

## Erratum

# **Genetic interactions between Cdk1-CyclinB and the Separase complex in *Drosophila***

Jun-Yuan Ji, Justin Crest and Gerold Schubiger *Development* **132**, 1875-1884.

Two errors on p. 1876 were not corrected before going to press.

The last paragraph of the Introduction should start, ‘The investigations of the Pim-Thr-Sse complex...’.

In the Materials and methods, under the sub-heading ‘Stocks’, the last mention of *histone-GFP* should read ‘*histone-GFP/+*’.

We apologise to the authors and readers for these mistakes.

# Genetic interactions between Cdk1-CyclinB and the Separase complex in *Drosophila*

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## Summary

Cdk1-CycB plays a key role in regulating many aspects of cell-cycle events, such as cytoskeletal dynamics and chromosome behavior during mitosis. To investigate how Cdk1-CycB controls the coordination of these events, we performed a dosage-sensitive genetic screen, which is based on the observations that increased maternal CycB (four extra gene copies) leads to higher Cdk1-CycB activity in early *Drosophila* embryos, delays anaphase onset, and generates a sensitized non-lethal phenotype at the blastoderm stage (defined as *six cycB* phenotype). Here, we report that mutations in the gene *three rows* (*thr*) enhance, while mutations in *pimples* (*pim*, encoding *Drosophila* Securin) or *separase* (*Sse*) suppress, the sensitized phenotype. In *Drosophila*, both *Pim* and *Thr* are known to regulate *Sse* activity, and activated *Sse* cleaves a Cohesin

subunit to initiate anaphase. Compared with the *six cycB* embryos, reducing *Thr* in embryos with more CycB further delays the initiation of anaphase, whereas reducing either *Pim* or *Sse* has the opposite effect. Furthermore, nuclei move slower during cortical migration in embryos with higher Cdk1-CycB activity, whereas reducing either *Pim* or *Sse* suppresses this phenotype by causing a novel nuclear migration pattern. Therefore, our genetic screen has identified all three components of the complex that regulates sister chromatid separation, and our observations indicate that interactions between Cdk1-CycB and the *Pim*-*Thr*-*Sse* complex are dosage sensitive.

Key words: Cdk1-CycB, *three rows*, Separase, Anaphase, *Drosophila*

## Introduction

In all eukaryotic cells, the Cohesin complex holds sister chromatids together until its removal initiates anaphase (Haering and Nasmyth, 2003). Sister chromatids are pulled apart either by shortening of spindle microtubules at the centrosomal region leading to poleward microtubule movement, or by disassembly of spindle microtubules at kinetochores (Maddox et al., 2002; Rogers et al., 2004). Defects of chromatid separation in somatic cells often lead to aneuploidy, which is associated with abnormal development and various cancers (Bharadwaj and Yu, 2004; Jallepalli and Lengauer, 2001). Defects of chromosomal separation in the germ line (during meiosis) may result in genetic diseases such as Down's syndrome (Petronczki et al., 2003; Pihan and Doxey, 2003). Therefore, accurate control of chromosomal separation is crucial for faithful transmission of genetic material in dividing cells.

Because Separase is the enzyme that disrupts the Cohesin complex, the reliability of chromatid separation depends on the precise control of Separase activity. One widely accepted model to explain how Separase activity is regulated is that Separase activity is inactive when bonded to Securin but is activated when Securin is degraded by the 26S proteasome. This process requires poly-ubiquitination of Securin by a highly conserved ubiquitin protein ligase APC/C (anaphase-promoting complex or cyclosome) (Murray, 2004; Zachariae and Nasmyth, 1999). This model is based on many genetic and

biochemical studies using yeasts, *Xenopus* egg extracts and mammalian cell lines (Haering and Nasmyth, 2003; Harper et al., 2002). However, several observations do not support the idea that Separase activity is only regulated by the inhibition of Securin. For example, the budding yeast Securin (*Pds1*) null mutation causes chromatid separation defects without cell cycle arrest (Yamamoto et al., 1996; Shirayama et al., 1999). Non-degradable *Drosophila* CycA causes metaphase arrest without inhibiting Securin (*Pim*) degradation (Leismann and Lehner, 2003), thus Securin destruction is not sufficient to induce anaphase in *Drosophila*. Furthermore, three types of mitotic cyclins in *Drosophila* are also degraded in succession: CycA is degraded at metaphase, CycB during anaphase and CycB3 during telophase (Huang and Raff, 1999; Jacobs et al., 1998; Lehner and O'Farrell, 1989; Parry and O'Farrell, 2001; Sigrist et al., 1995). Non-degradable cyclins result in blockage of cell-cycle progression at stages when the cyclins are normally degraded (Parry and O'Farrell, 2001; Sigrist et al., 1995; Su et al., 1998). Therefore, in addition to Separase activity regulated by Securin, the order of degradation of mitotic cyclins must also control proper anaphase initiation (Follette and O'Farrell, 1997; Parry and O'Farrell, 2001).

There are observations indicating that Separase activity is inhibited by Cdk1-CycB1 modification on Separase. For example, in *Xenopus*, a slightly above the physiological level of non-degradable CycB1 causes metaphase arrest because Cdk1-CycB1 either directly or indirectly phosphorylates

Separase, thereby inhibiting Separase activity (Stemmann et al., 2001). Similar observations have been made with Australian rat kangaroo PtK1 cells, and it was estimated that a 1.5- to 2-fold excess of CycB1 inhibits sister chromatid separation even when Securin degradation occurs (Hagting et al., 2002). Thus, it is likely that Separase activity is inhibited by both phosphorylation and binding to Securin in vertebrate cells (Hagting et al., 2002; Stemmann et al., 2001). However, it is not known whether Cdk1-CycA or Cdk1-CycB in *Drosophila* can modify Separase activity in a similar way.

In *Drosophila*, a novel protein Thr (encoded by *three rows*) is involved in regulating Separase (Sse) activity and Cohesin cleavage. *thr* mutant embryos have reduced rows of epidermal denticles, presumably caused by cell division defects (Nüsslein-Volhard et al., 1984). Thr protein forms a trimeric protein complex with Sse and Securin (Pim, encoded by *pimples*) (Leismann et al., 2000; Herzig et al., 2002). This three-protein complex is present but inactive during interphase and metaphase (Leismann et al., 2000; Jäger et al., 2001). Sse activation occurs when Pim is degraded shortly before anaphase begins. The Thr-Sse heterodimer is now active and presumably cleaves Drad21/Sccl, a subunit of the Cohesin complex in *Drosophila* (Vass et al., 2003; Warren et al., 2000). After chromatids separate, Thr is cleaved by Sse, which presumably inhibits Sse activity (Herzig et al., 2002).

The investigation of the Pim-Thr-Sse complex have provided us with a molecular description of the changes in this complex necessary to induce chromatid movements in anaphase (Herzig et al., 2002; Jäger et al., 2001; Jäger et al., 2004; Leismann et al., 2000). However, it is not known whether Cdk1-CycB interacts with Pim-Thr-Sse in a dose-dependent manner to act as a timer in regulating the onset of anaphase. Based on the observation that increasing maternal Cdk1-CycB activity causes abnormal nuclear distribution and morphology at cycle 14 interphase, we performed a loss-of-function genetic screen for modifiers of Cdk1-CycB (Ji et al., 2002). Here, we report genetic interactions between Cdk1-CycB and components of the Pim-Thr-Sse complex, and document the dosage effects of these factors in anaphase initiation and early embryonic development in *Drosophila*. Both CycB and Cdk1 are overexpressed in many human malignant cancers, such as colorectal, breast, liver and lung cancers (for examples, see Ito et al., 2000; Sarafan-Vasseur et al., 2002; Soria et al., 2000). Therefore, our results suggest that higher Cdk1-CycB activity in cancer cells may contribute to generating aneuploidy by directly affecting Separase activity and thereby the onset of anaphase.

