Positive feedback sharpens the anaphase switch

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At the onset of anaphase, sister-chromatid cohesion is dissolved abruptly and irreversibly, ensuring that all chromosome pairs disjoin almost simultaneously. The regulatory mechanisms that generate this switch-like behaviour are unclear. Anaphase is initiated when a ubiquitin ligase, the anaphase-promoting complex (APC), triggers the destruction of securin, thereby allowing separase, a protease, to disrupt sister-chromatid cohesion[1–4]. Here we demonstrate that the cyclin-dependent kinase 1 (Cdk1)-dependent phosphorylation of securin near its destruction-box motif inhibits securin ubiquitination by the APC. The phosphatase Cdc14 reverses securin phosphorylation, thereby increasing the rate of securin ubiquitination. Because separase is known to activate Cdc14 (refs 5 and 6), our results support the existence of a positive feedback loop that increases the abruptness of anaphase. Consistent with this model, we show that mutations that disrupt securin phosphoregulation decrease the synchrony of chromosome segregation. Our results also suggest that coupling securin degradation with changes in Cdk1 and Cdc14 activities helps coordinate the initiation of sister-chromatid separation with changes in spindle dynamics.

Securin is known to integrate multiple signalling pathways to delay anaphase in response to perturbations such as DNA damage[7,8]. We hypothesized that securin might also receive regulatory inputs that make separase activation more switch-like. To address this possibility, we used mass spectrometry to analyse the phosphorylation state of securin from mitotic budding yeast cells (Fig. 1a)[9]. We identified six phosphorylation sites, including four of the five sites corresponding to the consensus sequence of Cdk1 (S/T*-P) (see also Supplementary Fig. 1). Three Cdk1 sites were found in securin's carboxy (C)-terminal domain, which is known to interact with separase[10]; mutations at these sites are known to modulate securin's ability to bind separase and import it into the nucleus[11]. The fourth Cdk1 phosphopeptide, near the amino (N) terminus of securin, has not been characterized.

The N-terminal Cdk1 site is near the destruction-box of securin, a motif important for recognition of substrates by the APC[2–4,12]. To assess whether phosphorylation of this site affects securin ubiquitination by the APC, we tested the ability of purified APC to ubiquitinate purified securin before and after phosphorylation *in vitro* by Cdk1. We found that if securin was phosphorylated by Cdk1, the rate of ubiquitination by APC^{Cdc20} (the APC–activator complex that controls anaphase onset) was reduced tenfold (Fig. 1b, c) and the rate of ubiquitination by APC^{Cdh1} was reduced fivefold (Supplementary Fig. 2).

This regulatory mechanism may be conserved: *Drosophila* and human homologues of securin have S/T-P motifs near their destruction-boxes, and both were ubiquitinated less efficiently by the APC after phosphorylation by Cdk1 (Supplementary Fig. 3).

Cdc14 is a phosphatase that removes phosphates from Cdk1 substrates during anaphase and late mitosis[4,13–16]. We found that Cdc14 removed Cdk1-dependent phosphates from securin, as judged by the loss of a gel-mobility shift that results from phosphorylation (Fig. 1b; Supplementary Fig. 2a). Dephosphorylation reactivated securin as an efficient APC substrate.

