Securin is not required for cellular viability, but is required for normal growth of mouse embryonic fibroblasts

Junjie Mei*‡, Xingxu Huang* and Pumin Zhang*†

Sister chromatid separation depends on the release of cohesion by the activity of Esp1, a member of the caspase family [1, 2]. In budding yeast, Esp1p is kept inactive by its association with Pds1p, until the onset of anaphase, when Pds1p is ubiquitinated by the APC/Cdc20 complex [3–5] and subsequently degraded by the 26S proteasome. *Pds1* is not an essential gene in budding yeast, but is required for cell cycle arrest prior to anaphase in response to the disruption of spindle structures [6, 7]. Thus, *Pds1* mutant yeast cells display precocious sister chromatid separation in the presence of nocodazole [6]. Mammalian orthologs of yeast *Esp1* and *Pds1*, separin and securin, have been identified [8], and, as anticipated, a nondegradable mutant form of securin inhibits sister separation when added to mitotic *Xenopus* egg extracts [8]. Securin was also independently identified as *PTTG* (pituitary tumor transforming gene), a gene overexpressed in pituitary tumors [9]. The relationship between its overexpression in tumors and its control of sister chromatid cohesion remains ill defined. To explore securin function in mammals, we took a targeted gene disruption approach in mice. Here, we report that securin is neither essential for cell viability nor required for spindle checkpoint function, and mice lacking securin are viable and apparently normal, but mouse embryonic fibroblasts lacking securin grow abnormally in culture.

Addresses: *Department of Molecular Physiology and Biophysics and †Department of Biochemistry and Molecular Biology, Baylor College of Medicine, Houston, Texas 77030, USA.

Present address: ‡Department of Medicine, University of California, San Francisco, California 94143, USA.

