**Drosophila** securin destruction involves a D-box and a KEN-box and promotes anaphase in parallel with Cyclin A degradation

Oliver Leismann and Christian F. Lehner*
Department of Genetics, University of Bayreuth, 95440 Bayreuth, Germany
*Author for correspondence (e-mail: chle@uni-bayreuth.de)

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Cyclin A degradation

**Summary**

Sister chromatid separation during exit from mitosis requires separase. Securin inhibits separase during the cell cycle until metaphase when it is degraded by the anaphase-promoting complex/cyclosome (APC/C). In *Drosophila*, sister chromatid separation proceeds even in the presence of stabilized securin with mutations in its D-box, a motif known to mediate recruitment to the APC/C. Alternative pathways might therefore regulate separase and sister chromatid separation apart from proteolysis of the *Drosophila* securin PIM. Consistent with this proposal and with results from yeast and vertebrates, we show here that the effects of stabilized securin with mutations in the D-box are enhanced in vivo by reduced Polo kinase function or by mitotically stabilized Cyclin A. However, we also show that PIM contains a KEN-box, which is required for mitotic degradation in addition to the D-box, and that sister chromatid separation is completely inhibited by PIM with mutations in both degradation signals.

Key words: Pimples, Sister chromatid separation, Separase, Cyclin A, Polo, APC/C

**Introduction**

Securins are regulatory proteins that associate with a cysteine protease called separase (Nasmyth, 2002). Separase cleaves the Scc1/Mcd1/Rad21 subunit of the cohesin complex and thereby brings about the final resolution of sister chromatid cohesion during the metaphase-to-anaphase transition of mitosis. Securins from different eukaryotes are surprisingly divergent at the primary sequence level and their physiological importance varies. Deletion of the budding yeast (Pds1) and vertebrate (PTTG) securin genes results in increased rates of chromosome loss but is not necessarily lethal (Jallepalli et al., 2000; Stratmann and Lehner, 1996; Uhlmann et al., 2000). These particular securins therefore provide a first function that is absolutely required for sister chromatid separation, perhaps by assisting in separase folding or localization. In addition, all of the securins also appear to function as inhibitors of separase activity and are degraded during the metaphase-to-anaphase transition (Ciosk et al., 1998; Cohen-Fix et al., 1996; Funabiki et al., 1996b; Leismann et al., 2000; Stratmann and Lehner, 1996; Uhlmann et al., 2000; Zou et al., 1999). Mitotic securin degradation therefore contributes to the temporal control of separase activity.

Mitotic securin degradation involves polyubiquitinylation by a special ubiquitin ligase known as anaphase-promoting complex/cyclosome (APC/C) (for a review, see Peters, 2002). During mitosis, APC/C activity is controlled by the mitotic spindle checkpoint pathway. This checkpoint assures that securin protein remains stable until all chromosomes have reached the correct bi-orientation within the mitotic spindle. The fact that securin stabilization by the mitotic spindle checkpoint prevents premature sister chromatid separation has been clearly shown in budding yeast (Ciosk et al., 1998; Yamamoto et al., 1996). By contrast, premature sister chromatid separation has not been observed in human cells lacking securin when arrested during mitosis (Jallepalli et al., 2001), suggesting that sister chromatid separation in these cells involves additional securin-independent pathways.

In addition to mitotic securin degradation, other levels of regulation have been implicated in the temporal control of separase activity and of sister chromatid separation. In budding yeast, phosphorylation of the Scc1 cohesin subunit by the Cdc5/Polo-kinase provides regulation at the substrate level (Alexandru et al., 2001). After phosphorylation, Scc1 is a better substrate for the yeast separase Esp1. Regulated Scc1 phosphorylation therefore provides sufficient temporal control of sister chromatid separation when the securin Pds1 is absent and Esp1 is constitutively active throughout the cell cycle. Cohesin phosphorylation by Polo-like kinase also controls the separase-independent dissociation of cohesin complexes from higher eukaryotic chromosomes during mitotic prophase (Losada et al., 2002; Sumara et al., 2002).

In higher eukaryotes, where separase is required for the removal of those cohesin complexes that remain on chromosomes until the metaphase-to-anaphase transition (Hauf et al., 2001), cyclin-dependent kinase 1 (Cdk1) has been proposed to inhibit separase activity in parallel to securin (Stemmann et al., 2001). Separase is phosphorylated by cyclin-Cdk1 complexes and thereby inhibited in cells arrested by the mitotic spindle checkpoint. High levels of non-degradable
Cyclin B have been shown to inhibit sister chromatid separation in *Xenopus* egg extracts and in PK1 cells (Stemmman et al., 2001; Haging et al., 2002). In *Drosophila* embryos, expression of non-degradable Cyclin A, which is found exclusively in Cdk1(Cdc2) and not in Cdk2(Cdc2c) complexes, delays sister chromatid separation significantly (Jacobs et al., 2001; Parry and O’Farrell, 2001; Sigrist et al., 1995). Cdk1 inactivation resulting from the APC/C-dependent proteolysis of the mitotic cyclins at the metaphase-to-anaphase transition therefore presumably leads to separase activation, similar to securin degradation. Securin- and Cdk1-dependent separase regulation might be largely redundant, explaining why human cells display only very subtle defects in the absence of securin function.