To confirm that regulation depends on the canonical Cdk1 sites, we mutagenized securin to remove the N-terminal Cdk1 sites
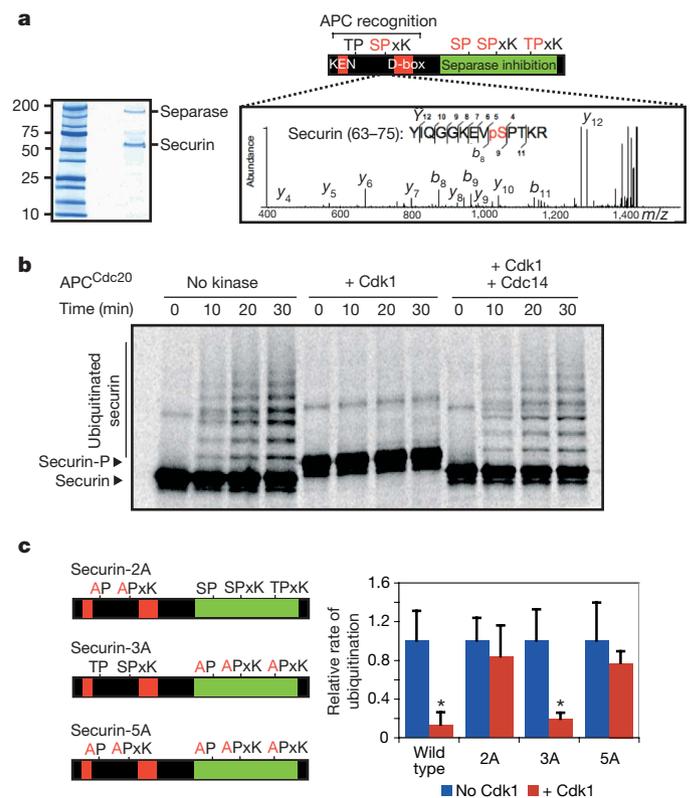

Figure 1 | Cdk1 and Cdc14 control the phosphorylation state of securin near its destruction-box and modulate the rate of securin ubiquitination.
a, Securin was purified from a benomyl-arrested strain carrying *SECURIN–3FLAG–6HIS* (left panel), and tandem mass spectrometry analysis resulted in the detection of ten phosphopeptides (Supplementary Fig. 1), including four peptides phosphorylated at Cdk1 consensus sites (red). One of these sites lies next to the securin destruction-box motif (the MS^3 spectrum of the phosphopeptide is shown to the right, with the phosphopeptide sequence most consistent with the data). **b**, [^{35}S]methionine-labelled securin was produced by translation *in vitro* and incubated with purified APC^{Cdc20} and other ubiquitination components. Before the reactions, as indicated, some samples were incubated with either purified Clb2–Cdk1 or with Clb2–Cdk1 and then Cdc14 sequentially. The substrate was purified away from kinase and phosphatase before addition of APC^{Cdc20}. **c**, Quantification of experiments conducted as in **b** using either wild-type securin, securin-2A (T27A, S71A), securin-3A (S277A, S292A, T304A) or securin-5A as substrate. Error bars, s.d. from three experiments. Asterisks, $P < 0.05$ (*t*-test) when comparing results with and without kinase.

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(securin-2A), the three C-terminal sites (securin-3A) or all five sites (securin-5A). Securin-2A and securin-5A were still recognized by the APC after incubation with Cdk1 (Fig. 1c and Supplementary Fig. 2b), whereas securin-3A ubiquitination was inhibited by Cdk1 (Fig. 1c). We conclude that the ability of Cdk1 and Cdc14 to control securin ubiquitination depends on one or both of the N-terminal Cdk1 sites.

Current models of anaphase regulation do not provide many opportunities for switch-like behaviour; in particular there is no known positive feedback in this system (Fig. 2a)[17,18]. It is therefore interesting that separase, in addition to cleaving cohesin, initiates the activation of Cdc14 in early anaphase[5,6]. Thus, our observation that Cdc14 can dephosphorylate securin, and thereby increase its efficiency as an APC substrate, leads to the potential for a positive feedback loop that is predicted to make anaphase more switch-like (Fig. 2b).

APC^{Cdc20} activation triggers the destruction of cyclins as well as securin (Fig. 2a, b). Thus, the kinases responsible for securin phosphorylation are partly inactivated at anaphase initiation, providing an additional mechanism by which securin destruction is promoted, and potentially increasing the robustness of this switch.

We explored a simple mathematical version of this model (Fig. 2c: see Supplementary Fig. 4 for details) by numerically determining the

steady-state levels of securin as a function of APC activity under a range of parameters. We did not include the effects of the APC on Cdk activity in the model, as it did not significantly affect the outcome of the simulations (Supplementary Fig. 5). To explore how the modulation of securin destruction might affect the abruptness of anaphase, we varied the effect of securin phosphorylation on the rate of its destruction. As we increased the inhibitory effect of phosphorylation from 1- to 100-fold, securin degradation became more switch-like (Fig. 2c).