Correspondence: Dr. Pumin Zhang
E-mail: pzhang@bcm.tmc.edu

Received: **20 April 2001**
Revised: **18 May 2001**
Accepted: **11 June 2001**

Published: **7 August 2001**

Current Biology 2001, 11:1197–1201

0960-9822/01/$ – see front matter

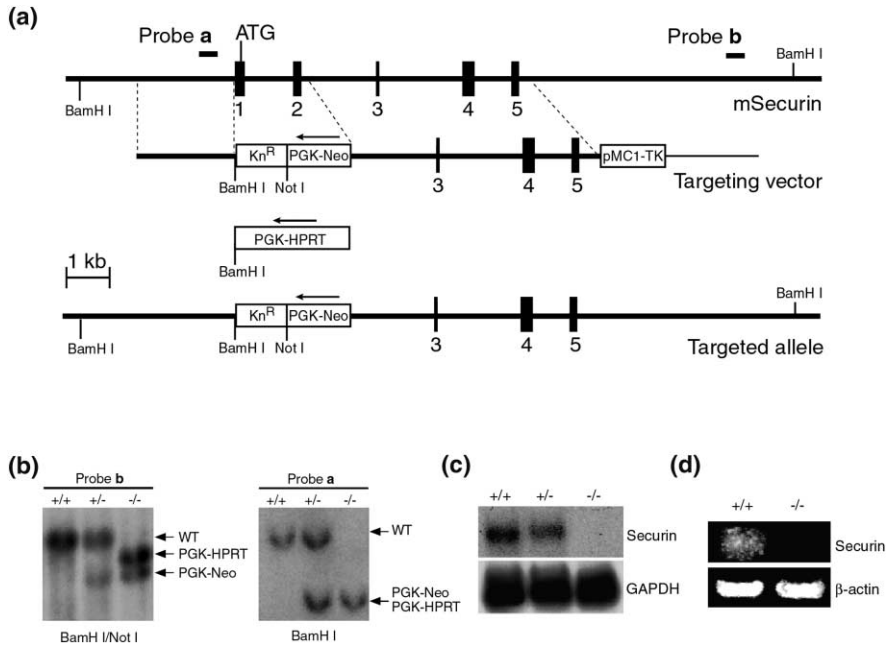
Results and discussion

We targeted the mouse securin gene through homologous recombination in mouse embryonic stem (ES) cells (Figure 1a). Several recombinant clones were obtained with a Neo replacement construct (Figure 1b). Given the potential importance of securin in regulating anaphase, we asked if the gene is essential. Therefore, we sought to generate ES cells that were null for securin. Through targeting the second allele in two independent securin+/− ES cell lines with a HPRT construct (Figure 1a), we obtained two securin-null ES cell lines (Figure 1b), and Northern blot analysis indicated the absence of securin expression in these cells (Figure 1c). Furthermore, in an RT-PCR assay with a forward primer located in exon 3 and a reverse primer located in exon 4, we failed to detect any sequences that were amplifiable by this pair of primers in securin mutants (Figure 1d). The two securin mutant ES cell lines behaved similarly to each other and grew at rates indistinguishable from wild-type cells (data not shown). Consistent with this, we found that the distribution of securin-null ES cells in each cell cycle phase (G1, S, and G2/M) was similar to that of wild-type cells (Figure 2a). These data indicate that securin is not essential for mouse embryonic stem cells.

In mammalian cells, nocodazole treatment induces the accumulation of securin proteins [10, 11], suggesting that it may be involved in the spindle checkpoint control. Therefore, we examined spindle checkpoint function in securin−/− ES cells. We used colcemid to disrupt microtubule assembly. In wild-type ES cells, colcemid treatment resulted in an apparently complete G2/M arrest within 12 hr (Figure 2a). However, securin-null ES cells also arrested with similar efficiency (Figure 2a). Furthermore, mitotic index analysis indicates similar percentages of cells with condensed chromosomes between wild-type and securin mutant ES cells, indicating that securin mutant cells did not exit mitosis prematurely (Figure 2b). These results demonstrate that the spindle checkpoint function is still intact in securin mutant ES cells.

By 24 hr of colcemid treatment, both wild-type and securin mutant cells had accumulated nearly identical numbers of cells with more than 4C DNA content, indicating that these cells were rereplicating their DNA without cytokinesis (Figure 2a). Accordingly, we saw a decrease in the mitotic indices in both wild-type and securin mutant ES cells (Figure 2b). The extent of the decease is the same between wild-type and securin mutant ES cells. This indicates that mouse ES cells, regardless of securin status, cannot maintain a prolonged metaphase arrest, in

Figure 1



The targeted disruption of securin in mouse ES cells. **(a)** A diagram of the securin disruption strategy. Two kinds of targeting vectors are shown, one utilizing PGK-Neo as a positive selection marker, and the other utilizing PGK-HPRT. Both vectors delete exons 1 and 2 of securin. pMC1-TK is placed at the end of homology for negative selection. Kn^R is the kanamycin resistance gene left over from the construction of the PGK-Neo targeting vector via homologous recombination in *E. coli*. Probes (a and b) for Southern analysis are indicated. **(b)** Southern blot analysis of genomic DNA isolated from wild-type (+/+), heterozygous (+/−), and homozygous (−/−) mutant ES cells. Restriction enzymes used to digest the DNA are indicated at the bottom of each blot. **(c)** Northern blot analysis of securin mRNA levels in total RNA isolated from wild-type, heterozygous, and homozygous mutant ES cells. The same blot was reprobed with GAPDH for a loading control. **(d)** RT-PCR analysis of total RNA isolated from the tail tissues of wild-type (+/+) and securin mutant (−/−) mice, with a forward primer located in exon 3 and a reverse primer located in exon 4.

agreement with the notion that mouse ES cells lack p53-mediated checkpoint controls [12].

Next, we examined sister cohesion in colcemid-treated cells. We found that sister chromatids maintained their cohesion at the centromere region in both wild-type and securin−/− cells with similar morphologies, even after 24 hr of treatment with the drug (Figure 3c,d), indicating that no sister separation had occurred. This is in sharp contrast to untreated cells in which the loss of centromeric cohesion was easily identifiable (Figure 3b). Regardless of securin status, colcemid treatment causes chromosomes to adopt a very different morphology. They appear shorter and thicker than normal metaphase chromosomes (Figure 3a), probably due to an adaptation to prolonged arrest in a condensed state.

Taken together, these data demonstrate that securin is not required for spindle checkpoint function in mouse ES cells.