In securin-expressing cells, mitotic sister chromatid separation is generally assumed to be strictly dependent on mitotic securin degradation. However, the corresponding evidence is from experiments involving overexpression of securin variants with mutant degradation signals. By contrast, our experiments in *Drosophila* embryos involving expression at physiological levels raised the possibility that sister chromatid separation might not depend on PIM degradation (Leismann et al., 2000). Therefore, we have further analyzed the role of PIM degradation.

We have previously shown that PIM contains a novel D-box variant that functions as a mitotic degradation signal (Leismann et al., 2000). D-boxes that form an RxxLxxxxN consensus sequence (Peters, 1999) were initially identified in various proteins (Pfleger and Kirschner, 2000; Peters, 2002). A KEN-box, can also mediate APC/C-dependent degradation of B-type cyclins where they are required for mitotic destruction. The D-box variant identified in PIM starts with a K instead of an R. Apart from the D-box, a different destruction signal, the KEN-box, can also mediate APC/C-dependent degradation of various proteins (Pfleger and Kirschner, 2000; Peters, 2002). A KEN-box has recently been shown to contribute to the mitotic securin degradation. However, we also show genetic interactions arguing for the presence of additional, securin-independent regulation of sister chromatid separation.

### Materials and Methods

**Drosophila stocks**

*Pim*1, pol110, UAS-pim-myc, UAS-Cdk1-myc, UAS-CycA-ΔI-53 and UAS-CycA-ΔI-170 (TF73) have been described previously (Donaldson et al., 2001; Jacobs et al., 2001; Kaspar et al., 2001; Leismann et al., 2000; Meyer et al., 2000; Stratmann and Lehner, 1996). UAS transgenes were expressed with arm-GAL4, da-GAL4, prd-GAL4 or nos-GAL4-GCNI-bcd3’UTR (Brand and Perrimon, 1993; Janody et al., 2000; Sanson et al., 1996; Wodarz et al., 1995). P[bsTub85D-FLP] was used for testis-specific expression of FLP recombinase (Golic et al., 1997).

UAS-*pim*emo-myc lines were generated with a pUAST construct (Brand and Perrimon, 1993). Its construction involved the introduction of the desired KEN-box mutations by enzymatic amplification using the plasmid pKS+*pim-myc* as a template. pKS+*pim-myc* was constructed by first ligating into the NotI and KpnI sites of pKS+ the regulatory, the coding and the 3’ region of *pim*1 enzymatically amplified from the pKS+S2P4B1 plasmid containing a genomic *pim*1 fragment (Stratmann and Lehner, 1996) using the primer pairs RS37 (5’T-ATAAGAATGGGGCCGCACACA-GGAGAGAGTTCTTAATGGTT-3’ and RS6 (5’T-TTATATTTTTTATTGG-3’), RS34 (5’T-GGATCACTTTAGTTTAAACAAGGAAA-3’), and RS35 (5’T-GAGAGATCTAGCCTTCCAA-GTGGACCAATG-3’), or RS38 (5’T-GTATGATCTTTATTTGTACCGATCTTTGTTTTAAA-3’) and RS39 (5’T-GGTTACCCGGCGTATCTTGCCGCAAC-3’), respectively, to yield pKS+*gpim*. These primers introduced a BamHI site immediately after the start codon, a BgIII site just before the stop codon, and mutations (Q3P, V197L) that do not affect PIM function (Stratmann and Lehner, 1996). The construction of pKS+*gpim-myc* was then completed by amplifying a fragment encoding six copies of the myc epitope with the primers RS68 (5’T-GGATCACTTTAGTTTAAACAAGGAAA-3’) and RS51 (5’T-GAGAGGCTGAGAAGATATGTTCTAACAG-3’), and inserting it into the BgIII site of pKS+*gpim*. Primers OL33 (5’T-GGAAACAGGGCCCGCTTTGTTTTAAAATACCCGAGCTGCTCTTGC-3’) and OL35 (5’T-GGAGTACGACAAGTCGCTTGGTTCCTACAG-3’), were then used for amplification of a first fragment from pKS+*gpim-myc* and OL32 (5’T-GGAAAGACGCGCCGGAGGCTTCCCTTACAG-3’) and OL2 (5’T-GCATCTAGAAAGTTTTTATGGTGCTTTATATTGCTT-3’), for a second fragment. Fragment 1 was digested with Asp718 and NotI, fragment 2 with NotI and XbaI. Both fragments were ligated into the Asp718 and XbaI sites of pUAST.