To investigate the relevance of our model *in vivo*, we created a yeast strain in which we specifically disrupted the putative positive feedback loop by replacing the securin gene with a mutant gene encoding securin-2A. This strain was viable at both 30 °C (the restrictive temperature for a *securinΔ* strain) and 37 °C, suggesting that securin-2A is functional in its ability to bind and fold separase. We measured the timing of securin-2A degradation in cells released from a G1 arrest and found no significant difference between the mutant and wild-type securin (Supplementary Fig. 6). We suspected, however, that asynchrony in the cell population in these experiments prevented the detection of small differences in the timing and abruptness of securin destruction. We therefore attempted to develop microscopic methods to measure securin destruction in single cells. Green fluorescent protein (GFP)-tagged securin was undetectable when expressed at normal levels, and overexpressed securin–GFP was detectable but delayed cells in late mitosis. It was possible, however, to assess the relative stabilities of securin and securin-2A in late mitosis by simultaneously overexpressing and analysing the levels of both proteins, each tagged with a different GFP variant. We found that mitotic securin-2A levels were lower than those of wild-type securin (Supplementary Fig. 7).

To analyse the consequences of securin phosphoregulation in more detail, we developed methods for the analysis of spindle dynamics and the abruptness of anaphase onset in single cells. To quantify spindle length, we tagged the spindle-pole body protein, Spc42, with dsRed. To visualize the rate and abruptness of chromosome segregation, we integrated *LAC* and *TET* operator arrays at two independent loci, *TRP1* and *URA3*, respectively, and expressed GFP-tagged Lac and Tet repressors in the cell to generate fluorescent marks at these loci[19,20]. Chromosomes IV (*TRP1-LACO*) and V (*URA3-TETO*) were thereby marked at loci that are close to the centromere (12 and 35 kilobases, respectively) but far enough away that they are not resolved until cohesin is cleaved by separase. One GFP-labelled locus was consistently brighter than the other, and we found that this dot disappeared upon addition of tetracycline, suggesting that the bright dot labels chromosome V (Supplementary Fig. 8).

We used a spinning-disk confocal microscope to obtain movies of anaphase at a time resolution of 10 s. These movies allowed us to determine the time lag between the segregation of one chromosome and that of a second, providing a direct measure of the abruptness of anaphase. In addition, we measured the rates and coordination of spindle elongation and chromosome segregation.

If there is any variation in the inherent sensitivity of each chromosome to separase activity—that is, if different chromatid pairs disjoin at different thresholds of separase activity—then the time lag between segregation of one pair of sister chromatids and another is a measure of the abruptness of separase activation (Supplementary Fig. 9). Indeed, we found that there was a reproducible time lag between the segregation of the two labelled chromosomes in our strain. In addition, the order of chromosome segregation was invariant: in 100% of cells, the bright dot on chromosome V segregated after the dot on chromosome IV. Quantification of 40 wild-type anaphases (Fig. 3a, d) revealed that the median time between the two chromosome segregation events (t_{seg}) was 90 s, with most cells clustered tightly around this value. This abrupt timing of anaphase depends on rapid activation of separase: a mutant with reduced separase function (*esp1-1*) displayed a less synchronous anaphase (median t_{seg} = 180 s).
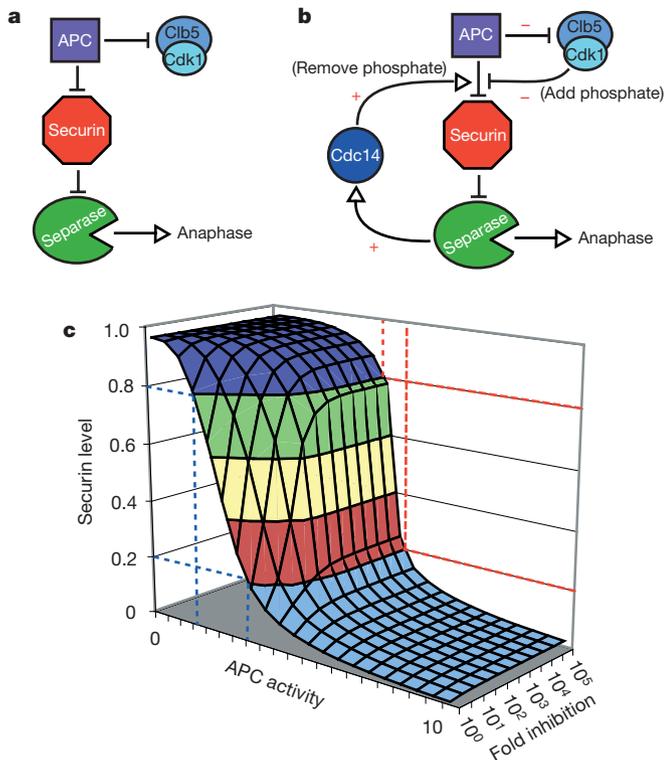

