Cyclin-B1-mediated inhibition of excess separase is required for timely chromosome disjunction

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Summary

Separase, the cysteine protease that cleaves cohesin and thereby triggers chromosome disjunction, is inhibited by both securin- and phosphorylation-dependent cyclin B1 binding. Using a novel phosphorylation-specific antibody, we show that mitotic-specific phosphorylation of human separase on S1126 is required to establish, but not maintain, cyclin B1 binding. Cells expressing a non-phosphorylatable S1126A mutant maintain cohesion early in mitosis, aligning their chromosomes. Cohesion is then synchronously lost 5 minutes ahead of schedule, without degrading securin or cyclin B1. This premature chromatid disjunction requires the catalytic activity of separase, indicating that it is dependent on cohesin cleavage. Single chromatids then attempt to realign but the lack of tension results in unstable kinetochore-microtubule interactions and Aurora-B-dependent spindle checkpoint activation. Separase mutants that cannot bind cyclin B1 but are phosphorylated on S1126 phenocopy separase S1126A, indicating that cyclin B1 binding, rather than phosphorylation, is the key inhibitory event. Significantly, by overexpressing separase S1126A, we have simultaneously overridden the two known inhibitory mechanisms. First, by elevating separase levels above securin, securin-mediated inhibition is alleviated. Second, by preventing phosphorylation, cyclin-B1-mediated inhibition is also alleviated. Surprisingly, however, cohesion is maintained during the early stages of mitosis, indicating the existence of another mechanism that either inhibits separase or protects its substrate during early mitosis.

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Introduction

Anaphase is marked by the synchronous loss of sister chromatid cohesion, allowing the replicated chromosomes to segregate to opposite ends of the cell, and thereby ensuring that cell division produces two genetically identical daughters. Elegant experiments in yeast have shown that the key regulatory step is the activation of separase, a cysteine protease that cleaves cohesin, the molecular string responsible for sister chromatid cohesion (Uhlmann et al., 1999; Uhlmann et al., 2000; Gruber et al., 2003). Separase activation must be carefully timed: activation before all the chromosomes are bi-oriented on the microtubule spindle risks the production of aneuploid daughters (Weaver and Cleveland, 2005). Prior to bi-orientation, separase is inhibited by a chaperone, securin (Ciosk et al., 1998). Following chromosome alignment, securin is ubiquitylated and then degraded, thus liberating and activating separase (Funabiki et al., 1996; Ciosk et al., 1998).

The mechanisms that regulate the loss of sister chromatid cohesion are largely conserved in vertebrates: separase is required for the separation of sister chromatids at anaphase (Kumada et al., 2006; Wirth et al., 2006), and securin binds and inhibits separase before being degraded just prior to sister chromatid separation (Zou et al., 1999; Hagting et al., 2002). However, to resolve sister chromatids in higher eukaryotes, the vast majority of arm cohesion is removed during prophase (Losada et al., 1998; Waizenegger et al., 2000; Losada et al., 2002; Hauf et al., 2005). This is mediated by two kinases, Plk1 and Aurora B, that phosphorylate the SA2 subunit of cohesin, thereby causing its dissociation from the chromosomes (Sumara et al., 2002; Gimenez-Abian et al., 2004; Hauf et al., 2005). During this process, the condensing sisters remain cohesed by virtue of residual cohesion, predominantly at the centromere. Here, cohesin is protected from the prophase pathway by Sgo1 (Salic et al., 2004; McGuinness et al., 2005), a protein originally identified as being required to maintain chromatid cohesion during meiosis I (Kitajima et al., 2004).

Despite securin's conserved role as an inhibitor of separase, it is not essential for maintaining cohesion in mammals. In particular, *Securin*$^{-/-}$ mice are viable and fertile (Mei et al., 2001; Wang et al., 2001) and human HCT116 cells lacking securin execute mitosis normally (Pfleghaar et al., 2005). Furthermore, in response to spindle toxins, cells that lack securin arrest with cohesed chromosomes (Jallepalli et al., 2001). This suggests that other mechanisms must contribute to separase regulation in higher eukaryotes. Indeed, two mechanisms for inhibiting separase have been described in *Xenopus* egg extracts. Early studies showed that a non-degradable cyclin B1 arrested extracts in late anaphase, whereas inhibitors of ubiquitylation blocked both mitotic exit and sister chromatid separation (Holloway et al., 1993). These experiments formed the conceptual basis for the existence of a degradable anaphase inhibitor, later found to be securin. However, subsequent work in this system has shown that sister chromatid separation is sensitive to the levels of non-

degradable cyclin B1: whereas low levels allow the separation of sister chromatids, high levels inhibit anaphase (Stemmann et al., 2001). This inhibition is mediated by the phosphorylation of separase on S1126, which in turn promotes the binding of cyclin B1 (Gorr et al., 2005). Significantly, the binding of securin and cyclin B1 to separase is mutually exclusive (Gorr et al., 2005).

Indirect evidence suggests that cyclin B1 also inhibits separase in mammalian cells: overexpression of a non-degradable cyclin B1 arrests cells in metaphase, despite the degradation of endogenous cyclin B1 and securin (Hagting et al., 2002; Chang et al., 2003; Herbert et al., 2003). Furthermore, there is evidence that both securin and cyclin B1 interact with separase in human cells (Gorr et al., 2005). However, whereas the biochemical evidence supporting the existence of a separase–cyclin-B1 complex is compelling, the physiological significance of this mechanism in regulating anaphase onset in somatic cells remains unclear. To directly test the significance, an allele encoding a non-phosphorylatable separase mutant (S1121A, the murine equivalent to S1126A) was knocked-in to mouse ES cells (Huang et al., 2005). It was predicted that if separase phosphorylation is required to prevent premature activation, then the non-phosphorylatable mutant would have a dominant effect, inducing a premature loss of cohesion. Surprisingly, however, there was no apparent phenotype: *Separase*$^{+/S1121A}$ cells were viable and divided normally (Huang et al., 2005). Even when the *S1121A* allele was knocked-in to *Securin*$^{-/-}$ ES cells, there was no gross phenotype: *Securin*$^{-/-}$*; Separase*$^{+/S1121A}$ cells were viable and only showed signs of chromatid separation following prolonged treatment with spindle toxins.

Here, we have addressed the role of separase phosphorylation in human somatic cells. First, we describe a novel antibody that specifically recognises human separase when phosphorylated on S1126. We confirm that human separase is phosphorylated on this residue in a mitosis-specific manner, and show that this phosphorylation is required to initiate, but not maintain, cyclin B1 binding. Second, we demonstrate that expression of a non-phosphorylatable separase mutant (S1126A) in excess of securin, has a dominant effect in human cells, inducing a premature loss of sister chromatid cohesion. However, despite overriding separase inhibition by both cyclin B1 and securin, cells expressing separase S1126A still maintain cohesion long enough for chromosomes to align. This suggests that a third mechanism, independent of separase inhibition by securin and cyclin B1, may prevent the loss of cohesion in early mitosis.

Results

Separase is phosphorylated on S1126 in human cells

Human separase is phosphorylated on S1126 in mitosis (Stemmann et al., 2001). However, the physiological significance of this in regulating anaphase in somatic cells is unclear. Therefore, to dissect the function of this phosphorylation event, we generated an antibody that recognises separase when phosphorylated on S1126 (Fig. 1A). Briefly, rabbits were immunised with an appropriate phosphorylated peptide and antibodies then affinity-purified based on their ability to bind either the phosphorylated or the unphosphorylated peptide (Fig. 1B). We therefore generated two anti-peptide antibodies, an anti-separase antibody and an anti-phosphorylated S1126 antibody (*P*-S1126). To test whether these antibodies recognised separase, we expressed in cells either wild-type separase (WT) or a non-phosphorylatable S1126A mutant (S:A), both as Myc-tagged fusions. We then immunoprecipitated the Myc-tagged proteins from mitotic-enriched cells and analysed them by immunoblotting. Whereas the anti-separase antibody recognised both WT and S:A, *P*-S1126 antibody only recognised WT separase (Fig. 1C). Importantly, the ability of *P*-S1126 to recognise WT separase was abolished when the immune-complex was treated with λ phosphatase (Fig. 3A, compare lanes 1 with 3). Thus, these observations confirm that separase is phosphorylated on S1126 in human cells, and demonstrate that the *P*-S1126 antibody represents a new tool to study separase regulation.

