZEN-4. *Mol. Biol. Cell*, 9, 2037-2049.
- Rappaport, R. (1978) Effects of continual mechanical agitation prior to cleavage in echinoderm eggs. *J Exp Zool*, 206, 1-11.

- Rappaport, R. (1997) Cleavage furrow establishment by the moving mitotic apparatus. *Dev Growth Differ*, 2, 221-6.
- Reynolds, N. & Ohkura, H. (2003) Polo boxes form a single functional domain that mediates interactions with multiple proteins in fission yeast polo kinase. *J Cell Sci*, 116, 1377-1387.
- Russell, P., (1998) Checkpoints on the road to mitosis. *Trends Biochem Sci*, 23, 399-402.
- Santamaria, A., Neef, R., Eberspacher, U., Eis, K., Husemann, M., Mumberg, D., Prectl, S., Schulze, V., Siemeister, G., Wortmann, L., Barr, F. A. & Nigg, E. A. (2007) Use of the Novel Plk1 Inhibitor ZK-Thiazolidinone to Elucidate Functions of Plk1 in Early and Late Stages of Mitosis. *Mol. Biol. Cell*, 18, 4024-4036.
- Satterwhite, L. L. & Pollard, T. D. (1992) Cytokinesis. *Curr Opin Cell Biol*, 4, 43-52.
- Saxton, W. M. & Mcintosh, J. R. (1987) Interzone microtubule behavior in late anaphase and telophase spindles.
- Schmidt, A. & Hall, A. (2002) Guanine nucleotide exchange factors for Rho GTPases: turning on the switch. *Genes Dev*, 16, 1587-1609.
- Seong, Y.-S., Kamijo, K., Lee, J.-S., Fernandez, E., Kuriyama, R., Miki, T. & Lee, K. S. (2002) A Spindle Checkpoint Arrest and a Cytokinesis Failure by the Dominant-negative Polo-box Domain of Plk1 in U-2 OS Cells. *J. Biol. Chem.*, 277, 32282-32293.
- Shirayama, M., Toth, A., Galova, M. & Nasmyth, K. (1999) APC<sup>Cdc20</sup> promotes exit from mitosis by destroying the anaphase inhibitor Pds1 and cyclin Clb5. *Nature*, 402, 203-207.
- Sisson, J. C., Field, C., Ventura, R., Royou, A. & Sullivan, W. (2000) Lava Lamp, a Novel Peripheral Golgi Protein, Is Required for *Drosophila melanogaster* Cellularization. *J Cell Biol*, 151, 905-918.
- Skoufias, D. A., Andreassen, P. R., Lacroix, F. O. B., Wilson, L. & Margolis, R. L. (2001) Mammalian mad2 and bub1/bubR1 recognize distinct spindle-attachment and kinetochore-tension checkpoints. *Proc Natl Acad Sci U S A*, 98, 4492-4497.
- Somers, W. G. & Saint, R. (2003) A RhoGEF and Rho Family GTPase-Activating Protein Complex Links the Contractile Ring to Cortical Microtubules at the Onset of Cytokinesis. *Dev Cell*, 4, 29-39.
- Straight, A. F., Field, C. M. & Mitchison, T. J. (2005) Anillin Binds Nonmuscle Myosin II and Regulates the Contractile Ring. *Mol Biol Cell*, 16, 193-201.
- Sudakin, V., Chan, G. K. T. & Yen, T. J. (2001) Checkpoint inhibition of the APC/C in HeLa cells is mediated by a complex of BUBR1, BUB3, CDC20, and MAD2. *J. Cell Biol.*, 154, 925-936.
- Sunkel, C. E. & Glover, D. M. (1988) Polo, a mitotic mutant of *Drosophila* displaying abnormal spindle poles. *J Cell Sci*, 89, 25-38.
- Takai, Y., Sasaki, T. & Matozaki, T. (2001) Small GTP-Binding Proteins. *Physiol. Rev.*, 81, 153-208.
- Tan, J. L., Ravid, S. & Spudis, J. A. (1992) Control of Nonmuscle Myosin by Phosphorylation. *Annu. Rev. Biochem*, 61, 721-59.
- Tatsumoto, T., Xie, X., Blumenthal, R., Okamoto, I. & Miki, T. (1999) Human ECT2 Is an Exchange Factor for Rho GTPases, Phosphorylated in G2/M Phases, and Involved in Cytokinesis. *J. Cell Biol.*, 147, 921-928.
- Townsley, F. M. & Ruderman, J. V. (1998) Proteolytic ratchets that control progression through mitosis. *Trends Cell Biol*, 8, 238-244.
- Trotter, J. A. & Adelstein, R. S. (1979) Macrophage myosin. Regulation of actin-activated ATPase, activity by phosphorylation of the 20,000-dalton light chain. *J. Biol. Chem.*, 254, 8781-8785.