Figure 2 | Modulation of securin ubiquitination by Cdk1 and Cdc14 gives rise to a potential positive feedback loop in the anaphase regulatory network. a, Simplified previous model for anaphase regulation in *S. cerevisiae*: the APC targets securin and some mitotic cyclins for destruction, which liberates separase to cleave cohesin and initiate anaphase. **b**, Modified model of anaphase control: Cdk1 phosphorylates securin, reducing the rate at which it is ubiquitinated by the APC. On accumulation of high levels of APC activity, some securin is destroyed, releasing a small amount of separase. Separase activates Cdc14, which dephosphorylates securin, increasing the rate at which it is ubiquitinated by the APC. Concomitantly, some mitotic cyclins are destroyed, reducing Cdk1 activity and allowing Cdc14 to dephosphorylate securin more efficiently. **c**, Set of numerical solutions to a simplified mathematical model of the network in **b** (but excluding cyclin degradation by the APC), showing the steady-state levels of securin remaining (*y*-axis) as APC activity is varied (*x*-axis) when securin ubiquitination is inhibited to various degrees by phosphorylation (*z*-axis) (see Supplementary Fig. 3 for details of model).

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If the positive feedback loop proposed in Fig. 2b is operating *in vivo*, then disruption of this loop should decrease the abruptness of anaphase. Indeed, the *securin-2A* mutant strain had a t_{seg} of 170 s, almost double that of wild-type cells (Fig. 3b, d).

Our model (Fig. 2b) predicts that positive feedback should be weakened by reducing Cdc14 activity or increasing Cdk1 activity. Consistent with this possibility, we observed less anaphase synchrony in *cdc14-1* mutant cells ($t_{seg} = 190$ s) and in cells expressing Clb5-Δdb, a partly stabilized cyclin mutant that lacks its destruction-box and thus generates high levels of Cdk1 activity in anaphase ($t_{seg} = 150$ s) (ref. 21). Defects in Cdc14 or Clb5 affect numerous processes in addition to securin degradation. Nevertheless, when taken together with the effects of the securin-2A mutant, these results support the hypothesis that phosphorylation of the N-terminal Cdk1 sites of securin creates a positive feedback loop that makes anaphase more switch-like.

We also analysed cells carrying a deletion of securin, the loss of which reduces separase activity and partly uncouples anaphase initiation from APC activity. Securin deletion dramatically reduced the synchrony of sister separation ($t_{seg} = 300$ s; Fig. 3c, d), arguing that a major role for securin is to increase the abruptness of the metaphase-to-anaphase transition.