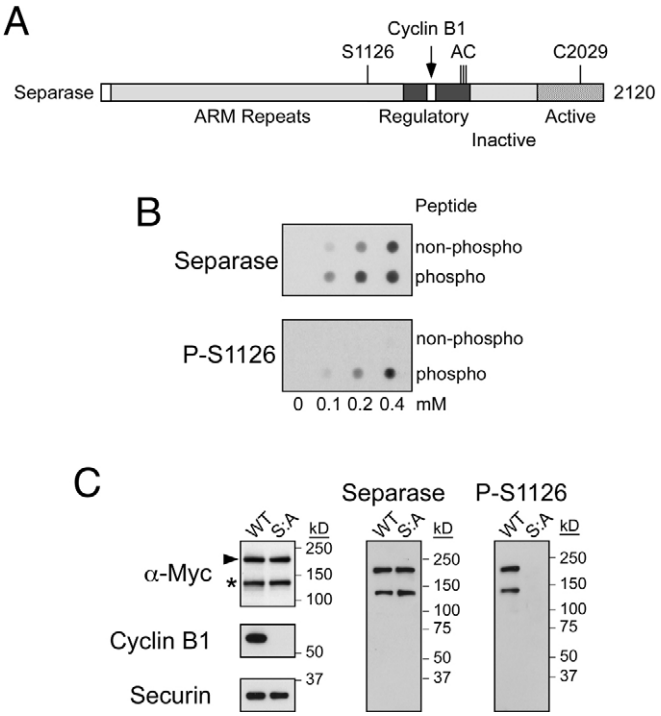

Fig. 1. Human separase is phosphorylated on S1126. (A) Schematic representation of human separase showing the ARM-repeat domain, the active and inactive caspase-like domains, and the regulatory region, which contains S1126 and the cyclin B1 binding domain. The catalytic cysteine C2029 and the auto-cleavage sites (AC) are shown. (B) Dot blot showing that the separase antibody recognises the phosphorylated and non-phosphorylated peptides, whereas the *P*-S1126 antibody only detects the phosphorylated peptide. (C) Immunoprecipitations of Myc-tagged proteins showing that the separase antibody recognises both WT and separase S:A, whereas the *P*-S1126 antibody only detects WT. The band marked by the arrow head represents full-length separase, the asterisk marks the N-terminal auto-cleavage product.

Separase S1126 phosphorylation is mitosis specific

To determine when separase is phosphorylated on S1126, cells expressing Myc-tagged WT or S:A separase were enriched in G1-S or mitosis by using thymidine or spindle toxins, respectively. Myc-tagged proteins were then immunoprecipitated and analysed by immunoblotting. Whereas

S:A separase was not phosphorylated under any of the conditions, phosphorylation of WT separase on S1126 was enriched in mitotic cells compared with G1-S cells (Fig. 2A, compare lane 1 with lanes 2 and 3). To confirm whether the same is true of the endogenous protein, we analysed asynchronous populations or cultures that had been treated with nocodazole to enrich for mitotic cells. Phosphorylated separase was detected in the lysates from the nocodazole-treated population but not the asynchronous culture (Fig. 2B, compare lanes 1 and 2). Furthermore, when we immunoprecipitated cyclin B1, phosphorylated separase could be detected in immune-complexes isolated from the nocodazole-treated population, but not the asynchronous culture (Fig. 2B, compare lanes 4 and 6). These data are therefore consistent with the notion that endogenous separase is phosphorylated and forms a complex with cyclin B1 in a mitosis-specific manner.

S1126 phosphorylation is required to initiate but not maintain cyclin B1 binding

In *Xenopus* egg extracts and transfected HEK 293 cells, separase phosphorylation on S1126 promotes binding of cyclin B1 (Gorr et al., 2005). When we immuno-purified WT separase from mitotic-enriched human cells, both securin and cyclin B1 were present (Fig. 1C and Fig. 2A), consistent with the notion that separase binds both securin and cyclin B1. However, whereas securin was present in the separase S:A immune-complex, cyclin B1 was not (Fig. 1C and Fig. 2A). In addition, when we immuno-purified cyclin B1 from transfected cells, WT separase but not the S:A mutant co-purified (data not shown). These observations confirm, therefore, that separase S1126 phosphorylation is indeed required for cyclin B1 binding in human somatic cells. Furthermore, whereas separase was present in the cyclin B1 immune complex, securin was not (Fig. 2B), consistent with the notion that binding of securin and cyclin B1 to separase are mutually exclusive (Gorr et al., 2005). In addition, exogenous WT separase was present in immunoprecipitates of endogenous cyclin B1, whereas securin was not (data not shown).

It has been suggested that S1126 becomes inaccessible to dephosphorylation following cyclin B1 binding (Gorr et al., 2005). Having generated an antibody that recognises separase when phosphorylated on S1126, we tested this notion directly. Myc-tagged WT and separase S:A were immunoprecipitated from mitotic-enriched cells and treated with λ phosphatase. In the absence of phosphatase, WT separase was phosphorylated on S1126 and remained bound to both securin and cyclin B1 when washed in low-salt buffer (Fig. 3A, lane 1). Consistent with the data shown in Fig. 1C and Fig. 2A, separase S:A bound only securin (Fig. 3A, lane 5). Importantly, however, treatment with λ phosphatase removed the phosphate from S1126, whereas cyclin B1 and securin binding were largely unaffected (Fig. 3A, lane 3). Cyclin B1 could be released with a high-salt wash (Fig. 3A, lanes 2 and 4), confirming that cyclin B1 was not irreversibly bound to the affinity matrix.

The observation that separase still binds cyclin B1 despite

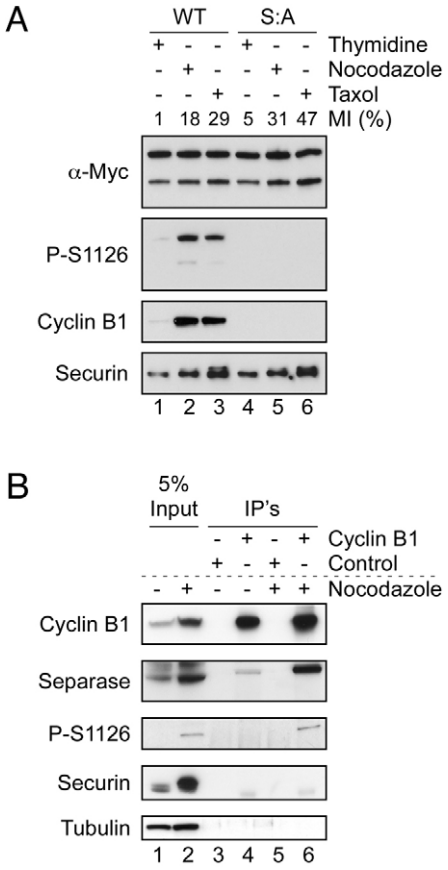

Fig. 2. S1126 phosphorylation is mitotic-specific. (A) Anti-Myc immunoprecipitates from cells synchronised with thymidine, nocodazole or taxol, showing that separase is phosphorylated and bound to cyclin B1 only in mitotic-enriched populations. MI represents the mitotic index (%) of the population. (B) Cyclin B1 immunoprecipitations from asynchronous or nocodazole-treated HeLa cells showing that phosphorylated separase is present in the immune complex isolated from the nocodazole-treated sample.