## Materials and methods

### Stocks

We used *Sevelen* as wild-type stock. The alleles of *three rows* (*thr*<sup>P9.8</sup>, *thr*<sup>13C4</sup>, *thr*<sup>3E22</sup>, *thr*<sup>5E16</sup>, *thr*<sup>BH9</sup>, *thr*<sup>1L62</sup> and *thr*<sup>5B22</sup>) were provided by S. Bray, A. Carpenter and D. Glover (University of Cambridge, UK), and C. Nüsslein-Volhard (Max-Planck-Institut für Entwicklungsbiologie, Tübingen, Germany). C. Lehner (University of Bayreuth, Germany) provided us with three amorphic *pimples* (*pim*) alleles *pim*<sup>2</sup>, *pim*<sup>3</sup> and *pim*<sup>4</sup>, the *separase* (*Sse*) amorphic allele *sse*<sup>13m</sup> and the deficiency *Df(3L)SseA* uncovering *Sse*. T. Schüpbach (Princeton University, USA) sent us *early*, *subito* and *halted* mutant alleles. The deficiency line *Df(2R)Pcl-W5* was provided by J. Kennison (National Institute of Child Health). All other mutant alleles

were obtained from the Bloomington Stock Center (<http://flystocks.bio.indiana.edu/>).

Genetic crosses and the nomenclature of genotypes have been described previously (Ji et al., 2002). Briefly, '*mutation/six cycB*' refers to embryos from females that were heterozygous for a specific mutant gene or deficiency, and also carried six copies of the *cycB*<sup>+</sup> gene. We used the description '*thr*<sup>1</sup>/*pim*<sup>1</sup>/*six cycB*' for flies with the genotype +; *thr*<sup>1</sup>, *pim*<sup>1</sup>, *cycB*<sup>+</sup>/*thr*<sup>+</sup>, *pim*<sup>1</sup>, *cycB*<sup>+</sup>; 2*P*[w<sup>+</sup> *cycB*<sup>+</sup>]; and '*thr*<sup>1</sup>/*sse*<sup>13m</sup>/*six cycB*' for the genotype +; *thr*<sup>1</sup>, *cycB*<sup>+</sup>/*thr*<sup>+</sup>, *cycB*<sup>+</sup>, 2*P*[w<sup>+</sup> *cycB*<sup>+</sup>]; *sse*<sup>13m</sup>/*sse*<sup>+</sup>, 2*P*[w<sup>+</sup> *cycB*<sup>+</sup>]. For time-lapse two-photon microscopy, embryos with the maternal genotype +; *cycB*<sup>+</sup>/*cycB*<sup>+</sup>; *histone-GFP*/+ were referred to as '*two cycB*' embryos or controls; +; *cycB*<sup>+</sup>/*cycB*<sup>+</sup>, 2*P*[w<sup>+</sup> *cycB*<sup>+</sup>]; *histone-GFP*/+ as '*four cycB*' embryos; +; *thr*<sup>1</sup>, *cycB*<sup>+</sup>/*thr*<sup>+</sup>, *cycB*<sup>+</sup>, 2*P*[w<sup>+</sup> *cycB*<sup>+</sup>]; *histone-GFP*/+ as '*thr/four cycB*' embryos; +; *pim*<sup>1</sup>, *cycB*<sup>+</sup>/*pim*<sup>+</sup>, *cycB*<sup>+</sup>, 2*P*[w<sup>+</sup> *cycB*<sup>+</sup>]; *histone-GFP*/+ as '*pim/four cycB*' embryos; and +; *cycB*<sup>+</sup>/*cycB*<sup>+</sup>, 2*P*[w<sup>+</sup> *cycB*<sup>+</sup>]; *sse*<sup>13m</sup>/*sse*<sup>+</sup>, *histone-GFP* as '*sse/four cycB*' embryos. The phenotypes of the *four cycB* and *six cycB* embryos are not different at cycle 10 and cycle 14 (Table 1). Compared with *two cycB* (wild type) embryos, we observed a similar increase of Cdk1-CycB kinase activity in *four cycB* and *six cycB* embryos (Ji et al., 2002; Stiffler et al., 1999).

### Phenotypic analyses

Phenotypes and cytological analyses at cycle 10 and cycle 14 were performed as described previously (Ji et al., 2002). The 95% confidence intervals were calculated by using either StatXact 4.0 (Cytel Software) or an online 'Sample Size Calculator' provided by Creative Research Systems (<http://www.surveysystem.com/sscalc.htm>). Embryo fixation, immunostaining (Baker et al., 1993), confocal microscopy (Stiffler et al., 1999) and live imaging with the two-photon microscopy (Ji et al., 2004) were performed as described before. Information from fixed embryos was used as an additional independent assay of live analyses.

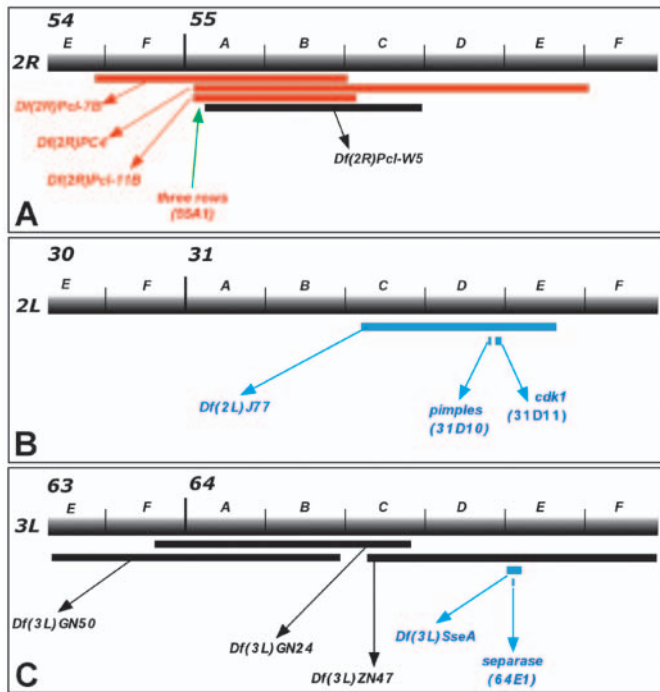
### Synthesis and injection of double-stranded RNA of *thr*

We prepared dsRNA as described (Kennerdell and Carthew, 1998). Both PCR strand fragments of the *thr* gene from positions 888 to 1442 were used as templates for simultaneous in vitro transcription using Ambion MEGAscript T7 kit. The complementary RNA products were annealed during the transcription reactions to form dsRNAs. About 250–370 pL of 5 μM dsRNA dissolved in injection buffer (5 mM KCl, 0.1 mM NaH<sub>2</sub>PO<sub>4</sub>, pH 7.5) was injected into the posterior part of histone-GFP embryos, as well as the buffer-only injection control. We also used RNAi to downregulate endogenous *bicoid* as a positive control and obtained the *bicoid* phenotype as reported (Kennerdell et al., 2002).