A second possible function for phosphoregulation of securin is the coordination of separase activation with changes in anaphase spindle behaviour. After APC[Cdc20] activation, partial cyclin destruction, together with Cdc14 activation by separase, results in dephosphorylation of a subset of Cdk1 substrates that trigger important changes in the behaviour of spindle and kinetochore proteins[4,22–25]. These changes must be coordinated with the cleavage of cohesin. We propose that this coordination depends in part on phosphoregulation of securin, which we predict delays securin degradation relative to that of cyclins, allowing more robust dephosphorylation of Cdk1
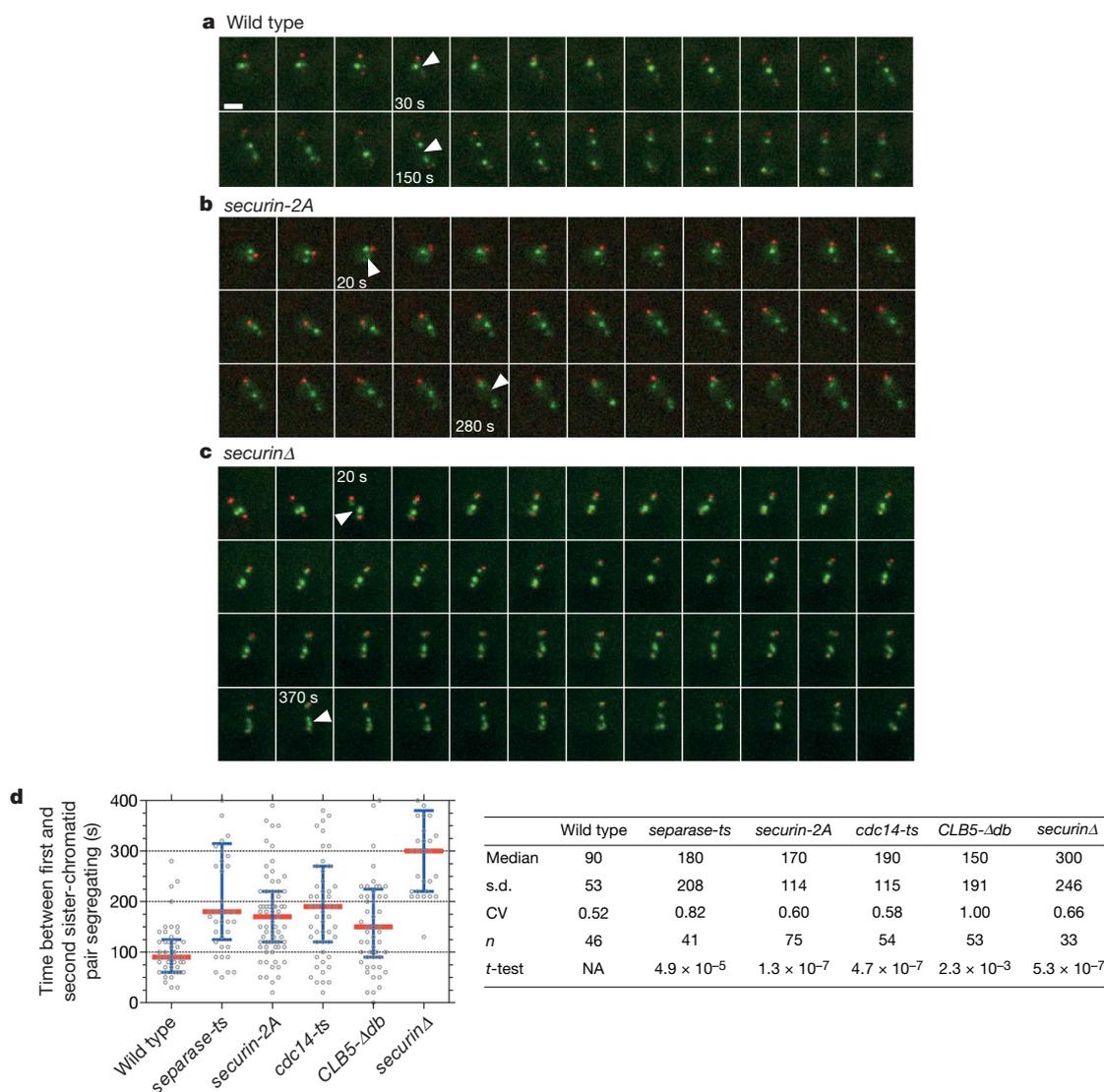