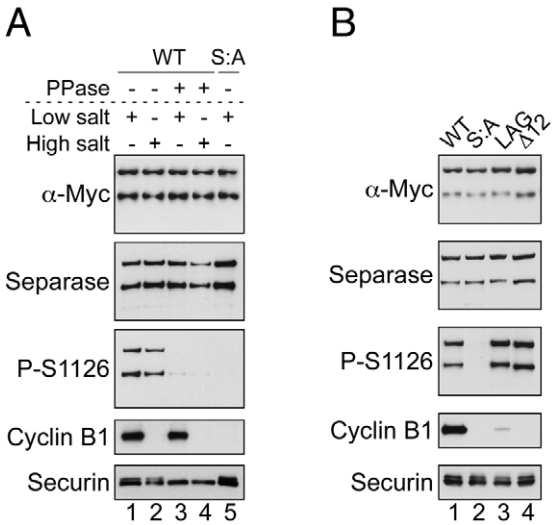

Fig. 3. S1126 phosphorylation is not required to maintain cyclin B1 binding. (A) Anti-Myc immunoprecipitates treated with λ phosphatase, then washed with low-salt or high-salt buffers. Notice that, despite being dephosphorylated on S1126, separase still binds cyclin B1. (B) Anti-Myc immunoprecipitates showing that, despite being phosphorylated, the LAG and Δ12 mutants do not bind cyclin B1.

being dephosphorylated suggests that the cyclin B1 binding site is distinct from S1126. Consistently, two separase mutations that abolish cyclin B1 binding, LAG and Δ12, are ~250 amino acids distal to S1126 (Gorr et al., 2005) (Fig. 1A). Therefore, to test whether S1126 phosphorylation and cyclin B1 binding are separable, we transfected cells with constructs expressing WT separase and the S:A, LAG and Δ12 mutants, all as Myc-tagged fusions. Myc-tagged proteins were then immunoprecipitated and analysed by immunoblotting. Whereas WT, S:A, LAG and Δ12 all bound securin, only WT bound cyclin B1 (Fig. 3B, compare lanes 1 and 2-4), confirming that the LAG and Δ12 mutations abolish cyclin B1 binding. Importantly, however, whereas separase S:A was not phosphorylated on S1126, the LAG and Δ12 mutants were (Fig. 3B, compare lanes 2 and 3,4), demonstrating that cyclin B1 binding and S1126 phosphorylation are separable. Indeed, taken together with the data from the phosphatase experiment (Fig. 3A), our observations suggest that, although separase phosphorylation is required for cyclin B1 binding, once bound, S1126 phosphorylation is not required to maintain the interaction.

Expression of non-phosphorylatable separase (S1126A) induces mitotic arrest

To probe the physiological significance of separase phosphorylation and cyclin B1 binding, we generated stable human cell lines expressing WT separase and the non-phosphorylatable S:A mutant. The transgenes were integrated at a pre-defined genomic locus by using FRT/Flp-mediated recombination, thus facilitating direct comparison of the transgenes. Separase expression was under tetracycline control (Fig. 4A), with expression becoming maximal after approximately 4 hours (not shown), thereby allowing us to study the first mitosis following induction. Notably, induction of separase increased securin levels (Fig. 4A). Because separase levels are reduced in *Securin*$^{-/-}$ cells (Jallepalli et al., 2001), it appears that separase and securin stabilise each other.

Expression of separase S:A had a profound effect on the cell cycle: 24 hours post-induction ~70% of cells contained ⩾4N DNA contents, consistent with a G2 and/or M defect (Fig. 4B). By contrast, induction of WT separase had little effect. To define the G2/M defect in separase S:A cells, we performed time-lapse microscopy and measured the time cells spent in mitosis. In the absence of tetracycline, cells spent ~30 minutes in mitosis (Fig. 4D and supplementary material, Table S1). Strikingly, however, upon induction 100% of the separase S:A cells underwent a prolonged mitotic arrest, spending >100 minutes in mitosis (283 minutes on average), before advancing to interphase, frequently without dividing. By contrast, induction of WT separase had only a marginal effect, with ~10% of cells spending >100 minutes in mitosis. Despite the prolonged mitotic delay observed in separase S:A cells, the mitotic index of the population increased only marginally 24 hours post-induction (Fig. 4B). This suggests that the non-mitotic cells with 4N DNA contents are either delayed in G2 or have advanced to G1 without dividing. Although separase cleavage products have recently been implicated in G2 progression (Papi et al., 2005), we noticed that following longer inductions, the vast majority of S:A cells accumulated DNA contents greater than 4N (data not shown). Therefore, although we cannot rule out the possibility that separase S:A induces a brief G2 delay, the large proportion of non-mitotic S:A cells with 4N DNA contents is likely to predominantly represent cells that have exited mitosis and returned to G1 without division.

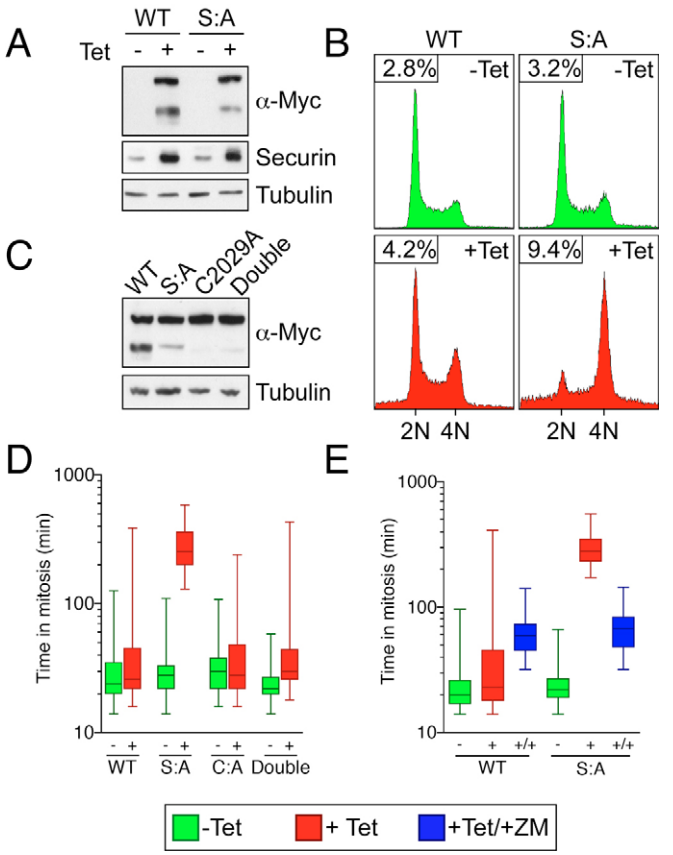

Fig. 4. The separase S1126A mutation induces mitotic arrest. (A) Immunoblot showing tet-induced expression of Myc-tagged WT and separase S:A. Notice the increase in securin following separase induction. (B) Histograms showing accumulation of cells with DNA content ⩾4N cells 24 hour post-induction of separase S:A. Numbers represent mitotic index as determined by MPM-2 staining. (C) Immunoblot of tet+ cells showing equivalent expression of WT, S1126A, C2029A and the S1126A-C2029A double mutant. (D,E) Bar graphs showing the time spent in mitosis based on phase-contrast time-lapse analysis.

In addition to being non-phosphorylatable, separase S:A does not bind cyclin B1 (Fig. 1C, Fig. 2A, Fig. 3A). We therefore asked whether the mitotic delay exhibited by separase S:A was caused by preventing S1126 phosphorylation or inhibiting cyclin B1 binding. To address this, we generated cell lines expressing the separase mutants Δ12 and LAG that can be phosphorylated on S1126 and bind securin, but do not bind cyclin B1 (Fig. 5A). Importantly, cells expressing Δ12 and LAG phenocopied separase S:A, accumulating 4N DNA contents (Fig. 5B) and undergoing prolonged mitotic arrest (Fig. 5C). Thus, the mitotic defect induced by separase S:A is caused by the inhibition of cyclin B1 binding, not the prevention of S1126 phosphorylation.