## Results

### Identification of the gene *three rows* as an enhancer in cytogenetic region 54–55

Compared with the *two cycB* control, *Drosophila* embryos with more maternal CycB (e.g. four extra copies of the maternal *cycB* gene dose) have a slightly lower hatching rate and a higher frequency of abnormal nuclear distribution and morphology at cycle 14 interphase. This non-lethal phenotype is referred to as '*the six cycB* phenotype' and was used to perform a dosage-sensitive genetic screen. We identified 12 enhancer and 12 suppressor deficiency lines (Ji et al., 2002). In cytogenetic map region 54–55, we identified three partially overlapping deficiencies that enhance the *six cycB* phenotype: *Df(2R)Pcl-7B*, *Df(2R)PC4* and *Df(2R)Pcl-11B* (Ji et al., 2002). They overlap between 55A1 and 55B9–C1. An additional deficiency line *Df(2R)Pcl-W5* (55A; 55C1–13) did not affect



**Fig. 1.** Cytogenetic map of *Drosophila* chromosome regions covering *thr* (A), *pim* (B) and *sse* (C). Bars designate deficiency lines: red bars are enhancers, blue bars are suppressors and black bars are deficiencies that do not alter the *six cycB* blastoderm phenotype. The cytogenetic region for *thr* (A) is based on published data by D'Andrea et al. (D'Andrea et al., 1993) and Philp et al. (Philp et al., 1993). The region covering *pim* (B) is based on information from FlyBase and the region that covering *sse* (C) is based on data from Jäger et al. (Jäger et al., 2001).

the *six cycB* phenotype (Fig. 1A; Table 1). Therefore, the enhancer is defined to region 55A.

The gene *three rows* (*thr*) is located at cytogenetic region 55A3 based on complementation testing (D'Andrea et al., 1993; Philp et al., 1993). To test whether reduction of *thr* enhances the *six cycB* blastoderm phenotype, we tested 10 different *thr* mutant alleles. All of them significantly enhanced the *six cycB* phenotypes, with significantly lower hatching rates (61% or less) and fewer normal cycle 14 blastoderm configurations (less than 27%, Table 1). The majority of the *thr/six cycB* embryos have abnormally large or small nuclei. They also fail to divide synchronously in the blastoderm cycles (data not shown). Furthermore, we frequently observed chromosomal bridges, uneven nuclear distributions, centrosomes without associated nuclei, and aneuploid nuclei

**Table 1. Analyses of the cycle 14 phenotype**

Genotype	<i>two cycB</i> background			<i>six cycB</i> background		
	Normal cycle 14 embryos (%)	95% C.I. (%) <sup>†</sup>	n <sup>‡</sup>	Normal cycle 14 embryos (%)	95% C.I. (%) <sup>†</sup>	n <sup>‡</sup>
Wild type	97	95-98	409			
<i>four cycB</i>				79	74-84	267
<i>six cycB</i>				74	71-77	790
<i>Df(2R)Pcl7B</i>	96	89-98	89	14*	5-31	28
<i>Df(2R)PC4</i>	93	88-97	135	0*	0-6	52
<i>Df(2R)Pcl11B</i>	74	66-80	160	15*	8-26	60
<i>Df(2R)PclW5</i>	87	82-92	215	67	62-72	295
<i>thr<sup>1</sup></i>	98	94-99	163	8*	4-13	138
<i>thr<sup>P9.8</sup></i>	91	87-95	224	3*	1-5	277
<i>thr<sup>13C4</sup></i>	ND	ND	ND	14*	10-18	270
<i>thr<sup>3E22</sup></i>	ND	ND	ND	24*	18-30	226
<i>thr<sup>5E16</sup></i>	86	81-91	212	13*	9-17	254
<i>thr<sup>BH9</sup></i>	90	86-94	227	9*	6-12	291
<i>thr<sup>DL62</sup></i>	ND	ND	ND	3*	2-6	232
<i>thr<sup>3</sup></i>	ND	ND	ND	26*	20-32	213
<i>thr<sup>S1B22</sup></i>	95	92-98	225	13*	9-18	219
<i>thr<sup>A07805b</sup></i>	92	88-96	184	26*	21-31	358
<i>Df(2L)J77</i>	89	83-94	148	86**	79-93	107
<i>pim<sup>1</sup></i>	97	94-99	212	96**	91-98	120
<i>pim<sup>2</sup></i>	70	64-76	224	73	65-81	118
<i>pim<sup>3</sup></i>	95	92-98	168	81**	75-87	151
<i>pim<sup>4</sup></i>	97	95-99	195	88**	83-93	151
<i>Df(3L)SseA</i>	97	95-99	204	91**	87-95	183
<i>sse<sup>13m</sup></i>	95	91-99	139	84**	77-91	108
<i>Ipim<sup>1</sup>, Ithr<sup>1</sup></i>	ND	ND	ND	44	38-50	232
<i>Isse<sup>13m</sup>, Ithr<sup>1</sup></i>	ND	ND	ND	64	59-69	317

\*The percentage of normal embryos is significantly lower than the percentage of *six cycB* embryos (Pearson's  $\chi^2$  test,  $P < 0.03$ ).

\*\*The percentage of normal embryos is significantly higher than the percentage of *six cycB* embryos (Pearson's  $\chi^2$  test,  $P < 0.03$ ).

<sup>†</sup>The 95% Confidence Interval (C.I.) for the percentage of normal cycle 14 embryos.

<sup>‡</sup>Number of embryos analyzed.

ND, not determined.



(data not shown). These abnormalities were more extreme than the ones observed in *six cycB* embryos, which indicate defects during M-phase or disrupted coordination between nuclear and cytoplasmic cycles (Table 1).

To exclude possible non-lethal dominant maternal effects that were independent of increased Cdk1-CycB activity, we analyzed the enhancer alleles in a *two cycB* background (*enhancer/two cycB*). We found that *thr* heterozygous embryos are not different from wild-type embryos in their hatching rate (data not shown) or in their blastoderm phenotype at cycle 14 (Table 1). Similar observations were made with the three enhancing deficiencies identified in cytogenetic region 54-55 (Table 1).

In addition to *thr*, we tested four other maternal effect mutations in the cytogenetic map region 54/55: *early* (*ey*, at cytogenetic region 54F6-55B12), *subito* (*sub*, at 54E7), *staufen* (*stau*, at 55B5-7) and *halted* (*hal*, at 55A1) (Schüpbach and Wieschaus, 1989). None of them enhanced the *six cycB* blastoderm phenotype at cycle 14 (data not shown).

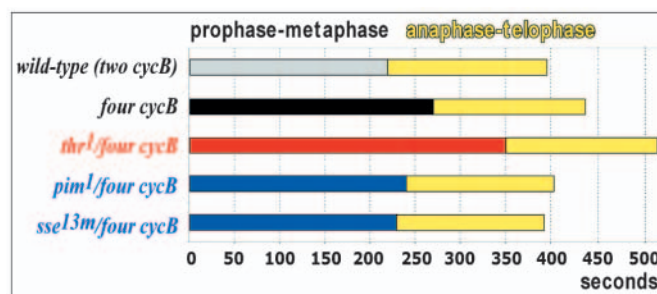
### Identification of the gene *pimples* and the gene *separase* as suppressors of the *six cycB* phenotype

Since *thr* is an enhancer of the *six cycB* phenotype, we tested whether Cdk1-CycB genetically interacts with other proteins related to Thr, such as Pim and Sse (Herzig et al., 2002; Jäger et al., 2001). We tested four alleles of *pim* and found that three of them (*pim*<sup>1</sup>, *pim*<sup>3</sup> and *pim*<sup>4</sup>) suppressed the *six cycB* phenotype at cycle 14 (Table 1). With the exception of *pim*<sup>2</sup>, reducing one copy of the other three *pim* alleles normalized the cycle 14 blastoderm phenotype (Table 1).