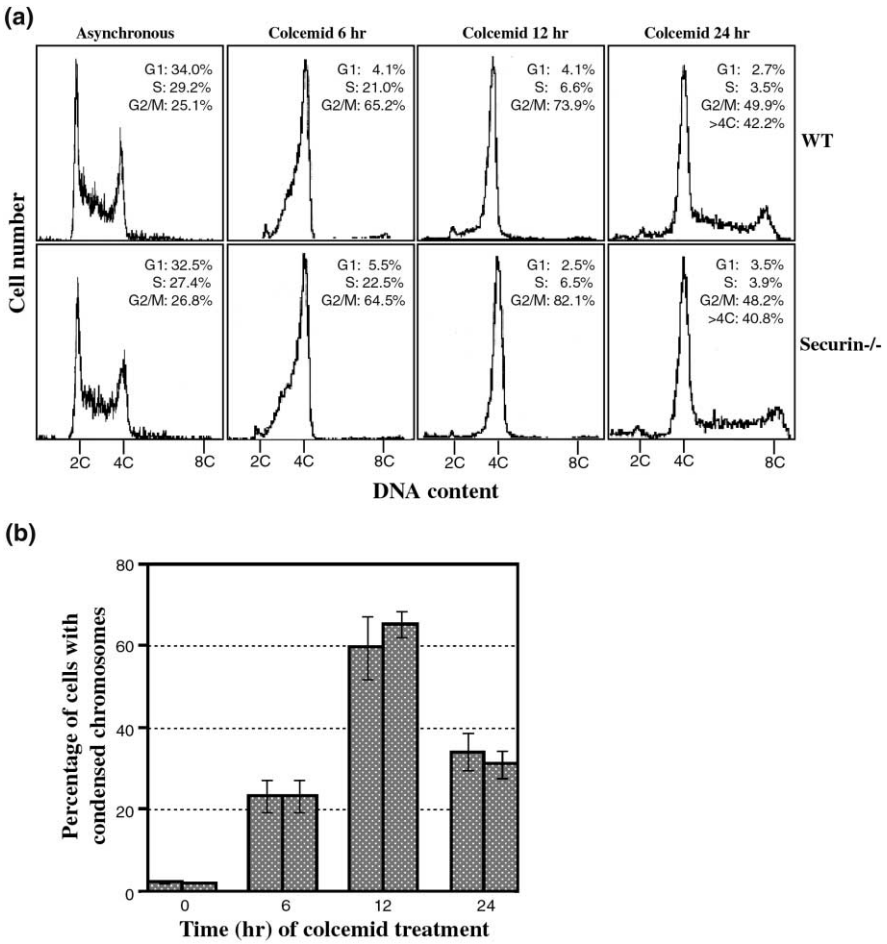
Since mouse embryonic stem cells are a unique cell type, we wondered if securin is required for other cell types. Therefore, we established mouse embryonic fibroblasts (MEF) from embryos obtained from intercrosses between two securin heterozygous mice, which were derived from a founder male with a germ line-transmitted mutant allele (Neo replacement). Securin heterozygous mice are fertile, and no gross anatomical defects are seen. At the time of embryo harvesting (E12.5–E18.5), no growth or structural abnormalities were apparent in securin−/− embryos when compared to wild-type embryos. Recently, we have obtained three securin-null mice of both sexes. Up to the age of 4 weeks, no apparent differences from wild-type littermates are detected in these mice.

In contrast to ES cells, however, securin−/− fibroblasts grew noticeably slower than both wild-type and heterozygous cells. Indeed, a growth curve analysis indicated a much slower growth rate by securin−/− cells than by both securin+/− and wild-type cells (Figure 4a). Securin +/− cells grew at a similar rate to wild-type cells (Figure 4a). This growth defect has been confirmed in three independent securin−/− MEF lines. To investigate if defects in cell cycle progression are responsible for the slowed growth, we analyzed the cell cycle phase distribution of securin-null cells. As shown in Figure 4b, securin−/− cells displayed a significant accumulation of cells in G2/M compared to wild-type cells, 41% versus 27%, or about a 52% increase, with a concurrent decrease in both G1 and S phases. This result largely explains the slower growth rate associated with securin deficiency.

We also investigated whether securin-null fibroblasts accumulate in mitosis. Wild-type fibroblasts exhibited a mitotic index of 0.85%, and securin-null cells exhibited an index of 0.80%. Thus, no increase in the number of cells in mitosis was found in securin-null cells, indicating that a lack of securin causes cells to accumulate in the G2 phase of the cell cycle. Among the cells undergoing mitosis, we found that wild-type and securin-null cells

Figure 2

The response of ES cells to colcemid treatment. **(a)** Cell cycle analysis. Wild-type and securin mutant ES cells, asynchronously growing or treated with colcemid for 6, 12, and 24 hr, were analyzed with flow cytometry. The percentage of each cell cycle phase is indicated. **(b)** Mitotic indices. The results are from three independent experiments.