Separase S:A induces premature loss of cohesion

To determine the cause of the mitotic defect in separase S:A

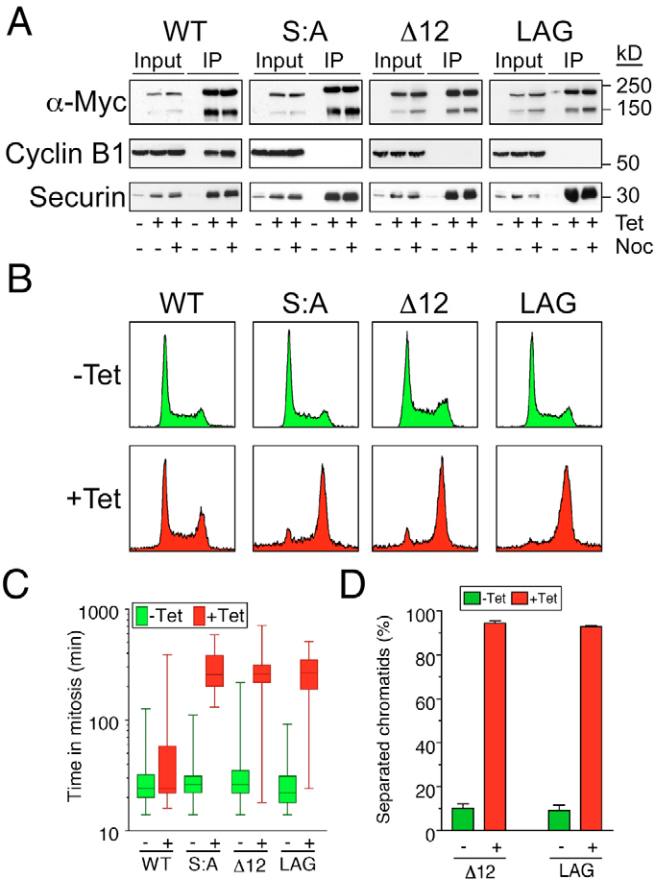

Fig. 5. Cyclin B1 binding is required to prevent premature activation of separase. (A) Immunoprecipitations of Myc-tagged proteins from cell lines harbouring four separase transgenes blotted as indicated. (B) DNA content profiles showing G2/M defect 24 hours post-induction of separase mutants. (C) Box-plots of phase-contrast time-lapse microscopy analysis showing mitotic arrest phenotype following induction of separase mutants that do not bind cyclin B1. WT and separase S:A are shown for comparison. (D) Bar graph quantitating the number of metaphase spreads with separated chromatids 8 hours post-induction.

cells, we analysed cells by fluorescent in situ hybridisation (FISH) and immunofluorescence microscopy. Consistent with a chromosome segregation defect, FISH of interphase nuclei 24 hours post-induction showed that many of the separase S:A cells had become tetraploid (Fig. 6A). Specifically, in a large proportion of separase S:A cells, chromosomes 7 and 8 were present at twice the modal number of controls (Fig. 6B). Notice that because bi-nucleated and multi-nucleated cells were not counted in this experiment, the proportion of tetraploid cells is likely to be under-estimated. Indeed, the DNA content profiles indicate that the majority of separase S:A cells were tetraploid by this time point (Fig. 4B). Strikingly, metaphase FISH revealed that chromosomes in separase S:A cells had dissolved sister chromatid cohesion. Specifically, although ~85% of WT spreads contained chromosomes with paired chromatids (Fig. 6C,D), >90% of separase S:A spreads displayed a 'scattered chromatid' phenotype, indicating that separase S:A induces premature loss of sister chromatid cohesion. This notion is supported by immunofluorescence analysis: whereas normal mitotic figures were prominent in WT cells, separase S:A cells typically exhibited a 'pseudo-anaphase' phenotype, with kinetochores frequently clustered near the spindle poles (Fig. 6E,F). However, these kinetochores stained strongly for Bub1 and Aurora B, indicating the spindle checkpoint had not been silenced. In addition, Aurora-B-positive midzone structures were absent, and separase S:A cells stained positive for cyclin B1 (Fig. 2F). Therefore, separase S:A cells lose cohesion and stabilise cyclin B1. Cells expressing the LAG and Δ12 mutants also exhibited the 'scattered chromatid' phenotype (Fig. 5D), confirming that the phenotype is due to the lack of cyclin B1 binding, rather than an inability to be phosphorylated.

These observations suggest that separase mutants that cannot bind cyclin B1 become prematurely active, cleaving cohesin and thereby triggering sister chromatid separation before the spindle checkpoint is silenced. Then, because separated chromatids cannot bi-orient and come under tension (Stern and Murray, 2001; Dewar et al., 2004), futile rounds of microtubule capture and release ensue, thus sustaining the spindle checkpoint and stabilising cyclin B1, yielding a prolonged mitotic arrest. This explanation of the S:A phenotype makes two predictions. First, mitotic arrest induced by separase S:A should be dependent on its proteolytic activity. Consistently, mutation of the catalytic cysteine (C2029, Fig. 1A) nullified the ability of separase S:A to induce mitotic arrest (Fig. 4D and supplementary material, Table S1). Second, because Aurora B destabilises kinetochore-microtubule interactions that do not yield tension, the arrest should depend on Aurora-B-kinase activity (Tanaka et al., 2002; Hauf et al., 2003). Consistently, when separase S:A cells were exposed to ZM447439, an Aurora B inhibitor (Ditchfield et al., 2003), mitotic exit was accelerated (Fig. 4E and supplementary material, Table S1).

Separase S:A cells align chromosomes before undergoing premature disjunction

To define when separase S:A cells dissolve cohesion, we performed time-lapse microscopy using cells expressing GFP-tagged histone. 74% of WT cells completed mitosis normally, achieving metaphase within ~14 minutes, then segregating their chromosomes ~9 minutes later (Fig. 7A,D,E; supplementary material, Table S2 and Movie 1). By contrast, all the cells expressing separase S:A behaved abnormally (Fig. 7D). Although these cells aligned most chromosomes on a metaphase plate, they then rapidly disjoined their sister chromatids, on average 5 minutes before cells expressing WT separase (Fig. 7B,C,E; supplementary material, Table S2 and Movies 2 and 3). Note, the ability of separase S:A cells to align their chromosomes prior to disjunction is not dependent upon how long cells had expressed the transgene: cells that entered mitosis ~20 hours after the addition of tetracycline behaved indistinguishably from those cells that entered after ~4 hours (data not shown).

The subsequent behaviour of these cells is consistent with other reports describing loss of cohesion in the absence of cyclin degradation (e.g. Parry et al., 2003). Specifically, we observed separated chromatids reforming unstable metaphases, with chromatids falling off and then realigning. This persisted for several hours before cells exited mitosis (Fig. 7B, supplementary material, Movie 2). The alignment of separated

chromatids is not unprecedented and probably occurs via merotelic orientations (Parry et al., 2003).

Although 26% of WT cells are scored as 'abnormal' in Fig. 7D, it is important to stress that, in contrast to S:A cells, all the WT cells underwent a normal delay between chromosome alignment and separation (Fig. 7E). However, whereas 5% underwent a metaphase arrest, 11% segregated their chromosomes normally before undergoing a brief telophase delay (Fig. 7D). Only the remaining 10% of WT cells appeared to lose cohesion in a global manner followed by prolonged mitotic arrest. Thus, consistent with the data in Figs 4-6, the vast majority of WT cells (~85%) successfully segregated their chromosomes and then exited mitosis (Fig. 7D). Clearly, therefore, expression of the S:A mutant induces a very different phenotype when compared with WT separase. Specifically, expression of separase S:A accelerates chromatid disjunction, thereby activating the spindle checkpoint and preventing mitotic exit. Thus, the binding of cyclin B1 to separase is required to couple sister chromatid disjunction with mitotic exit.

Securin degradation is not required for premature disjunction in separase S:A cells

Although separase S:A cells undergo a premature loss of sister chromatid cohesion, we were struck by the fact that cohesion is maintained long enough for most chromosomes to align (e.g. Fig. 7B). One possible explanation is that, prior to chromosome alignment, separase S:A is inhibited by securin. The degradation of securin might then activate separase S:A, triggering sister chromatid disjunction. To test this, we analysed securin by immunofluorescence microscopy. In interphase, securin localised diffusely throughout the cell in the absence of tetracycline. However, upon induction of either WT or S:A, both separase and securin colocalised in the cytoplasm (Fig. 8A). This cytoplasmic sequestration of securin suggests that all the securin in the cell is bound to separase. In mitosis, securin was present in metaphase (Fig. 8A). Consistent with securin degradation normally being required for sister chromatid separation, it was not detectable during anaphase in uninduced cells or cells expressing WT separase. Importantly however, separase S:A cells that had undergone a premature loss of cohesion still contained high levels of securin (Fig. 8B).