The *pim*<sup>2</sup> allele was generated by X-ray and has a small in-frame deletion that leads to a protein missing amino acids 110-114 (Jäger et al., 2001). It was proposed that the small deletion in Pim specifically abolishes the binding to Sse but does not result in destabilization or complete misfolding of the mutant Pim<sup>2</sup> protein (Jäger et al., 2001). The other three *pim* alleles (*pim*<sup>1</sup>, *pim*<sup>3</sup> and *pim*<sup>4</sup>) are nucleotide substitutions that lead to defective mRNA splicing (*pim*<sup>1</sup>) or premature translation stop (*pim*<sup>3</sup> and *pim*<sup>4</sup>) (Stratmann and Lehner, 1996). Homozygous embryos of all four *pim* alleles show the same zygotic phenotype: failure of sister chromatid separation in the centromeric region at cycle 15 and cycle 16 (Leismann et al., 2000; Stratmann and Lehner, 1996).

We investigated why the *pim*<sup>2</sup> allele behaved differently in the presence of elevated CycB when compared with the other three amorphic *pim* alleles. We found that the hatching rates of both *pim*<sup>2</sup>/*two cycB* and *pim*<sup>2</sup>/*six cycB* embryos were significantly lower than *two cycB* or *six cycB* embryos, but the frequencies of normal cycle 14 blastoderm embryos were not different from *six cycB* embryos (Table 1). These results and further analyses with this allele at cycle 10 and cycle 14 (see below) suggest that the *pim*<sup>2</sup> allele is semi-dominant (see Discussion).

Previously, we observed that *Df(2L)J77* and the neomorphic allele *cdk1*<sup>D57</sup> (at cytogenetic map position 31D11), but not the null allele of *cdk1*, suppressed the *six cycB* phenotype (Fig. 1B), thus we concluded that another suppressor gene was present within *Df(2L)J77* whose product genetically interacted with Cdk1-CycB (Ji et al., 2002). The gene *pim* maps to cytogenetic region 31D10 and is uncovered by *Df(2L)J77* (31C; 31E7), suggesting that *Df(2L)J77* indeed uncovers at least two suppressor genes (Fig. 1B).



**Fig. 2.** The onset of anaphase is regulated by both Cdk1-CycB activity and the Pim-Thr-Sse complex in a dose-dependent manner. The durations of cell-cycle phases are measured by analyzing time-lapse recordings using two-photon microscopy in embryos with different levels of CycB and different components of Pim-Thr-Sse complex between cycles 6 and 11. The duration of prophase-metaphase and anaphase-telophase do not change between cycle 6 and 11 (Ji et al., 2004), thus data are averages of the duration from 10-20 time-lapse recordings of each genotype between cycle 6 and 11. Prophase-metaphase duration is 220 seconds in wild-type embryos (51 measurements from 20 embryos, s.d.=30 seconds); 270 seconds in *four cycB* embryos (51 measurements from 18 embryos, s.d.=40 seconds); 350 seconds in *thr*<sup>1</sup>/*four cycB* embryos (24 measurements from 11 embryos, s.d.=60 seconds); 240 seconds in *pim*<sup>1</sup>/*four cycB* embryos (33 measurements from 10 embryos, s.d.=30 seconds) and 230 seconds in *sse*<sup>13m</sup>/*four cycB* embryos (52 measurements from 11 embryos, s.d.=30 seconds). When compared with *four cycB* embryos, the differences caused by lower *thr*, *pim* or *Sse* levels are significant ( $P<0.0002$ ). There is no significant difference in the duration of anaphase-telophase in the five genotypes (duration varies between 160-180 seconds).

To further test the genetic interaction between Cdk1-CycB and proteins of the Pim-Thr-Sse complex, we tested the *separase* (at 61E1) null allele *sse*<sup>13m</sup> and the deficiency *Df(3L)SseA* (Fig. 1C) (Jäger et al., 2001). The allele *sse*<sup>13m</sup> has a deletion of four bases leading to a frame shift and premature translation stop, thus the mutant Sse<sup>13m</sup> protein lacks the invariant cysteine that is involved in catalysis (Jäger et al., 2001). As shown in Table 1, both *Df(3L)SseA* and *sse*<sup>13m</sup> have the same suppressive effect on the *six cycB* phenotype at cycle 14, supporting the idea that *sse*<sup>13m</sup> is an amorphic allele (Jäger et al., 2001).

### Dose-dependent interaction between Cdk1-CycB and Pim-Thr-Sse

To genetically confirm that Cdk1-CycB interacts with the Pim-Thr-Sse complex in regulating a common process, we combined the enhancer (*thr* mutation) with suppressor mutations in double heterozygous combinations. We generated mothers that had six copies of the *cycB*<sup>+</sup> gene and were heterozygous for both Thr and Pim (referred as *thr*<sup>1</sup>/*pim*<sup>1</sup>/*six cycB*), or both Thr and Sse (referred as *thr*<sup>1</sup>/*sse*<sup>13m</sup>/*six cycB*). We found that compared with the *thr*/*six cycB* embryos, double heterozygous embryos (*thr*<sup>1</sup>/*pim*<sup>1</sup>/*six cycB* and *thr*<sup>1</sup>/*sse*<sup>13m</sup>/*six cycB*) were normalized at cycle 14 (Table 1) and had increased hatching rates (data not shown). Thus, reducing either Pim or Sse partially suppressed the enhancing effects caused by lower Thr in the *six cycB* background, probably by restoring stoichiometry among the Pim-Thr-Sse heterotrimer complex. Taken together, our genetic analyses demonstrate a dosage-

dependent interaction between Cdk1-CycB and the Pim-Thr-Sse complex.

### The level of Thr, Pim, Sse and CycB affects anaphase onset

In *four cycB* and *six cycB* embryos, we observed a higher Cdk1-CycB activity and a delayed onset of anaphase (Ji et al., 2002; Ji et al., 2004). Because components of the Pim-Thr-Sse complex are involved in regulating sister chromatid separation, we asked whether reducing the levels of each of these proteins had any effect on the onset of anaphase. We analyzed the duration of cell-cycle phases by using a two-photon laser-scanning microscope. Reducing Thr in embryos with more maternal CycB (*thr<sup>1</sup>/four cycB*) led to significantly longer prophase-metaphase duration (Fig. 2). By contrast, reducing either Pim or Sse in embryos with more maternal CycB significantly shortened the prophase-metaphase time,

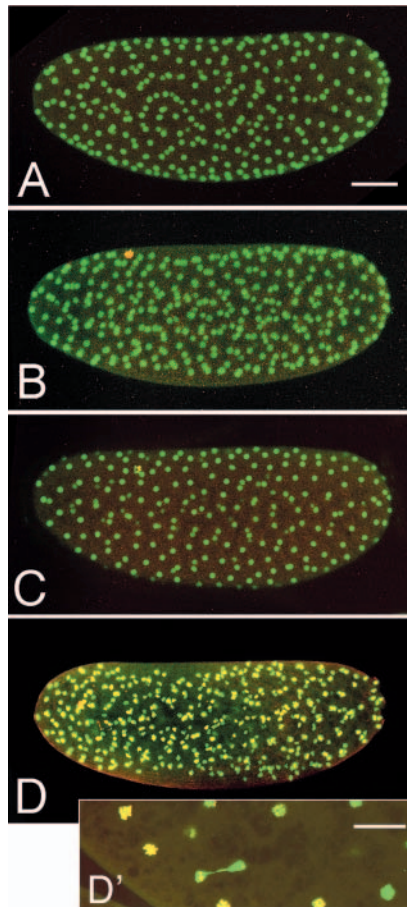
normalizing the timing of anaphase onset. Furthermore, varying the dosage of Thr, Pim or Sse in embryos that have higher levels of CycB had no effect on interphase (data not shown) and anaphase-telophase duration (Fig. 2), suggesting that the levels of these proteins specifically define when anaphase begins.