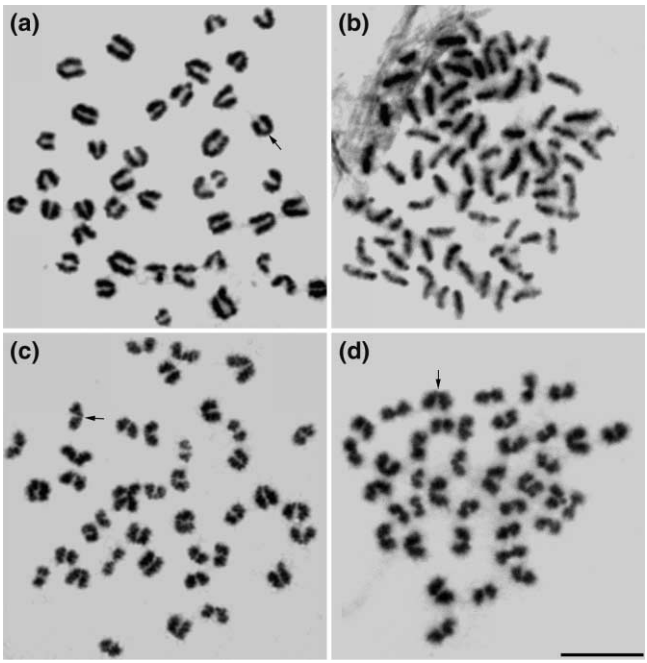


display nearly identical distributions of cells in metaphase versus cells in anaphase (referred to as cells with separated sister chromatids), indicating that there is an apparent normal metaphase-to-anaphase transition in the absence of securin (Figure 4c).

When the fibroblasts were treated with colcemid, we saw a similar G2/M arrest in both wild-type and securin-null cells (Figure 4b). As with ES cell results, mitotic indices and metaphase spread analyses did not show any differences between wild-type and securin-null fibroblasts in response to colcemid treatment (data not shown), indicating that securin is not required for spindle checkpoint function in mouse embryonic fibroblasts either.

The results presented in this study indicate that mouse securin is not an essential gene and is not required for spindle checkpoint control, either in ES cells or embryonic fibroblasts. In sharp contrast, the deletion of *Mad2* kinase, an upstream component of the spindle checkpoint, causes much more severe phenotypes, including the premature separation of sister chromatids [11, 13]. *Mad2*-deficient mice survive fewer than 6.5 days of embryogenesis [13], whereas securin-null mice survived up to 4 weeks after birth and did not display gross abnormalities. One possible explanation is that there are securin homologs that may function in the absence of securin. Indeed, Chen et al. [14] have reported the presence of two additional *PTTG* homologs in the human genome. These two homologs are intronless and are ~90% identical to human securin in their amino acid sequence. However, the presence of similar homologous genes in mice is questionable for two reasons. First, we failed to detect them on Southern blots using securin cDNA as a probe, and no securin-related messenger RNA was revealed to be present in our securin-null cells by Northern blot analysis. Second, there are no securin-like sequences, other than securin itself, in the mouse EST database. Therefore, other mechanisms are responsible for the control of the metaphase-to-anaphase transition in the absence of securin. There are several possibilities. First, it is possible that the cleavage of centromeric Scc1 depends not just on separin activity but on other events too, for example, the phosphorylation status of Scc1 itself. Secondly, separin activity could be regulated

Figure 3



The chromosome spread of wild-type and mutant ES cells. **(a)** Normal metaphase chromosomes from wild-type unsynchronized ES cells. **(b)** A portion of chromosome spread from wild-type ES cells demonstrating sister separation. **(c)** The chromosome spread from 24-hr colcemid-treated wild-type ES cells. **(d)** The chromosome spread from 24-hr colcemid-treated securin−/− ES cells. The arrows indicate cohesion in the centromere region. The scale bar indicates 50 μm.

independently of securin. Finally, in HeLa cells, okaid acid treatment causes centromeres to disjoin although the arms are fully separated [15], suggesting that phosphatase activity is required for centromere separation.