- Uhlmann, F., Lottspeich, F. & Nasmyth, K. (1999) Sister-chromatid separation at anaphase onset is promoted by cleavage of the cohesin subunit Scc1. *Nature*, 400, 37-42.
- Waizenegger, I. C., Giménez-Abián, J. F., Wernic, D. & Peters, J.-M. (2002) Regulation of Human Separase by Securin Binding and Autocleavage. *Curr Biol*, 12, 1368-1378.
- Watanabe, N., Madaule, P., Reid, T., Ishizaki, T., Watanabe, G., Kakizuka, A., Saito, Y., Nakao, K., Jockusch, B. M. & Narumiya, S. (1997) p140mDia, a mammalian homolog of *Drosophila* diaphanous, is a target protein for Rho small GTPase and is a ligand for profilin. *EMBO J*, 16, 3044–3056.
- Wheatley, S. P. & Wang, Y. (1996) Midzone microtubule bundles are continuously required for cytokinesis in cultured epithelial cells. *J. Cell Biol.*, 135, 981-989.
- Wong, O. K. & Fang, G. (2007) Cdk1 phosphorylation of BubR1 controls spindle checkpoint arrest and Plk1-mediated formation of the 3F3/2 epitope. *J. Cell Biol.*, 179, 611-617.
- Yamada, T., Hikida, M. & Kurosaki, T. (2006) Regulation of cytokinesis by mgcRacGAP in B lymphocytes is independent of GAP activity. *Exp Cell Res*, 312, 3517-3525.
- You, X., Nguyen, A. W., Jabaiah, A., Sheff, M. A., Thorn, K. S. & Daugherty, P. S. (2006) Intracellular protein interaction mapping with FRET hybrids. *Proc Natl Acad Sci*, 103, 18458-18463.
- Yu, H. (2002) Regulation of APC-Cdc20 by the spindle checkpoint. *Curr Opin Cell Biol*, 14, 706-714.
- Yuan, X.-B., Jin, M., Xu, X., Song, Y.-Q., Wu, C.-P., Poo, M.-M. & Duan, S. (2003) Signalling and crosstalk of Rho GTPases in mediating axon guidance. *Nat Cell Biol*, 5, 38-45.
- Yuce, O., Piekny, A. & Glotzer, M. (2005) An ECT2-centralspindlin complex regulates the localization and function of RhoA. *J. Cell Biol.*, 170, 571-582.
- Zavortink, M., Contreras, N., Addy, T., Bejsovec, A. & Saint, R. (2005) Tum/RacGAP50C provides a critical link between anaphase microtubules and the assembly of the contractile ring in *Drosophila melanogaster*. *J Cell Sci*, 118, 5381-5392.
- Zhao, W.-M. & Fang, G. (2005) MgcRacGAP controls the assembly of the contractile ring and the initiation of cytokinesis. *Proc Natl Acad Sci U S A*, 102, 13158-13163.

## **SUPPLEMENTAL MATERIALS ON INCLUDED CD**

### **Video SV1**

A wild type dividing 3rd instar larval brain cell expressing Venus-tagged RacGAP. RacGAP can be seen localised to the site of cleavage furrow formation which then constricts down to a midbody.