Interestingly, just reducing the dosage of these proteins (in heterozygous embryos of *thr*, *pim* or *Sse*) did not change the onset of anaphase (data not shown), suggesting that Pim, Thr or Sse are haplosufficient in the *two cycB* background. We observed this interplay between Cdk1-CycB and Thr-Pim-Sse complex in the presence of increased Cdk1-CycB activity.

To test whether Thr levels affected anaphase onset without increasing Cdk1-CycB activity, we injected dsRNA of *thr* into *two cycB* embryos labeled with histone-GFP. We found that compared with embryos injected only with buffer, embryos injected with *thr* dsRNA at cycle 6 have a significantly delayed onset of anaphase at cycle 12 and cycle 13 (26% and 41% longer prophase-metaphase, respectively), but not before cycle 12. This observation indicates that RNAi process takes about 60 minutes to downregulate endogenous *thr* mRNA, and that reducing Thr alone can postpone the onset of anaphase in *two cycB* embryos as well.

### Cycle 10 phenotype of *thr/six cycB* and *pim/six cycB* embryos

The earliest mitoses in the *Drosophila* embryo have been well studied previously. The first four cycles occur in the interior and slightly towards the anterior end of the embryo. Between cycles 4-7, nuclei move along the anteroposterior axis of the embryo in a process known as 'axial expansion' (Baker et al., 1993). Later, during cycles 8-10, nuclei are pushed to the



**Fig. 3.** The cycle 10 phenotype of *two cycB* (A), *six cycB* (B), *sse<sup>13m</sup>/six cycB* (C, suppressor) and *thr<sup>1</sup>/six cycB* (D,D', enhancer) embryos. Embryos were stained with antibodies against histone (green) and phosphorylated histone H3 (red); areas stained with both appear yellow. In *two cycB*, *six cycB* and suppressor embryos, all nuclei are in interphase (green), except for polar body nuclei arrested in metaphase of cycle 1 (yellow in B,C). The enhancer (D,D') embryos have many nuclei in mitosis (yellow). Note that there are many micro/macro-nuclei and chromosomal bridges, as well as asynchronous mitoses, in *thr/six cycB* embryos (D'). The images are projections of ~20 optical sections with a 3- $\mu$ m interval. Scale bars: in A, 50  $\mu$ m; in D', 10  $\mu$ m.

**Table 2.** Analyses of the cycle 10 phenotype

Genotype	Normal cycle 10 embryos (%)	95% C.I. (%) <sup>†</sup>	n <sup>‡</sup>
Wild type	97	96-98	97
<i>six cycB</i>	20	11-29	74
<i>pim<sup>1</sup>/six cycB</i>	95*	89-100	55
<i>pim<sup>2</sup>/six cycB</i>	17	0-34	18
<i>pim<sup>3</sup>/six cycB</i>	86*	73-99	28
<i>pim<sup>4</sup>/six cycB</i>	78*	65-89	41
<i>sse<sup>13m</sup>/six cycB</i>	89*	72-100	12
<i>Df(3L)SseA/six cycB</i>	68*	54-82	41
<i>thr<sup>1</sup>/six cycB</i>	5**	0-12	38
<i>thr<sup>P9.8</sup>/six cycB</i>	2**	0-6	45
<i>thr<sup>13C4</sup>/six cycB</i>	5**	1-9	107
<i>thr<sup>3E22</sup>/six cycB</i>	1**	0-3	162
<i>thr<sup>5E16</sup>/six cycB</i>	8**	1-15	63
<i>thr<sup>BH9</sup>/six cycB</i>	6**	1-11	71
<i>thr<sup>IL62</sup>/six cycB</i>	5**	0-14	22
<i>thr<sup>3</sup>/six cycB</i>	11**	6-16	153
<i>thr<sup>SJB22</sup>/six cycB</i>	5**	0-12	41
<i>thr<sup>K07805b</sup>/six cycB</i>	8**	3-13	103

\*The percentage of normal embryos is significantly higher than the percentage of *six cycB* embryos (Pearson's  $\chi^2$  test,  $P < 0.03$ ).

\*\*The percentage of normal embryos is significantly lower than the percentage of *six cycB* embryos (Pearson's  $\chi^2$  test,  $P < 0.03$ ).

<sup>†</sup>The 95% Confidence Interval (C.I.) for the percentage of normal cycle 10 embryos.

<sup>‡</sup>Number of embryos analyzed.



cortex by the expanding microtubule network, a process known as ‘cortical migration’ (Baker et al., 1993). Because nuclei migrate to the cortex almost simultaneously in *two cycB* embryos (Foe and Alberts, 1983), embryos fixed at cycle 10 have somatic nuclei evenly distributed at the cortex in 97% of the embryos (Fig. 3A, Table 2).

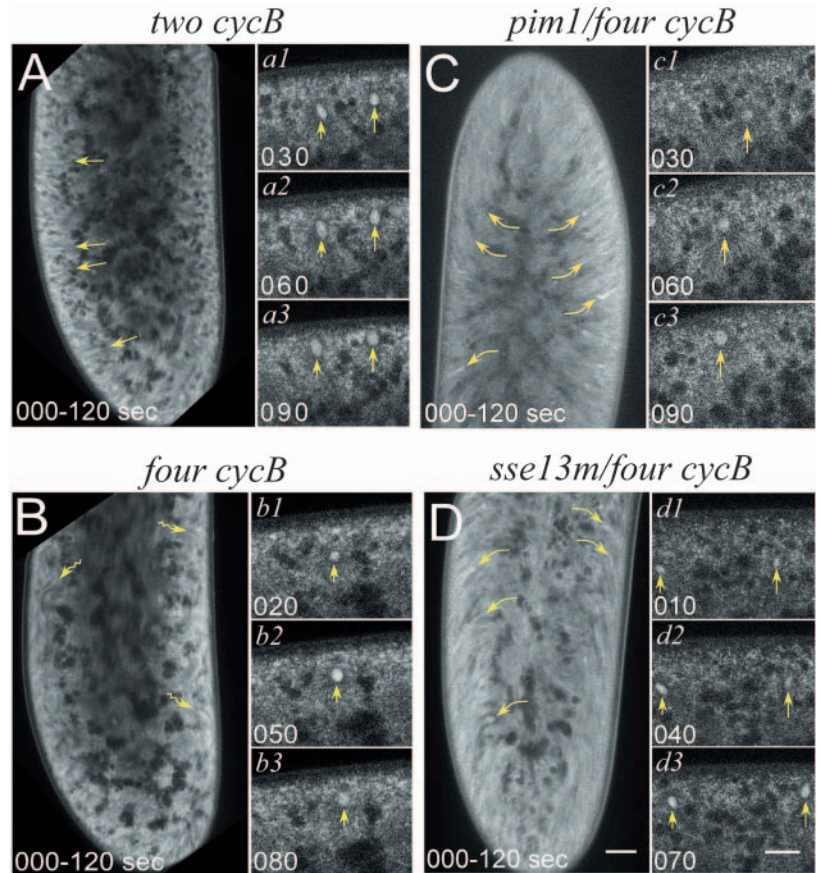
Comparing *six cycB* embryos with wild-type controls, we reported that higher maternal Cdk1-CycB activities lead to slower nuclear movement during both axial expansion and cortical migration (Stiffler et al., 1999). However, slower nuclear movement does not affect the nuclear distribution at cycle 7 (C. Trusty, J.-Y.J. and G.S., unpublished). Moreover, at cycle 10 more than 80% of the *six cycB* embryos had nuclei at the anterior-medial region that do not reach the cortex simultaneously (Fig. 3B) (Ji et al., 2002). This abnormal nuclear distribution defines the ‘cycle 10 phenotype’, and results from a defect in nuclear cortical migration (Ji et al., 2002). Thus for cycle 10 phenotype, *six cycB* embryos deviate from *two cycB* embryos after cycle 8 and before 10.