a Wild type

b *securin-2A*

c *securinΔ*

d

	Wild type	*separase-ts*	*securin-2A*	*cdc14-ts*	*CLB5-Δdb*	*securinΔ*
Median	90	180	170	190	150	300
s.d.	53	208	114	115	191	246
CV	0.52	0.82	0.60	0.58	1.00	0.66
n	46	41	75	54	53	33
t-test	NA	4.9×10^{-5}	1.3×10^{-7}	4.7×10^{-7}	2.3×10^{-3}	5.3×10^{-7}

Figure 3 | Modulation of securin ubiquitination by Cdk1 is required for an abrupt anaphase. a, A strain containing *SPC42-dsRed:NAT; trp1::256×LACO:TRP1; his3::CUP1-GFP-LACI:HIS3; ura3::112×TETO:URA3; leu2::TETR-GFP:LEU2* has red spindle poles and GFP dots marking chromosomes IV and V. Each panel in this montage represents 10 s in a time-lapse sequence during progression through anaphase. Arrowheads indicate the first and second chromosome separation. Each panel is a maximum intensity projection of a 7-μm stack. Note that the daughter spindle pole can be difficult to visualize owing to slow maturation of the dsRed fluorophore. Scale bar, 2 μM. **b**, Anaphase onset in a *securin-2A* strain. **c**, Anaphase onset in a *securinΔ* strain. **d**, Anaphase synchrony (time between the first and second chromosomes segregating) in approximately 40 cells of wild-type, separase mutant (*esp1-1*), securin-2A, cdc14-1, CLB5-Δdb and securinΔ strains. The time of sister-chromatid separation was defined as the first time point when chromatids separated and did not re-anneal in subsequent frames. The red line is the median and the blue bars indicate the inter-quartile range. The table below shows the median, s.d. and coefficient of variation (CV; s.d. divided by the mean) for each strain. NA, not applicable. The movies corresponding to panels **a**–**c** are found in Supplementary Movies 1–3, respectively.

substrates by the time cohesin cleavage begins. We also hypothesize that switch-like activation of separase generates switch-like activation of Cdc14, resulting in robust and coordinated dephosphorylation of the many factors controlling anaphase spindle behaviour.

We therefore investigated anaphase dynamics in wild-type cells and in cells with perturbed securin regulation. In wild-type cells (Fig. 4a), anaphase was robust and stereotypical, as illustrated by the spindle-pole and chromosome tracking data in Fig. 4b. Each pair of sister chromatids segregated rapidly and unidirectionally (quantified in Fig. 4c), and the spindle elongated robustly in two phases: a rapid initial phase (spindle rate I) and a slower second phase (spindle rate II), as described previously[19]. Spindle elongation initiated at the same time as chromosome disjunction (Fig. 4a), illustrating the tight temporal coordination of cohesin cleavage with changes in spindle dynamics.

We next analysed anaphase in the *securin-2A* strain, in which we predict the normal delay of separase activation is lost, resulting in cohesin cleavage early relative to dephosphorylation of Cdk1 substrates. We also analysed the effects of the *CLB5-Δdb* mutation, which is likewise predicted to introduce a delay in the dephosphorylation of Cdk1 substrates relative to separase activation. Finally, we analysed *securinΔ* cells, where separase activation is less tightly linked to changes in Cdk1 activity. We observed a wide range of anaphase defects in all mutants (Fig. 4). In a subset of *securin-2A* cells, the disjunction of chromatids was not coordinated with elongation of the spindle, chromosomes failed to segregate processively and the rates of chromatid separation at anaphase A were significantly reduced (Fig. 4a, c). Spindle defects were more pronounced in *CLB5-Δdb* and *securinΔ* cells, in which the spindle frequently failed to elongate or elongated and then collapsed back to a shorter length (Fig. 4a). Our results are consistent with a general role for securin in the coordination of chromosome disjunction with changes in the

phosphorylation state of Cdk1 substrates on the spindle and kinetochores. They also support the specific hypothesis that modulation of securin ubiquitination by phosphorylation helps coordinate the events of anaphase.