Although this suggests that securin had not been degraded prior to sister chromatid separation in separase S:A cells, immunofluorescence analysis can only demonstrate that a protein has disappeared, not whether degradation has been initiated. Indeed, because separase induction elevates securin levels (Fig. 4A), we could not rule out the possibility that a partial reduction in securin was sufficient to liberate enough separase to trigger chromatid disjunction. Therefore, to determine whether any securin degradation occurs prior to sister chromatid separation in S:A cells, we measured securin levels by time-lapse fluorescent imaging. To visualise both securin and the chromatin, we created a stable cell line co-expressing a securin-dsRed fusion protein and GFP-histone. We then transiently transfected these cells with a tetracycline-responsive separase S:A construct. This allowed us to co-induce separase S:A and securin-dsRed. Consistent with the immunofluorescence analysis, securin-dsRed was distributed throughout the cell, but then accumulated in the cytoplasm following

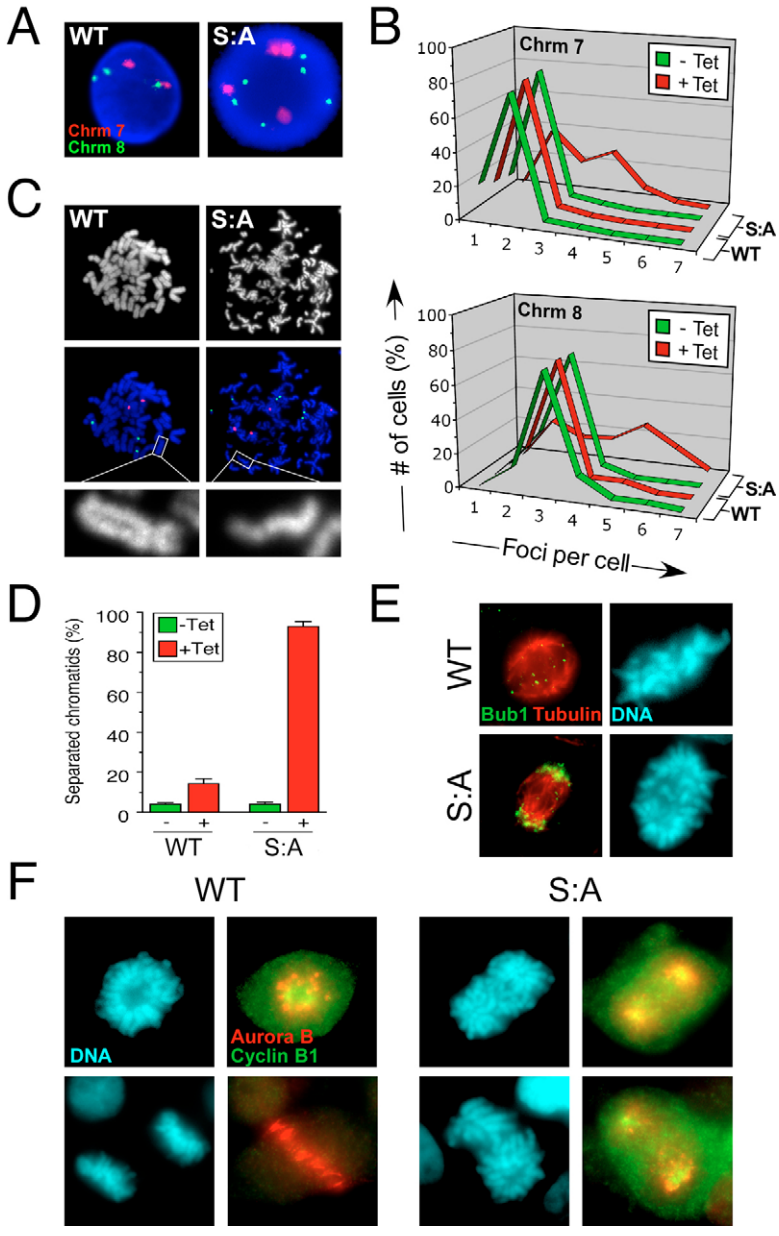

Fig. 6. Separase S1126A induces premature loss of sister chromatid cohesion. (A) Interphase FISH images of WT and separase S:A cells 24 hour post-induction. (B) Histograms scoring number of FISH foci per cell showing that separase S:A induces aneuploidy. (C) Metaphase spreads 8 hours post-induction showing separated sister chromatids in a separase S:A cell. (D) Bar graph quantitating the number of metaphase spreads with separated chromatids in WT and separase S:A populations 8 hours post-induction. (E,F) Immunofluorescence analysis of mitotic cells expressing either WT or separase S:A, stained as indicated.

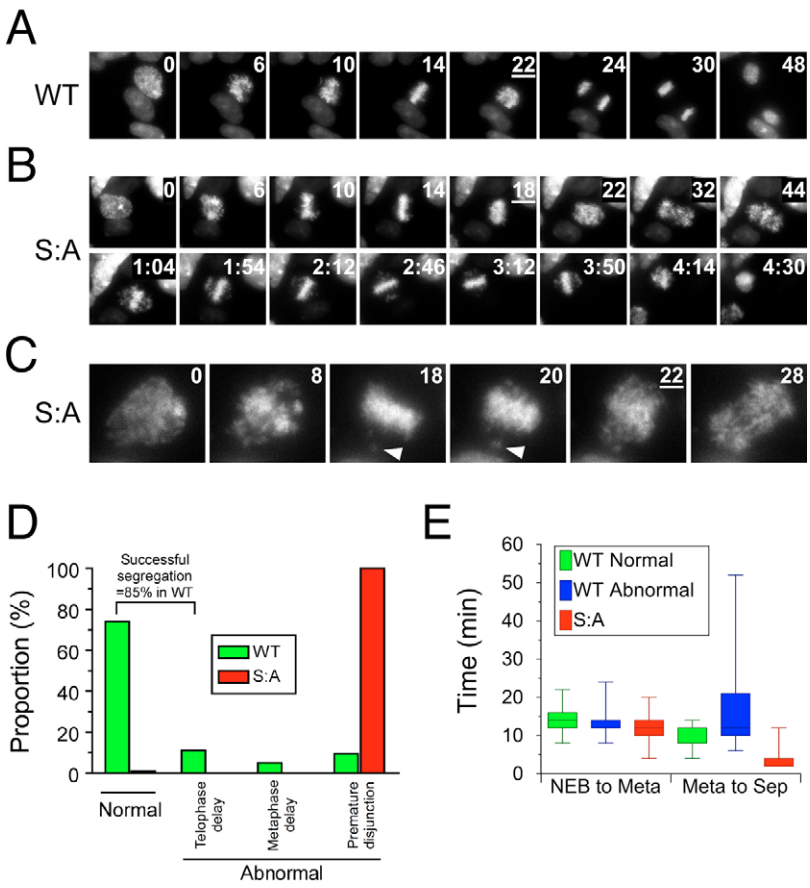

Fig. 7. Separase S1126A uncouples chromatid disjunction from mitotic exit. (A) GFP histone time-lapse analysis showing a normal mitosis in a WT separase cell. (B) Prolonged mitosis in a separase S:A cell, showing rapid chromatid disjunction followed by realignment of separated chromatids. (C) Separase S:A cell showing chromatid disjunction with unaligned chromosomes (arrow heads). (D) Quantitation of normal and abnormal mitoses in WT and separase S:A populations. (E) Bar graphs showing the time from nuclear envelope breakdown to metaphase and from metaphase to sister chromatid separation. Cells expressing WT separase are sub-divided into those that performed a normal mitosis (green), or an abnormal mitosis (blue), whereas all separase S:A cells are plotted together (red). Notice that metaphase in separase S:A cells is defined as the point prior to chromatid disjunction when most or all of the chromosomes have aligned.