Despite being dispensable for cellular viability and spindle checkpoint control, securin is required for the optimal growth of mouse embryonic fibroblasts. We speculate that this effect may reflect securin's positive interaction with separin in promoting cell cycle progression [1, 16, 17]. Alternatively, other unspecified functions of securin may be responsible for the observed delay in G2. Indeed, a transcriptional activation function has been proposed for securin as *PTTG* [18]. It is possible that the positive role of securin in cell cycle progression might be the sole reason why many tumor cells overexpress this gene. It remains to be tested if overexpression of securin shortens the G2/M transition.

So far, securin has been studied as loss of function mutations in budding (the *Pds1* gene) and fission (the *Cut2* gene) yeast, in *Drosophila* (the *Pimples* gene), and in the mouse (this study). Phenotypically, these four securins both resemble and differ from each other. First, neither mouse securin nor *Pds1* is essential [7], whereas both *Cut2* and *Pimples* are essential genes [19–21]. The essentiality lies in the requirement for both genes for the separation of sister chromatids [20, 22]. Second, mouse securin is not required for spindle checkpoint control, a property seemingly shared by *Cut2* [23], whereas *Pds1* is an important component of the checkpoint [6]. It is unknown if Pimples is required for this checkpoint [21]. Finally, all four securins seem to play a positive role in cell cycle progression. Thus, the securin/separin pathway has diversified during evolution and reflects different strategies used by various organisms in controlling the metaphase-to-anaphase transition.

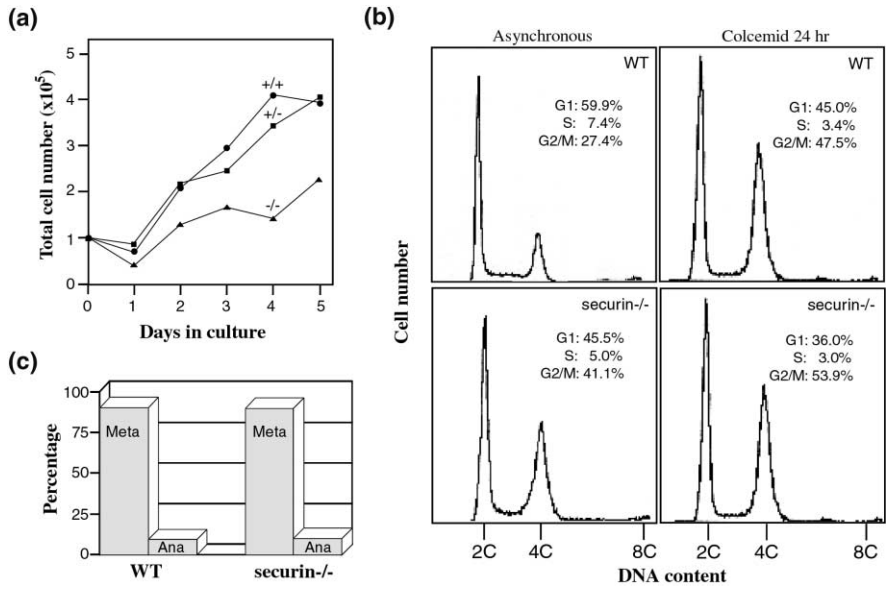
Materials and methods

Generation of securin KO ES cells and mice

A mouse securin cDNA was used to isolate genomic clones from a λKO library in which each genomic insert is already linked to the negative

Figure 4

Phenotypic analyses of securin-null fibroblasts. **(a)** A growth curve assay of wild-type, securin heterozygous, and securin-null mouse embryonic fibroblasts. **(b)** A cell cycle profile of asynchronously growing and colcemid-treated wild-type and securin-null fibroblasts. The percentage of each cell cycle phase is indicated. **(c)** A diagram showing the percentages of metaphase (Meta) versus anaphase (Ana) of mitotic cells in asynchronously growing wild-type and securin mutant fibroblasts.



selection marker TK (Zhang et al., unpublished data). Homologous recombination in *E. coli* was employed to generate the Neo targeting vector (Zhang et al., unpublished data). As a result, there is a bacterial selection marker (Kn^R) present next to PGK-Neo (Figure 1a). The second targeting vector was constructed with standard subcloning methods. We replaced Kn^R/PGK-Neo with the PGK-HPRT cassette. Linearized targeting vectors were electroporated into AB2.2 ES cells, and double-selected clones were screened for homologous recombination via Southern blot analysis. PGK-Neo recombinant ES clones were injected into C57BL/6 blastocysts to produce chimeric mice. One such male mouse gave germ line transmission of the targeted allele.