To test whether Thr, Pim or Sse affects nuclear cortical migration, we analyzed the cycle 10 phenotype in fixed *pim/six cycB* and *sse/six cycB* embryos. We observed that reducing Pim with the amorphic *pim* alleles *pim*<sup>1</sup>, *pim*<sup>3</sup> and *pim*<sup>4</sup> in the *six cycB* genetic background suppressed the *six cycB* phenotype at cycle 10 and cycle 14; the *pim*<sup>2</sup> allele had no suppressing effect (Tables 1, 2). Reducing Sse with either the amorphic allele *sse*<sup>13m</sup> or the deficiency *Df(3L)SseA* normalized the cycle 10 phenotype (Fig. 3C, Table 2). Therefore, both *pim* and *Sse* alleles suppressed the *six cycB* phenotype at both cycle 10 and cycle 14.

The cycle 10 phenotype in *thr/six cycB* embryos (Fig. 3D) was noticeably worse than in *six cycB* embryos (Fig. 3B, Table 2). In addition to the defects of nuclear cortical migration (Table 2), we frequently observed asynchronous mitoses (Fig. 3D'), macro/micro-nuclei and chromosomal bridges before cycle 10 (Fig. 3D'), suggesting that the enhancing effect of lower maternal Thr occurs prior to cycle 10.

### Effects of lower levels of Pim or Sse on nuclear movement

The observations made with fixed materials led us to focus on when and how cortical migration is normalized in *pim/six cycB* and *Sse/six cycB* embryos. For this, we analyzed in vivo time-lapse recordings of embryos labeled with histone-GFP and focused on cycles 9 and 10. We defined velocity and pattern of nuclear migration in *two cycB*, *four cycB*, *pim/four cycB* and *sse/four cycB* embryos. In all four genotypes, nuclear cortical migration was initiated at telophase of cycle 9 and ends ~1.5 min into early interphase of cycle 10. However, the velocity and pattern of nuclear migration were different. During cortical migration, nuclei moved slower in *four cycB* embryos (6.2±3.3 µm/min, 28 measurements from 8 embryos) than in wild-type embryos (8.8±3.2 µm/min, 26 measurements from 9 embryos), confirming our previous observation using DIC microscopy



**Fig. 4.** Pattern of nuclear cortical migration from late telophase at cycle 9 to early interphase of cycle 10. A projection of time-lapse recordings collected with 10-second intervals reveals the patterns. Genotypes of the embryos are labeled above images; posterior (A,B,D) and anterior (C) parts of the embryos are shown. Arrows (A-D) trace the direction of nuclear movement over 120 seconds. Note the straight movement in A, the meandering pattern in B, and the curved movement in C and D. Insets (a1-a3, b1-b3, c1-c3 and d1-d3) are images with 30-second intervals during cortical migration. Images in a1, b1, c1 and d1 begin at slightly different time-points, because most nuclei move in and out of focal planes during the recording. Scale bars: in D, 40 µm for A-D; in d3, ~10 µm for all insets.

(Stiffler et al., 1999). Compared with both the *four cycB* and *two cycB* embryos, significantly faster nuclear movements were observed in both *pim*<sup>1</sup>/*four cycB* embryos (11.1±3.2 µm/min, 30 measurements from 9 embryos) and *sse*<sup>13m</sup>/*four cycB* embryos (12.5±3.6 µm/min, 42 measurements from 10 embryos).

We also observed that the paths of nuclear movement were different. In *two cycB* embryos, nuclei migrated perpendicular to the cortex in straight paths (Fig. 4A) (Foe and Alberts, 1983). In *four cycB* embryos, nuclei moved to the cortex like ‘drunken soldiers’: they moved in meandering lines towards the cortex (Fig. 4B). By contrast, we observed that nuclei migrated in curved paths and at an angle to the cortex in both *pim*<sup>1</sup>/*four cycB* (Fig. 4C) and *sse*<sup>13m</sup>/*four cycB* (Fig. 4D) embryos. The same novel nuclear migration pattern was also observed with *pim*<sup>3</sup>/*four cycB* and *Df(3L)SseA/four cycB* embryos (data not shown). These observations indicate that faster nuclear movement and a novel cortical migration pattern contribute to the normal cycle 10 phenotype in both *pim*<sup>1</sup>/*four cycB* and *sse*<sup>13m</sup>/*four cycB* embryos.

### Effects of Sse or Pim levels on microtubule morphology

Previously, we reported that cortical migration of nuclei at the early interphase of cycles 8 and 9 is microtubule dependent (Baker et al., 1993). In addition, reducing the maternal levels of *chickadee* or *scrambled* in *six cycB* embryos suppressed both the astral microtubule phenotype observed at cycles 5-7 and cortical migration (Ji et al., 2002). These observations indicate that in the rescued background, a normalized microtubule network is responsible for the rescued cortical migration.

To test whether normalized microtubule function underlies the normalized cycle 10 phenotype in *pim/six cycB* and *Sse/six cycB* embryos, we analyzed microtubule morphology during early interphase of cycle 9. At this stage, we observed an extensive microtubule network within *two cycB* embryos (Fig. 5A). Increasing maternal CycB (*six cycB* embryos) reduced the microtubule network (Fig. 5B, also compare Fig. 5A' with 5B'). However, in *pim<sup>1</sup>/six cycB* and *sse<sup>13m</sup>/six cycB* embryos the microtubule morphology was restored (Fig. 5C, compare Fig. 5C' with Fig. 5B'). Similar differences in microtubule configurations were seen at cycles 7 and 8. Thus the normalized microtubule network could account for the faster nuclear movement during cortical migration.

If changing Pim or Sse has a general effect on microtubule morphology, these effects may also be observed in other cell-cycle phases. To test this, we analyzed the aster microtubule morphology in metaphase embryos. We had previously observed that 40% of metaphase spindles from cycle 5 to 7 had no visible astral microtubules in *six cycB* embryos, which was a significantly higher number than in wild-type embryos (16%) (Ji et al., 2002). The same observation was made at cycle 9 (compare Fig. 5A'' and 5B''). Surprisingly, we found that reducing Sse in the *six cycB* background (in either *sse<sup>13m</sup>/six cycB* or *Df(3L)SseA/six cycB* embryos) led to an even more reduced astral microtubule morphology (Fig. 5C'') at cycle 9. Similarly, reducing Pim with any *pim* allele had either no

effect, or led to a reduction in astral microtubules (data not shown). Therefore, levels of Sse and Pim have different effects on microtubule morphology depending on the cell-cycle phase: restoration in interphase but not in metaphase. These and the observations described above suggest that suppression of the cycle 10 phenotype occurs by normalizing the microtubule networks in interphase and by altering cortical migration path.