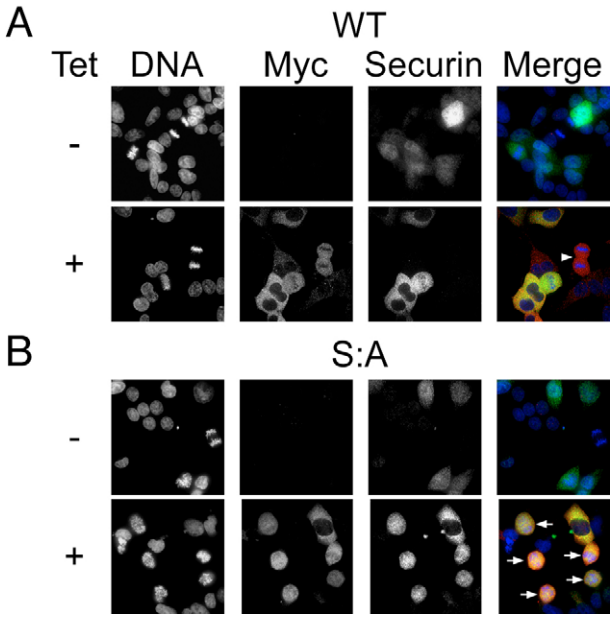

Fig. 8. Separase overexpression sequesters securin in the cytoplasm. Immunofluorescence images showing that tet-induced expression of WT separase (A) and separase S:A (B) results in the endogenous securin accumulating in the cytoplasm of interphase cells. Notice that, whereas securin is not present in anaphase WT cells (arrow head), it is detectable in separase S:A cells that have undergone a premature loss of cohesion (arrows).

expressing of exogenous separase (Fig. 9A,B). In control cells, and consistent with previous reports (Hagting et al., 2002), securin-dsRed degradation initiated following chromosome alignment at metaphase (Fig. 9A; supplementary material, Movie 4). Sister chromatid separation then initiated as soon as degradation was complete. By contrast, separase S:A cells underwent premature sister chromatid disjunction without any appreciable decrease in securin-dsRed (Fig. 9B; supplementary material, Movie 5). Indeed, securin-dsRed levels remained high during the subsequent prolonged mitotic arrest, before finally being degraded as cells exited mitosis. This indicates that separase S:A becomes prematurely active in the absence of securin degradation.

Securin overexpression rescues the separase S:A phenotype

The observation that premature disjunction in separase S:A cells occurs independently of securin degradation suggests that separase is overexpressed relative to securin, despite the fact that endogenous securin levels increase upon induction of separase (Fig. 4A). Consistently, securin becomes sequestered in the cytoplasm upon expression of the separase transgenes (Fig. 8). However, the S:A phenotype cannot simply be due to overexpression because when WT separase is expressed to the same level, mitosis is mainly normal (Fig. 7). We therefore reasoned that, although both WT and separase S:A were overexpressed with respect to securin, the WT was inhibited by cyclin B1 binding but the S:A mutant was not. This model

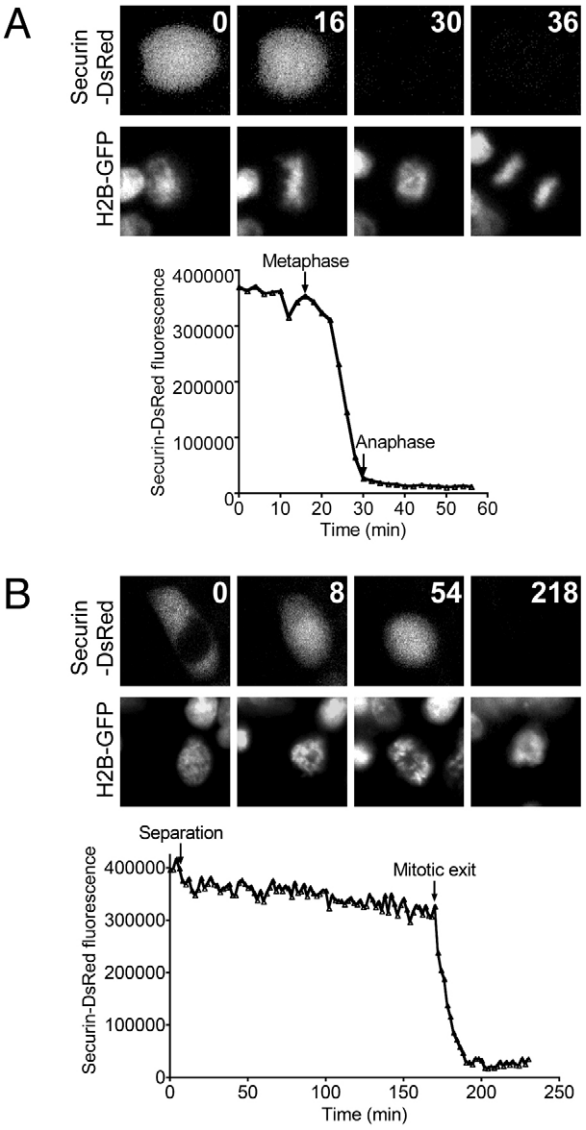

Fig. 9. Securin degradation is not required for the premature loss of cohesion in separase S:A cells. Time-lapse sequences and pixel-intensity measurements showing securin-dsRed fluorescence in (A) control or (B) Separase-S:A-expressing cells. Whereas securin-dsRed is degraded prior to chromatid disjunction in the control cell, it is not degraded until mitotic exit in the separase S:A cell.

predicts that, if securin is overexpressed together with separase S:A it should rescue the S:A phenotype by restoring the co-ordination of sister chromatid disjunction with mitotic exit. To test this, separase S:A cells expressing a GFP-histone were transfected with tetracycline-responsive constructs expressing securin-dsRed fusions. This allowed us to (1) co-induce separase S:A and securin-dsRed, (2) identify transfected cells, (3) monitor securin levels and, (iv) visualise chromosome alignment and segregation.

Expression of dsRed alone had no effect: all S:A cells prematurely lost cohesion and arrested (Fig. 10A,C and supplementary material, Movie 6). By contrast, securin-dsRed expression rescued the S:A phenotype in 44% of cells: perfect metaphases formed, then sister chromatid disjunction and mitotic exit occurred normally (Fig. 10B,C and supplementary material, Movie 7). Importantly, these cells typically expressed lower levels of securin-dsRed. Consistent with previous observations (Hagting et al., 2002), cells that expressed higher levels typically underwent a 'cut' phenotype, with cytokinesis and mitotic exit occurring without chromatid disjunction (supplementary material, Movie 8). This indicates that the ability of low levels of securin-dsRed to rescue the separase S:A phenotype depends on timely securin degradation. Indeed, when we expressed non-degradable securin-dsRed fusions, normal anaphases were not observed and the frequency of cut phenotypes increased to >70% (Fig. 10B,C and see supplementary material, Movies 9 and 10).

Discussion

Securin is not essential in mammalian cells (Jallepalli et al., 2001; Mei et al., 2001; Wang et al., 2001), suggesting that other mechanisms must regulate separase activation. Elegant biochemical experiments in *Xenopus* egg extracts have shown that separase is phosphorylated in mitosis, and then recruits cyclin B1, thus inhibiting separase (Stemmann et al., 2001; Gorr et al., 2005). Here, we show that this mechanism also operates to inhibit separase in human cells. We confirm that human separase is phosphorylated on serine S1126 and show that this is required to establish but not maintain cyclin B1 binding. In addition, we show that expression of a non-phosphorylatable separase mutant in excess of securin, uncouples sister chromatid disjunction and mitotic exit. Thus, the inhibition of excess separase by cyclin B1 binding is required for timely chromosome disjunction.

Cyclin B1 binding inhibits separase in mitosis

To maintain genome stability, separase must not be activated until all the chromosomes are bi-oriented. As a result, eukaryotes have evolved multiple mechanisms to inhibit separase. In interphase, two mechanisms are apparent. First, separase is cytoplasmic (Fig. 8) and is thus physically isolated from cohesin. Second, separase is bound to an inhibitor, securin. In mitosis, a further mechanism restrains separase, namely phosphorylation on S1126, which recruits cyclin B1, which in turn inhibits separase (Stemmann et al., 2001; Gorr et al., 2005) (Fig. 5). This mechanism may act to 'mop up' any excess separase that is not bound by securin. Consistent with this notion, the binding of securin and cyclin B1 to separase are mutually exclusive. In addition, when separase is expressed in excess of securin, cyclin B1 binding becomes essential to prevent premature loss of sister chromatid cohesion (Figs 4 and 5).