We assessed the accuracy of anaphase in our GFP-labelled yeast strain. Each cell in this strain is expected to have two GFP dots before anaphase, and cells containing more than two dots are products of a mis-segregation event. We found that loss of phosphoregulation of securin in the *securin-2A* mutant led to an elevated rate of chromosome mis-segregation, as did inappropriate Cdk1 activity in the *CLB5-Δdb* mutant (Fig. 4d).

We conclude that positive feedback in securin regulation promotes accurate chromosome segregation. Positive feedback could generate bistability in anaphase control[17,18], ensuring that the cell is irreversibly committed to complete sister-chromatid separation. Synchronous sister separation may have several benefits: for example, if disjunction of the first chromatid pair is sensed by the spindle assembly checkpoint, then rapid separation of the remaining sisters would reduce the danger of inappropriate checkpoint activation before anaphase is complete. In addition, the initiation of chromosome segregation might cause local perturbations to the spindle, making it important to transition rapidly to the segregation mode of spindle function to avoid mechanical catastrophe.

Our studies support the possibility that each sister chromatid pair separates at a distinct threshold of separase activity (Supplementary Fig. 9). Such differences in separase sensitivity would help explain the observation that sister chromatids separate in a specific order, as we observed in yeast and as previously observed in plant and animal cells[26,27]. The ordered segregation of chromatids provides a mechanism to maintain nuclear architecture through successive generations[27]. However, the existence of distinct separase thresholds provides a challenge for the rapid execution of anaphase. The most
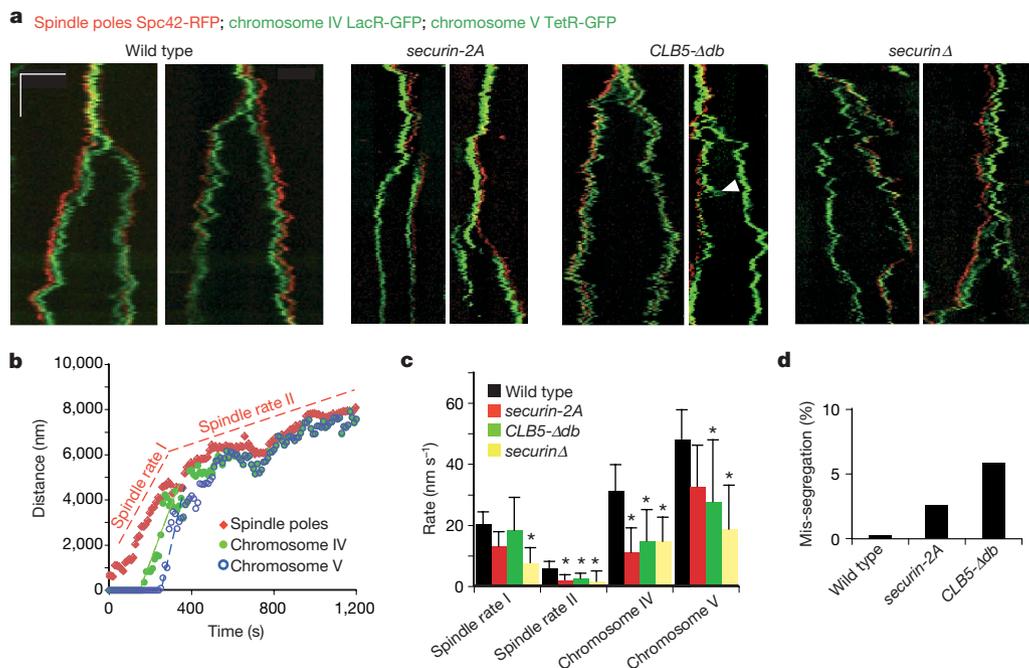

Figure 4 | Modulation of securin ubiquitination by Cdk1 helps coordinate anaphase onset with changes in spindle dynamics. a, Kymographs from wild-type and mutant cells. The *x* axis is a one-dimensional projection along the spindle axis (scale bar, 5 μm) and the *y* axis is time (scale bar, 5 min; total time, 30 min). **b**, Spindle length (red diamonds) and chromosome segregation (green dots, chromosome IV; blue circles, chromosome V) in a wild-type anaphase, using a spot-tracking algorithm[30]. Rates of the two phases of spindle elongation are highlighted by red dashed lines; initial rates of chromatid segregation are highlighted with green and blue dashed lines.