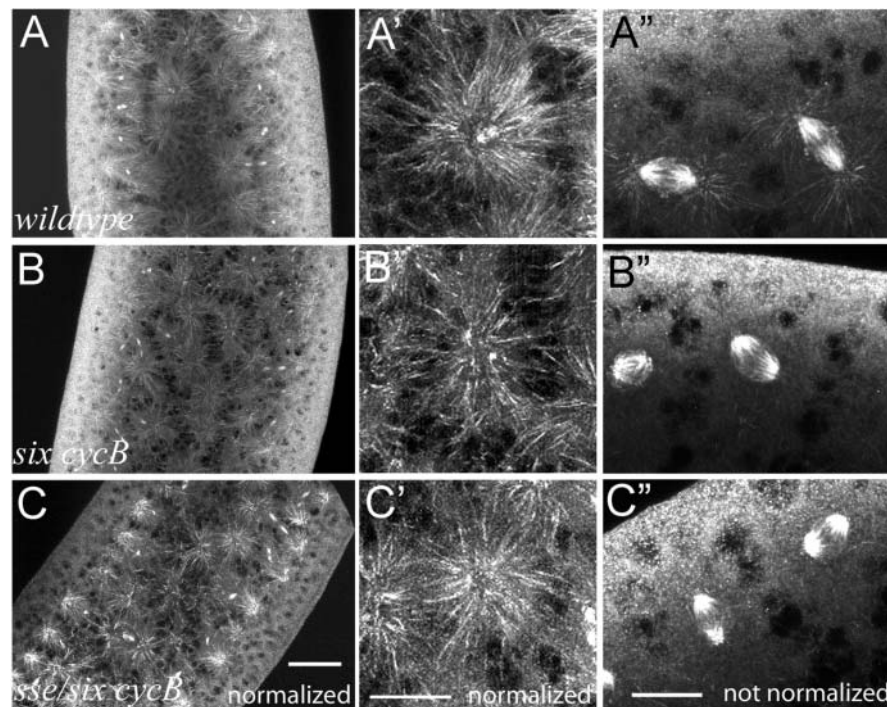
Overall, we conclude that reducing either *pim* or *Sse* suppressed the *six/cycB* phenotype at both cycle 10 and cycle 14 by normalizing the onset of anaphase, restoring microtubule morphology in interphase and by inducing faster nuclear movement during cortical migration. By contrast, reducing *thr* enhances the *six/cycB* phenotype at both cycle 10 and 14 by further delaying the initiation of anaphase.

### Discussion

#### Enhancement of the *six cycB* phenotype by reducing maternal *thr* levels

Increasing maternal Cdk1-CycB activity leads to defective mitoses, indicating a disruption in the coordination between the nuclear and cytoplasmic cycle (Ji et al., 2002). Nevertheless, these embryos develop to adults. Also, higher Cdk1-CycB activity causes shorter microtubules, and longer metaphase but shorter anaphase (Ji et al., 2002). These observations suggest that a slight delay of anaphase initiation may result in slightly disrupted coordination between nuclear and cytoplasmic events, such as chromatid separation and microtubule dynamics. Thus, in the *six cycB* genetic background, mutations that worsened the defect in coordination were identified as enhancers, whereas mutations that rectified the defects were identified as suppressors (Ji et al., 2002).

Indeed, further reducing maternal *thr* by one copy in embryos with higher Cdk1-CycB activity led to an even greater delay of anaphase onset (Fig. 2), resulting in more frequent and severe nuclear defects. We propose that a greater delay of anaphase onset is the result of fewer Thr-Sse dimers, thereby causing an increase in the time taken to cleave Cohesin. This idea



**Fig. 5.** The morphology of the microtubule network at cycle 9 interphase when nuclei move to the cortex (A-C, shown at higher magnification in A'-C'), and astral microtubule morphology at cycle 9 metaphase (A''-C''). (A-A'') *two cycB* embryo; (B-B'') *six cycB* embryo; (C-C'') *sse<sup>13m</sup>/six cycB* embryo. These embryos were stained with an antibody against tubulin to label microtubules and with rhodamine-conjugated anti-histone H1 antibody to label nuclei for precise staging (not shown). Images in A-C and A'-C' are projections of 11 optical sections with a 1.5- $\mu$ m interval. Images in A''-C'' are projections of six sections with 1- $\mu$ m intervals. Note that interphase microtubules in the *sse<sup>13m</sup>/six cycB* embryo (C,C') are stronger than those in *two cycB* embryos (A,A'), but the astral microtubules in metaphase *sse<sup>13m</sup>/six cycB* embryos (B'',C'') are not different from *six cycB* embryos (B'',C''). Scale bars: in C, 40  $\mu$ m for A-C; in C', 20  $\mu$ m for A'-C'; in C'', 10  $\mu$ m for A''-C''.



is based on the observation that the majority of the *thr/six cycB* embryos had many macro/micro-nuclei, and had disrupted synchrony and chromosomal bridges both before and after cycle 10 (Fig. 3D'), which indicates that these defects result from abnormal chromatid separation. This scenario would explain why *thr* becomes haplo-insufficient in the presence of higher Cdk1-CycB activity (*six cycB* background, Table 1 and Fig. 2), but not in the wild-type (*two cycB*) background (Table 1).

### Suppression of the *six cycB* phenotype by reducing *pim* levels

Sse and Cdk1-CycB activities have opposite effects on the onset of anaphase: higher Sse activity leads to earlier anaphase onset whereas higher Cdk1-CycB delays it. If this is so, reducing Pim, the inhibitor of Sse (Leismann et al., 2000; Jäger et al., 2001), would lead to slightly earlier activation of Sse than in *six cycB* embryos, and thus correct the timing of anaphase initiation (Fig. 2).

Alternatively, both Pim and CycB need to be degraded to initiate anaphase (Peters, 2002; Pickart, 2001), thus reducing *pim* in a *six cycB* genetic background might suppress the *six cycB* phenotype if Pim and CycB compete for destruction by the ubiquitin/proteasome system. Both CycB and Securin contain a similar N-terminal sequence motif, known as the 'destruction box' (Peters, 2002). The idea that CycB and Securin compete for degradation is supported by the observation that the N-terminal fragments of CycB and Securin compete with the full-length protein for the destruction machinery in yeast (Funabiki et al., 1996). According to this scenario, Pim degradation would be delayed in *six cycB* embryos because more CycB needs to be degraded. Reducing Pim, as in *pim/six cycB* embryos would relieve the inhibition of Pim on Sse, thus suppressing the *six cycB* phenotype.

Both scenarios could explain why reducing Pim in embryos with higher Cdk1-CycB normalizes anaphase onset (Fig. 2). However, additional assumptions are necessary for the second hypothesis. For example, it is not known whether Pim degradation is affected by its binding with Thr and/or Sse, or by levels of Thr and/or Sse. Interestingly, there are indications that degradation of Securin may be affected by its binding with Separase in human cells (H. Zou, personal communication).

How do we explain the dominant effect of the *pim*<sup>2</sup> allele (Table 1 and Table 2)? Since Pim<sup>2</sup> can still bind to Thr even though it does not bind to Sse (Jäger et al., 2001), Pim<sup>2</sup> may inhibit Thr by titrating it into an ineffective Pim<sup>2</sup>-Thr complex that cannot recruit Sse (Jäger et al., 2001). Accordingly, Pim<sup>2</sup> would inactivate both Pim and Thr, thus it might have a phenotype similar to that seen with other *pim* alleles when they were combined with a *thr* mutation (Table 1).

### Suppression of the *six cycB* phenotype by reducing Sse levels

Lehner and colleagues have proposed that after the active Thr-Sse heterodimer cleaves the Cohesin subunit, Thr itself is cleaved by Sse, which presumably inactivates Sse at the end of anaphase (Herzig et al., 2002). Because of this negative feedback, Thr-Sse heterodimer activity is likely to be limited to a short time after anaphase begins. It is not known whether Thr is cleaved by the same Sse molecule that it binds or by another Thr-Sse dimer. A similar negative-feedback

mechanism in Separase regulation was found in *Xenopus* and human cells, where Separase undergoes auto-cleavage. However, the cleaved fragments are still active and remain associated, thus the function of the auto-cleavage in regulating anaphase onset is not resolved (Waizenegger et al., 2002; Zou et al., 2002).