Rather than simply 'mopping up' excess separase, cyclin B1 binding might also contribute to the regulation of anaphase. Indeed, the cyclin-B1–separase complex is present at endogenous levels in mitotic cells (Gorr et al., 2005) (Fig. 2B), suggesting that this mechanism is physiological relevant during normal mitosis. Interestingly, separase becomes dephosphorylated in anaphase, leading to the suggestion that a phosphatase activates separase (Stemmann et al., 2001). Importantly, however, our data indicate that dephosphorylation probably activates separase. Using a novel antibody which specifically recognises separase phosphorylated on serine 1126, we demonstrate that S1126 can be efficiently

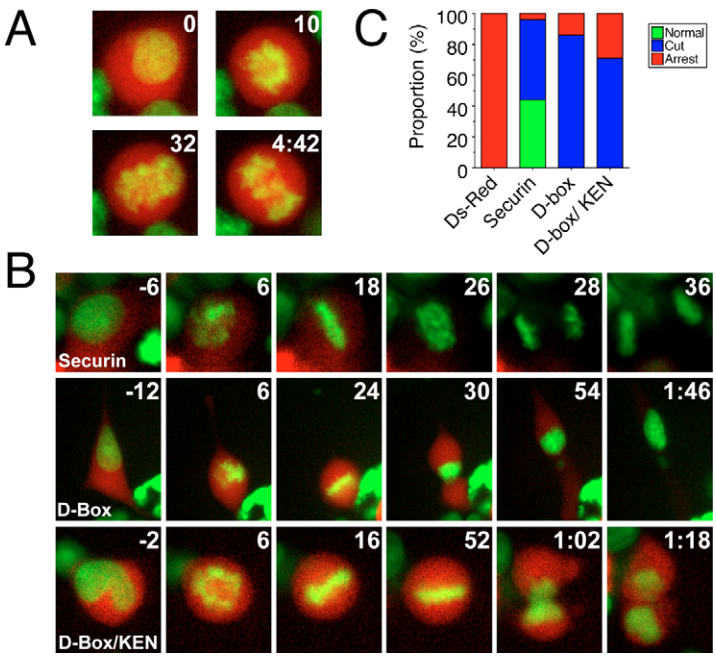

Fig. 10. Increasing securin levels restores coordination of mitotic events in separase S1126A cells. (A) Time-lapse sequence of a separase S:A cell expressing dsRed showing premature loss of cohesion. (B) Time-lapse sequences of separase S:A cells expressing securin-dsRed proteins, showing rescue and cut phenotypes. (C) Quantitation of phenotypes following expression of securin-dsRed fusions in separase S:A cells.

dephosphorylated when cyclin B1 is bound to separase. Significantly, this does not disrupt cyclin B1 binding (Fig. 3A). Thus, although separase phosphorylation is required for cyclin B1 binding once bound, S1126 phosphorylation is not required to maintain the interaction. A possible explanation for these data is that S1126 phosphorylation induces a conformational change in separase that allows cyclin B1 binding. Once bound, cyclin B1 maintains the new conformation, such that, even if S1126 is dephosphorylated, cyclin B1 remains bound and separase is kept inactive. Therefore, rather than dephosphorylation activating separase, it seems more likely that cyclin B1 degradation is required to liberate and activate separase. Consistently, several studies show that overexpression of a non-degradable cyclin B1 mutant can arrest cells at metaphase (Hagting et al., 2002; Chang et al., 2003). Taken together with these data, our observations suggest that degradation of both securin and cyclin B1 activates separase in mammalian cells.

Separase S:A overrides both securin and cyclin B1 inhibitory mechanisms

To dissect the physiological significance of separase phosphorylation and cyclin B1 binding, we compared the effects of expressing WT separase with a non-phosphorylatable S1126A mutant. Quantitative immunoblotting indicates that the exogenous separase was expressed 10- to 20-fold over endogenous (not shown). Despite the fact that endogenous securin levels rose in response to transgene induction (Fig. 4A), three observations indicate that – in this model system – separase became overexpressed with respect to securin. First, securin becomes sequestered in the cytoplasm when the separase transgenes are induced (Fig. 8). Second, separase S:A cells prematurely lose cohesion in the absence of securin degradation (Figs 8 and 9). Third, overexpression of securin rescues the S:A phenotype (Fig. 10). However, mitosis was entirely normal in ~75% of cells expressing WT separase (Figs 4 and 7). This implies that the excess WT separase not bound to securin was not active. Although this could be because securin binding is required for separase activity (Jallepalli et al., 2001), our data indicate that the overexpressed WT separase was held in check by cyclin B1. Specifically, when the separase mutants that cannot bind cyclin B1 were overexpressed to similar levels, cells underwent a premature loss of sister chromatid cohesion, suggesting a premature activation of separase. Thus, by overexpressing the separase S:A mutant, we have simultaneously overridden two inhibitory mechanisms. First, by elevating separase levels above securin, securin-mediated inhibition is alleviated. Second, by preventing S1126 phosphorylation, cyclin B1-mediated inhibition is also alleviated.

Under these conditions, we observed a premature loss of sister chromatid cohesion during a normal mitosis (Fig. 7). This is in contrast to a recent study using mouse ES cells where *Securin*$^{-/-}$; *Separase*$^{+/S1121A}$ cells only showed signs of chromatid separation after prolonged treatment with spindle toxins (Huang et al., 2005). One possible explanation is that, because securin and separase appear to stabilise each other (Jallepalli et al., 2001) (Fig. 4A), *Securin*$^{-/-}$ cells might contain a lower level of non-phosphorylatable separase that can only trigger a loss of cohesion during a prolonged mitotic arrest.

Chromosomes align despite overriding two separase inhibitory mechanisms

Although overexpression of separase S:A overrides both securin- and cyclin B1-mediated inhibition of separase, chromosomes still align at metaphase prior to disjoining (Fig. 7B). Because human cells that lack cohesion fail to align their chromosomes due to disjunction in or prior to prophase (Toyoda and Yanagida, 2006), it appears, therefore, that cohesion is maintained during the early stages of mitosis in separase S:A cells. This is independent of Sgo1, because Sgo1 repression does not cause an earlier loss of cohesion in

separase S:A cells (data not shown). Thus, two possible explanations may account for chromosome alignment prior to disjunction. First, although separase is prematurely active, its substrate may not be amenable to cleavage. Indeed, budding yeast cells that lack securin maintain cohesion until Scc1 is phosphorylated by the polo-like kinase Cdc5 (Alexandru et al., 2001). Although there appears to be no obvious phenotype associated with expressing non-phosphorylatable Scc1 mutants in humans cells (Hauf et al., 2005), it is conceivable that, even if separase is prematurely active in S:A cells, it cannot cleave its substrate. Second, if overexpression of separase S:A overrides both securin- and cyclin-B1-mediated inhibition of separase, then it is possible that a third mechanism keeps separase inactive during the early phases of mitosis. Interestingly, when we treated separase S:A cells with the proteasome inhibitor MG132, 72 % of cells sustained chromatid cohesion for at least 30 minutes (A.J.H. and S.S.T., unpublished data). This observation suggests that if a third mechanism does inhibit separase, it is switched off by proteolysis.

What advantage does the cyclin B1 inhibitory mechanism offer?

Considering the disastrous consequences of prematurely losing sister chromatid cohesion, it is perhaps not surprising that cells employ multiple mechanisms to inhibit separase. What is less clear, however, are the relative contributions of these mechanisms during a normal mitosis are. Is the cyclin B1 mechanism simply a backup, or does it play an active role in regulating anaphase? Our data clearly show that, when separase is overexpressed relative to securin, the cyclin B1 mechanism becomes essential. However, if securin levels can increase in response to extra separase (Fig. 4A), why is the cyclin B1 mechanism necessary? Although securin upregulation may serve to limit the amount of excess free-separase, we suspect that this mechanism can only operate within certain limits. Indeed, overexpression of securin has a disastrous consequence on mitosis, leading to highly aneuploid cells (Hagting et al., 2002), (A.J.H. and S.S.T., data unpublished). Consistently, securin is an oncogene and is overexpressed in many cancers (Pei and Melmed, 1997). Therefore, upregulating securin beyond a certain level is probably detrimental. One solution to this problem would be to employ a second mechanism, namely cyclin B1 binding, to inhibit separase if it became too abundant.