### **Video SV2**

A *polo Mad2* double mutant dividing 3rd instar larval brain cell expressing Venus-tagged RacGAP. RacGAP decorates the spindle microtubules and never localises to the equator.

**Figure S1.** RacGAP50C localizes to the contractile ring in dividing neuroblast cells.

Wild-type third instar larval brains undergoing anaphase were stained for actin (A and red in D) to visualise the contractile ring, RacGAP50C (B and green in D) and DAPI (C and blue in D) to visualize DNA. RacGAP50C co-localizes with actin at the cell cortex in the contractile ring (D).

**Figure S2.** Analysis of mutations to specifically disrupt the interaction between

RacGAP50C and Polo Kinase. A) Constructs of RacGAP50C with deletion of or mutations at specific residues were tested by yeast Two-hybrid assay for their interaction with Polo Kinase, Pav-KLP and Pebble. Three RacGAP50C constructs containing deletions of amino acids 149 to 155, 104 to 168 and 1 to 363 did not bind Polo Kinase. However, these constructs also did not bind Pav-KLP. B) Mutating two conserved amino acid residues in the intermediate domain of Polo Kinase (369 and 370 KP to AA) abolished the interaction between Polo Kinase and RacGAP50C. C) Mutation of residues 369-370 disrupts localization of Polo-GFP in dividing cells.

Stage 10 embryo expressing Polo(KP→AA)-GFP stained for GFP (A and green in C) and Phospho-Histone3 (B and red in C) shows no localization of mutant Polo to the spindle poles or cleavage furrow (arrowed). In contrast, Polo-GFP retaining wild type residues 369-370 localizes normally and rescues cytokinesis in *polo mad2* mutant 3<sup>rd</sup> instar larval brain cells (D-F).

**Figure S3.** A) FACS analysis of wild type and *polo mad2* mutant third instar larval brains. Dissociated brain cells were stained with Hoechst 33342 and their DNA content analysed by flow cytometry. This is a representative plot from four analyses, each containing five brains. Wild type cells (black line) show a large population with unreplicated chromosomes (marked G1) and a small peak showing cells that have replicated DNA but not yet divided (G2). In *polo mad2* brains (dashed red line) many cells contain sub-G1 amounts of DNA, representing apoptotic cells and debris. There is an additional peak of cells with greater than G2 amounts of DNA, representing hyperploid cells that have failed division and re-replicated the DNA. B) Percentage of *polo mad2* mutant cells in each stage of mitosis compared to that of wild-type cells. *polo mad2* and wild-type neuroblast cells were stained with anti-Tubulin to identify microtubules and Hoechst to visualize DNA. For each of the samples, the percentage of cells in each of the stages of metaphase, anaphase and telophase per field of view was graphed as mean with 95% CI.

**Figure S4.** RacGAP50C and Pav-KLP localise normally in *mad2* mutant cells.

Third instar larval brains mutant for *mad2* undergoing anaphase were stained for RacGAP50C (A and red in C), Pav-KLP (D and red in F) and DAPI (B, E, and green in C and F) to visualize visualize DNA. Both RacGAP50C and Pav-KLP localize to the midzone of the cell between the dividing daughter nuclei (in C and F respectively).

**Figure S5.** Microtubule structure is not affected in *polo mad2* double mutants.

Third instar larval brains double mutant for *polo* and *mad2* were stained with anti-tubulin antibody (red) to visualize the microtubules, and anti-PH3 antibody (Blue) to visualize DNA in dividing cells. A) Metaphase. A relatively normal bipolar spindle (arrows) is formed in *polo mad2*mutants (lower panels) compared to heterozygous controls (upper panels). B) Anaphase. *polo mad2*mutant cells (lower panels) undergo anaphase, attempting to separate the chromosomes using a microtubule spindle that is not drastically defective at this stage compared to heterozygous controls (upper panels).