Southern and Northern blot analyses were performed according to standard protocols. RT-PCR assays were done with a kit from Invitrogen Life Technologies. The forward primer that was used was 5′-TGATGAT GCCTACCCAGA-3′, and the reverse primer was 5′-CCAGATGCA GCAGCTTCT-3′. The primer pair for β-actin was purchased from Promega.

Cell culture and cell cycle analysis

ES cells were cultured on a layer of feeder cells in DMEM containing 15% fetal bovine serum (FBS) supplemented with penicillin and streptomycin (growth medium). For the extraction of total RNA, ES cells were cultured feeder-free by supplementing LIF in growth medium to avoid RNA contamination from feeder cells. Mouse embryonic fibroblasts were obtained as described [24] and genotyped. Cells were cultured in DMEM containing 10% FBS and antibiotics. Cells were used before passage 3. Colcemid (500 ng/ml) was added to the culture media to induce metaphase arrest.

Cell cycle distribution was analyzed using flow cytometry analysis with standard protocols. The percentage of the G1, S, and G2 population was measured with the Coulter (II) software (Beckman). For growth curve analysis, 1×10^5 cells were seeded in a 35-mm tissue culture dish and harvested each day for 5 days. Each time point was an average of three dishes.

Metaphase spread

Cells were trypsinized, swollen in hypotonic solution (0.3% sodium citrate/0.224% KCl) for 30 min at 37°C, and fixed in Carnoy's fixative (1 part glacial acetic acid:3 parts methanol) with several changes. Fixed cells were dropped onto positively charged glass slides (Fisher Scientific) and dried overnight. Dried cells were stained with Gimesa/Wright stain (0.8 g Wright stain/0.1 g Gimesa stain in 400 ml methanol) for 3 min, washed in phosphate buffer (11 ml Gimesa/Wright stain plus 67 ml 8.3 mM [pH7.2] phosphate buffer), and rinsed with distilled water. Due to low mitotic figures in fibroblasts, over 10^4 cells were counted to get a statistically meaningful mitotic index. The mitotic index in ES cells was measured as described [25]

Acknowledgements

We thank W.J. Harper and S.J. Elledge for critical reading of the manuscript, S. Webb for help in ES cell culture and blastocysts injection, and J. Dragoo and X. An for excellent technical help. P.Z. is supported by a National Institutes of Health grant.