In addition to inhibiting excess separase, cyclin B1 binding might play a more active role in anaphase regulation. Whereas securin-mediated inhibition may be the predominant mechanism in some cell types, there may be special situations where the cyclin B1 mechanism is particularly important. For example, during meiosis separase must be re-inhibited following separation of homologous chromosomes in anaphase I (Terret et al., 2003). This occurs without any physical isolation of separase from its substrate. Rapid and stable re-inhibition of separase may thus require both cyclin B1 and securin-mediated inhibition. Consistently with this notion, the inhibition of human separase by CSF-arrested *Xenopus* egg extracts depends upon S1126 phosphorylation (Fan et al., 2006).

The existence of two mechanisms to inhibit separase raises the question as to which one arose first and what was the evolutionary advantage of acquiring the second. One possibility is that the ancestral proto-eukaryote employed a mitotic cyclin not only to drive mitotic entry and exit, but also to inhibit separase prior to anaphase. However, separase inhibits MPF activity (Gorr et al., 2005), suggesting that suppressing separase and promoting mitosis may have been antagonistic functions, placing a limit on the fidelity of genome transmission. Evolution of securin would therefore have liberated cyclin from the role of inhibiting separase prior to mitosis, thereby allowing cyclin levels to regulate the G2-M transition. However, retaining the ability of cyclin B1 to inhibit separase not only provides a safety mechanism in case of securin failure, but also allows sister chromatid disjunction and mitotic exit to remain coupled.

The existence of two separase inhibitors may then, in turn, have allowed some eukaryotes to completely liberate cyclin from inhibiting separase altogether. Importantly, chromatid disjunction and mitotic exit are temporally resolved in budding yeast (Lew and Burke, 2003), with these transitions being regulated by separate checkpoints – one ensuring accurate chromosome segregation, the other ensuring the spindle is positioned in the mother-daughter bud neck. Thus, the evolution of a second separase inhibitor may have also facilitated the uncoupling of chromosome segregation and mitotic exit, thereby allowing the evolution of budding lifestyles in fungi.

Materials and Methods

Generation of specific antibody against phosphorylated S1126

Two synthetic peptides based on the human separase sequence, flanking serine residue 1126, were generated using standard methods (Moravian-biotechnology, Czech Republic). The phospho-peptide CAPSTNS(*SP*)PVLKTK was then crosslinked to BSA and injected into rabbits (Moravian-biotechnology). Antibodies specifically recognising the phosphorylated peptide were affinity-purified from the third bleed by using the phospho-peptide crosslinked to SulfoLink Coupling Gel following the manufacturer's recommendations (Pierce Biotechnology). Antibodies that recognised the unphosphorylated sequence were affinity-purified following a similar process but using the unphosphorylated peptide (CAPSTNSSPVLKTK).

Generation of stable cell lines

Stable, isogenic cell lines expressing separase mutants were generated using the FRT/Flp-mediated recombination as described previously (Tighe et al., 2004). Briefly, the human separase open reading frame, a kind gift from Jan-Michael Peters (IMP, Vienna), was cloned into a pcDNA5/FRT/TO based vector (Invitrogen) modified to contain an N-terminal Myc-epitope tag. The S1126A, C2029A, Δ12 and LAG mutations were generated by site-directed mutagenesis (QuickChange, Stratagene). Notice that the Δ12 and LAG mutations reside in the cyclin B1 binding domain (Fig. 1A), and are as described (Gorr et al., 2005). Resulting vectors were co-transfected into Flp-In™ TRex™-293 cells with pOG44, a plasmid encoding the Flp recombinase. After selection in hygromycin, colonies were pooled, expanded and transgene expression was induced with 1 μg/ml tetracycline. Flp-In™ TRex™-293 were used for all the experiments unless stated otherwise in the figure legends. Other small molecules were as described previously (Ditchfield et al., 2003) and used at the following final concentrations: nocodazole, 0.2 μg/ml; MG132, 20 μM; and ZM447439, 2 μM. All cell culture conditions were as described previously (Taylor et al., 2001). To enrich for mitotic cells, cultures were treated overnight with either taxol or nocodazole.

Antibody techniques

For immunoblot analysis, soluble cell proteins or immune complexes were resuspended in sample buffer, separated by SDS-PAGE, blotted onto nitrocellulose membranes (BioRad) and then probed with the following antibodies: 4A6 (mouse anti-Myc, Upstate, 1:2000); TAT-1 (mouse anti-tubulin, 1:10,000); mouse anti-cyclin B1 (Upstate, 1:2000); DCS-280 (mouse anti-securin, Abcam, 1:250). For immunoprecipitations, cells were lysed and soluble extracts prepared as described (Morrow et al., 2005). Anti-Myc (4A6) antibodies or anti-cyclin B1 (Upstate) coupled to protein G Sepharose (Amersham) were added to the supernatant and collected by centrifugation. To dephosphorylate separase in vitro, immune complexes bound to sepharose beads were incubated with λ phosphatase (NEB) at 30°C for 30 minutes then washed in buffer containing either 100 mM or 1000 mM

NaCl, designated low and high salt, respectively. Immunofluorescence analysis was performed as described (Taylor et al., 2001) using the following antibodies: SB1.3 (sheep anti-Bub1, 1:1000) (Taylor et al., 2001); TAT-1 (1:100); SAB.1 (sheep anti-Aurora B, 1:2000) (Ditchfield et al., 2003); GNS1 (mouse anti-cyclin B1, Abcam, 1:100); DCS-280 (1:100).

Cell biology

Flow cytometry was carried out as described (Ditchfield et al., 2003) using a CyAn™ (DakoCytomation). All DNA content profiles were measured 24 hours post-induction. For time-lapse analysis, stable cell lines were either analysed by phase-contrast microscopy alone or, to visualise the chromatin, a Flp-In™ TRex™-293 line expressing a GFP-histone H2B fusion protein was generated (Anthony Tighe and S.S.T., unpublished). Phase-contrast and fluorescence images were collected every two minutes using a Zeiss Axiovert 200 as described previously (Morrow et al., 2005). *XY*-point visiting and acquisition of *Z*-sections was performed using a PZ-2000 automated stage (Applied Scientific Instrumentation). To determine mitotic timing, tetracycline was added for four hours and then at least 50 cells were analysed over the subsequent 16 hours. Mitotic timing data are presented as box-and-whisker plots generated with Prism 4 (GraphPad), where the boxes show the median and interquartile range, whereas the whiskers show the entire range. In the time-lapse sequences, numbers refer to the time in minutes after nuclear-envelope breakdown. The underlined number indicates the first frame that clearly shows sister chromatid separation. Metaphase spreads were carried out as described (Tighe et al., 2004), whereas FISH with α-satellite probes specific for chromosomes 7 and 8 was performed according to the manufacturer's instructions (Qbiogene). When determining the number of metaphase spreads with separated chromatids, at least 50 spreads were counted in three experiments; values in bar graphs represent the mean ± s.e.m.

Securin experiments

The human securin open reading frame, the D-box mutant and the D-box/KEN box mutant (Hagting et al., 2002), kind gifts from Jon Pines (Gurdon Institute, Cambridge, UK), were cloned into pcDNA5/FRT/TO/Myc modified to include a C-terminal dsRed monomer tag (Clontech). Resulting vectors were then either transiently transfected into the HEK-293-based separase cell lines using the calcium phosphate method according to manufacturers instructions (ProFection®, Promega), or stably transfected in to the HEK-293 GFP-histone H2B line as described above. Time-lapse analysis were performed as described above. Kinetics of securin-dsRed degradation was measured by defining an area around the cell and quantifying the integrated pixel intensity minus background, at each time point. Degradation profiles were then plotted using Prism 4 (GraphPad).

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