If our hypothesis that levels of Thr and Pim affect the onset of anaphase by modifying Sse activity is correct, we expect Sse to be an enhancer. However, both the amorphic allele *sse*<sup>13m</sup> and the deficiency *Df(3L)SseA* are suppressors (Table 1). This presents a challenge. We propose two scenarios to explain this unexpected result. First, if cleavage of Thr by Thr-Sse inactivates Sse (Herzig et al., 2002), we speculate that both Thr-Sse heterodimers and Sse monomers have protease activity to cleave Thr bound to Sse. If so, compared with *six cycB* embryos, reducing Sse in *sse/six cycB* embryos would reduce the concentration of Thr-Sse, and thus the cleavage of Thr and the inactivation of Sse would take longer. The delay in Sse inactivation could have similar effects as increasing Thr-Sse levels (i.e. Separase activity) does, helping to overcome the inhibitory effect of a higher Cdk1-CycB activity on sister chromatid separation. Our explanation of the effect of Separase activity on the onset of anaphase is consistent with observations that depletion of a Cohesin subunit Drad21/Sccl in *Drosophila* cultured cells and embryos by RNAi leads to premature chromatid separation and abnormal spindle morphology (Vass et al., 2003), suggesting that the onset of anaphase is defined by the cleavage efficiency of Drad21/Sccl.

Alternatively, the suppressive effect of *Sse* could be caused by Sse possessing functions other than the ability to cleave the Cohesin subunit. This possibility is supported by the following observations in budding yeast. (1) Besides cleaving Securin, Separase can also cleave the kinetochore and the spindle associated protein Slk19 at the onset of anaphase. Cleaved Slk19 localizes to the spindle midzone and is required to maintain spindle stability in anaphase, preventing elongated spindle from breaking down prematurely (Sullivan et al., 2001). (2) Separase may also promote phosphorylation of Net1, the inhibitor of phosphatase Cdc14, thereby causing the release of Cdc14 from the nucleolus, a key step in mitotic exit (Sullivan and Uhlmann, 2003). It is still an open question whether Separase has additional substrates (Pellman and Christman, 2001). Although it is not known whether similar mechanisms also occur in *Drosophila*, it is possible that the suppressive effect we observed by reducing Sse may be caused by affecting the exit of mitosis through other Sse targets.

### Effects of Sse and Pim on cytoskeleton stability and nuclear migration pattern

We observed that reducing either Pim or Sse restores the microtubule morphology in interphase, but not in metaphase (Fig. 5, Table 2). In these embryos, nuclei show a faster and novel pattern in cortical migration, but this still leads to a normal nuclear distribution at cycle 10 (Fig. 4 and Table 2). Although it is not clear whether levels of Separase, Securin or APC/C modulate microtubule stability, it has been observed that Separase, Securin and components of the APC/C complex co-localize with spindle microtubules. For examples, in budding yeast, phosphorylated Pds1 (Securin) binds with Esp1 (Separase) and the complex is targeted to the spindle apparatus (Agarwal and Cohen-Fix, 2002). In *Drosophila*, both Sse and

Pim co-localize with spindle microtubules (Herzig et al., 2002). Furthermore, components of APC/C, such as CDC16 and CDC27, co-immunoprecipitate with microtubules in *Drosophila* embryos (Huang and Raff, 1999). Finally, Securin co-localizes with mitotic spindles in HeLa cells (Hagting et al., 2002).

Based on these observations, several hypotheses may explain the dosage effects of Pim and Sse on microtubule morphology at different cell-cycle phases. The most compelling one is that if CycB and Pim compete for polyubiquitination by APC/C on microtubules, reducing Pim may lead to faster CycB degradation, resulting in the restoration of microtubule morphology in interphase compared with in *six cycB* embryos. By contrast, because there is no degradation of either Pim or CycB in metaphase, the effect of degradation competition between Pim and CycB is absent, thus explaining why astral microtubule morphology is not restored in *pim/six cycB* embryos. As mentioned earlier, if Sse levels affect Pim degradation, reducing either Pim or Sse could have similar effects on CycB degradation. Thus we speculate that the interplay between the different kinetics of Cdk1-CycB activity and Separase activity over the cell cycle may contribute to the different effects of Sse/Pim dosage on microtubule stability.

To understand why reducing either Pim or Sse led to faster nuclear movements and a different nuclear migration pattern, the mechanics involved in the process of cortical migration need to be considered. Two major cytoskeletal networks are reorganized during this process: microtubules are stabilized in late telophase and early interphase, which pushes nuclei to the cortex (Baker et al., 1993); and the microfilament network is denser in the cortex than in the interior (von Dassow and Schubiger, 1994). Thus, the velocity and pattern of nuclear movement will be defined both by the pushing force generated by microtubules and by the resistance generated by the microfilament matrix.

In embryos with more Cdk1-CycB, microtubules become less stable (Ji et al., 2002). This may generate a weaker force to push nuclei to the cortex, resulting in the slower and less direct nuclear movement that we observed. When we further reduce either Pim or Sse, microtubule morphology is restored in early interphase (Fig. 5). This may contribute to the observation of faster nuclear cortical migration than in the *six cycB* embryos. However, why do nuclei in *Sse/four cycB* or *pim/four cycB* embryos move even faster than in *two cycB* embryos? This observation is puzzling to us. The simple explanation would be that the microtubule network is more robust in *Sse/four cycB* or *pim/four cycB* embryos than in *two cycB* embryos. Previously, we suggested a model in which microtubule and microfilament networks antagonistically interact with each other, and suggested that Cdk1-CycB activity negatively affects this interaction in early *Drosophila* embryos (Ji et al., 2002). Accordingly, a more robust microtubule network would result in a weaker microfilament network, presumably reducing the resistance for nuclear movement because of the less dense microfilament matrix in the extended cortex. The novel pathway of nuclear movement may reflect the disrupted balance between microtubule and microfilament networks because of the over-corrected microtubules in interphase. Consistent with this scenario, we also observed dramatic global cytoplasmic movements in *pim<sup>1</sup>/four cycB* and *sse<sup>13m</sup>/four cycB* embryos (Fig. 5C) during

the nuclear cortical migration. Thus, an increased microtubule network and the less dense microfilament matrix might account for accelerated nuclear movements.

Our genetic screen has identified modifiers of the *six cycB* phenotype (Ji et al., 2002). The studies have documented an interplay between Cdk1-CycB, microtubules and microfilaments. Here, we report three new modifiers that affect the *six cycB* phenotype. One of them, *thr*, is an enhancer. Interestingly, when the enhancer *thr* is combined with the suppressor *quail* (which encodes a villin-like protein), we find that the *six cycB* phenotype is restored (J.C. and G.S., unpublished). This indicates that, at least at the genetic level, the amount of Cdk1-CycB modulates many parameters of gene products regulating nuclear behavior and cytoskeletal stability.

Progress in developmental genetics requires the functional analyses of genes, which is best addressed by the description of pleiotropic phenotypes. We observed that increasing Cdk1-CycB in combination with decreasing Pim or Sse almost completely corrects the onset of anaphase and normalizes nuclear distribution at cycle 10. What is not expected and could only be observed by combining live analysis with data from fixed embryos is that microtubule configuration is corrected to wild type in interphase but not metaphase, and that a novel nuclear cortical migration pattern appears. Because this phenotype is only observed in combination with excessive Cdk1-CycB, we suggest using the term 'heterosis combined with epistasis' to describe the microtubule phenotype. Such a mechanism may have a selective advantage and therefore might occur in other slightly deleterious genetic combinations.

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