References

1. Ciosk R, Zachariae W, Michaelis C, Shevchenko A, Mann M, Nasmyth K: **An ESP1/PDS1 complex regulates loss of sister chromatid cohesion at the metaphase to anaphase transition in yeast.** *Cell* 1998, **93:**1067-1076.
2. Uhlmann F, Wernic D, Poupart MA, Koonin EV, Nasmyth K: **Cleavage of cohesin by the CD clan protease separin triggers anaphase in yeast.** *Cell* 2000, **103:**375-386.
3. Shirayama M, Toth A, Galova M, Nasmyth K: **APC(Cdc20) promotes exit from mitosis by destroying the anaphase inhibitor Pds1 and cyclin Clb5.** *Nature* 1999, **402:**203-207.
4. Visintin R, Prinz S, Amon A: **CDC20 and CDH1: a family of substrate-specific activators of APC- dependent proteolysis.** *Science* 1997, **278:**460-463.
5. Fang G, Yu H, Kirschner MW: **Direct binding of CDC20 protein family members activates the anaphase-promoting complex in mitosis and G1.** *Mol Cell* 1998, **2:**163-171.
6. Yamamoto A, Guacci V, Koshland D: **Pds1p, an inhibitor of anaphase in budding yeast, plays a critical role in the APC and checkpoint pathway(s).** *J Cell Biol* 1996, **133:**99-110.
7. Yamamoto A, Guacci V, Koshland D: **Pds1p is required for faithful execution of anaphase in the yeast, *Saccharomyces cerevisiae*.** *J Cell Biol* 1996, **133:**85-97.
8. Zou H, McGarry TJ, Bernal T, Kirschner MW: **Identification of a vertebrate sister-chromatid separation inhibitor involved in transformation and tumorigenesis.** *Science* 1999, **285:**418-422.
9. Pei L, Melmed S: **Isolation and characterization of a pituitary tumor-transforming gene (PTTG).** *Mol Endocrinol* 1997, **11:**433-441.
10. Waizenegger IC, Hauf S, Meinke A, Peters JM: **Two distinct pathways remove mammalian cohesin from chromosome arms in prophase and from centromeres in anaphase.** *Cell* 2000, **103:**399-410.
11. Michel LS, Liberal V, Chatterjee A, Kirchwegger R, Pasche B, Gerald W, *et al.*: **MAD2 haplo-insufficiency causes premature anaphase and chromosome instability in mammalian cells.** *Nature* 2001, **409:**355-359.
12. Schmidt-Kastner PK, Jardine K, Cormier M, McBurney MW: **Absence of p53-dependent cell cycle regulation in pluripotent mouse cell lines.** *Oncogene* 1998, **16:**3003-3011.
13. Dobles M, Liberal V, Scott ML, Benezra R, Sorger PK: **Chromosome missegregation and apoptosis in mice lacking the mitotic checkpoint protein Mad2.** *Cell* 2000, **101:**635-645.
14. Chen L, Puri R, Lefkowitz EJ, Kakar SS: **Identification of the human pituitary tumor transforming gene (hPTTG) family: molecular structure, expression, and chromosomal localization.** *Gene* 2000, **248:**41-50.
15. Ghosh S, Paweletz N, Schroeter D: **Failure of centromere separation leads to formation of diplochromosomes in next mitosis in okadaic acid treated HeLa cells.** *Cell Biol Int* 1993, **17:**949-952.
16. Jensen S, Segal M, Clarke DJ, Reed SI: **A novel role of the budding yeast separin Esp1 in anaphase spindle elongation. Evidence that proper spindle association of esp1 is regulated by pds1.** *J Cell Biol* 2001, **152:**27-40.
17. Kumada K, Nakamura T, Nagao K, Funabiki H, Nakagawa T, Yanagida M: **Cut1 is loaded onto the spindle by binding to Cut2 and promotes anaphase spindle movement upon Cut2 proteolysis.** *Curr Biol* 1998, **8:**633-641.
18. Dominguez A, Ramos-Morales F, Romero F, Rios RM, Dreyfus F, Tortolero M, *et al.*: **hpttg, a human homologue of rat pttg, is overexpressed in hematopoietic neoplasms. Evidence for a transcriptional activation function of hPTTG.** *Oncogene* 1998, **17:**2187-2193.
19. Uzawa S, Samejima I, Hirano T, Tanaka K, Yanagida M: **The fission yeast cut1+ gene regulates spindle pole body duplication and has homology to the budding yeast ESP1 gene.** *Cell* 1990, **62:**913-925.
20. Funabiki H, Kumada K, Yanagida M: **Fission yeast Cut1 and Cut2 are essential for sister chromatid separation, concentrate along the metaphase spindle and form large complexes.** *EMBO J* 1996, **15:**6617-6628.
21. Leismann O, Herzig A, Heidmann S, Lehner CF: **Degradation of *Drosophila* PIM regulates sister chromatid separation during mitosis.** *Genes Dev* 2000, **14:**2192-2205.
22. Stratmann R, Lehner CF: **Separation of sister chromatids in mitosis requires the *Drosophila* pimples product, a protein degraded after the metaphase/anaphase transition.** *Cell* 1996, **84:**25-35.
23. Yanagida M: **Cell cycle mechanisms of sister chromatid separation; roles of Cut1/separin and Cut2/securin.** *Genes Cells* 2000, **5:**1-8.
24. Deng C, Zhang P, Harper JW, Elledge SJ, Leder P: **Mice lacking p21CIP1/WAF1 undergo normal development, but are defective in G1 checkpoint control.** *Cell* 1995, **82:**675-684.
25. Liu Q, Guntuku S, Cui XS, Matsuoka S, Cortez D, Tamai K, *et al.*: **Chk1 is an essential kinase that is regulated by Atr and required for the G(2)/M DNA damage checkpoint.** *Genes Dev* 2000, **14:**1448-1459.