When the daughter spindle pole was difficult to detect because of the slow maturation of the dsRed fluorophore, the position of the associated sister chromatid was used as a proxy for spindle-pole position after anaphase A. **c**, Average rates of spindle elongation and sister-chromatid separation in wild-type and mutant cells. Error bars, s.d. (*n* = 10 cells). Asterisks, *P* < 0.05 (*t*-test) when comparing the wild type with the mutant. **d**, Rate of chromosome mis-segregation in wild-type and mutant cells. Only cells with more than the expected number of GFP dots were scored as a mis-segregation event.

effective mechanism for achieving chromosome segregation that is both ordered and abrupt is to make separase activation rapid and switch-like.

METHODS SUMMARY

Yeast strains. *Saccharomyces cerevisiae* strains were derivatives of a W303 diploid strain containing: *SPC42-dsRed:NAT; trp1::256× LACO:TRP1/trp1-1; his3::CUP1-GFP-LACI:HIS3/his3-11; ura3::112× TETO:URA3/ura3-1; leu2::TETR-GFP:LEU2/leu2-1.* This strain was created by mating strain LH557 (*Mat a; trp1::256× LACO:TRP1; his3::CUP1-GFP-LACI:HIS3; SPC42-dsRed-NAT*) with LH554 (*Mat alpha; leu2::TETR-GFP:LEU2; ura3::112× TETO:URA3; SPC42-dsRed-NAT*).

Protein purification. Securin–3FLAG–6HIS was purified by the tandem affinity method[9]. APC, Ubc4, Uba1 and Cdh1 were purified as described[28]. Clb2 was expressed from a 2μ P_{GAL1}-*TAP* plasmid, and Clb2–Cdk1 complexes were purified using a C-terminal TAP tag on Clb2 (ref. 29). GST–Cdc14 was purified from bacteria[15]. Securin and Cdc20 were expressed in the TNT reticulocyte lysate-coupled transcription/translation system (Promega) with a C- and N-terminal TEV-ZZ tag, respectively, purified rapidly on M-270 epoxy dynabeads (Invitrogen) coupled to IgG, and eluted with TEV protease.

Mass spectrometry. Identification of phosphorylation sites was performed using a modular mass spectrometric tool as described[9].

Ubiquitination assays. Ubiquitination assays were performed as described[28], except where securin was pre-incubated with Cdk1–Clb2 in a 10-μl reaction volume containing 25 mM HEPES pH 7.4, 150 mM NaCl, 10% glycerol, 0.1 mg ml^{-1} BSA, 1 mM ATP, and 10 mM MgCl$_2$ for 20 min at room temperature. Kinase reactions were terminated by incubation for 5 min with 50 mM EDTA. In some cases, Cdc14 was then added to this mixture for a further 20 min at room temperature before ubiquitination assays.

Microscopy. All microscopy was performed in the University of California, San Francisco, Nikon Imaging Center using a TE2000U inverted microscope (Nikon) with Yokogawa CSU22 spinning disk confocal illumination (Solamere Technology Group) and a Cascade II CCD Camera (Photometrics). Stacks of seven images 1 μm apart were acquired every 10 s for 30 min at 25 °C. Images were acquired using micro-manager software (http://micro-manager.org/) and analysed using ImageJ (http://rsb.info.nih.gov/ij/) with the SpotTracker2D plugin (http://bigwww.epfl.ch/sage/soft/spottracker/gasser.html).

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