The transgenes *gpim-myc* and *gpimemo-myc*, in which expression is directed by the *pim* regulatory region, have been described previously (Leismann et al., 2000; Stratmann and Lehner, 1996). For the generation of *gpimemo* lines, we first enzymatically amplified a fragment from pKS+*gpimemo-myc* with the primers RS37 (5’T-TTCAATACGTAGCCCGGCCC-3’) and OL59 (5’T-CAGGAAACACCGCGGATTATTGCTG-3’). The resulting 300 bp amplification product with the D-box mutations was used as a primer, which was then extended after hybridization to the plasmid pKS+S2P4B1. The newly synthesized strands were annealed and ligated, yielding pKS+S2P4B1*emo*. Its insert was excised by BamHI and transferred into the germ line transformation vector pCaSpeR 4 (Pirotta, 1988).

To generate the *gstop>pim*, *gstop>pimeemo* and *gstop>pimeemo-myc* lines, we first constructed pKS+S5*FRT*gpim*3’ by inserting a fragment with a FLP recombinase target sequence (FRT) amplified from the plasmid pKB345 (kindly provided by K. Basler, University of Zurich) with the primers RS62 (5’T-GGAGGATCGTACGTGAGGATTTCCAAGGAGAC-3’) and RS63 (5’T-GCGGGATCCATTTTATGTTACGGCTAAAGGAGACG-3’). The amplification product containing the 1500 bp cassette followed by the downstream FRT site was digested with BgIII and BamHI into the BamHI site of pKS+*gpim*. pKB345 contains a 2.4 kb Asp718 fragment with 3’TUTR and transcriptional terminator sequences from the heat-shock protein gene, hsp70 (stop cassette) flanked by two FRT sites (Struhl and Basler, 1993). For the introduction of the stop cassette along with the second FRT site into pKS+S5*FRT*gpim*3’, we first transferred the Asp718 fragment from pKB345 into pKS+, in which the BamHI site had been destroyed by religation after filling the restricted site. The BamHI site within the insert fragment of the resulting plasmid was also eliminated. The plasmid was then used as a template for enzymatic amplification with the primers OL83 (5’T-GGCCGGATCCATTTCAGGCCCGCTTAAGGCCG-3’) and RS67 (5’T-GCGGGATCCATTTTATGTTACGGCTAAAGGAGACG-3’). The amplification product containing the 1500 bp cassette followed by the downstream FRT site was digested with BgIII and BamHI and ligated into the BamHI site of pKS+S5*FRT*gpim*3’. Ligation in the correct orientation resulted in pKS+S5*FRT*gpim*3’. The plasmid with an intact stop cassette flanked by two FRT sites (Struhl and Basler, 1993). For the introduction of the stop cassette along with the second FRT site into pKS+S5*FRT*gpim*3’, we first transferred the Asp718 fragment from pKB345 into pKS+, in which the BamHI site had been destroyed by religation after filling the restricted site. The BamHI site within the insert fragment of the resulting plasmid was also eliminated. The plasmid was then used as a template for enzymatic amplification with the primers OL83 (5’T-GGCCGGATCCATTTCAGGCCCGCTTAAGGCCG-3’) and RS67 (5’T-GCGGGATCCATTTTATGTTACGGCTAAAGGAGACG-3’). The amplification product containing the 1500 bp cassette followed by the downstream FRT site was digested with BgIII and BamHI and ligated into the BamHI site of pKS+S5*FRT*gpim*3’.
template pUASTpimkena-myc. To add the D-box mutations, the resulting product was used as a primer for an additional polymerase chain reaction in combination with a second primer OL8 (5'-ATTAGTAGTACAAAGATACCTAGC-3') and the template pKS+ gpimdba-myc. The fragment with the kena and dba mutations was used to replace the wild-type sequence in pKS+ stop>gpim either by using BamHI and SnaBI (in the case of g>stop>pimkenadba) or BamHI and BglII (in the case of g>stop>pimkenadba–myc). The final pCaSpeR 4 constructs were again obtained by transposing Asp718-NotI fragments. All constructs were verified by DNA sequencing and used for Drosophila germ line transformation according to standard procedures.

**Fig. 1.** Polo kinase and stabilized Cyclin A modify the PIMdba phenotype. (A-F) DNA staining at stage 14 reveals the presence of many large polyploid abnormal nuclei in the CNS of embryos expressing the stabilized securin PIMdba if they are also polo mutant (E,F; pimdba polo−). By contrast, at this stage, cells in the CNS are hardly affected in polo mutants that do not express PIMdba (C,D; polo−) or in the polo+ siblings that express PIMdba (A,B; pimdba). B, D and F show high-magnification views with the CNS from the embryos displayed in A, C and E, respectively. (G–J) Using prd-GAL4 and UAS-CycAΔ1-53, stabilized Cyclin A was expressed in alternating embryonic segments. Expressing segments are indicated by arrowheads in G and I or by white lines in H and J, which display high-magnification views of epidermal regions. DNA staining indicates that the metaphase delay caused by stabilized Cyclin A is prolonged in embryos that also express the stabilized securin PIMdba under the control of the pim+ regulatory region. Compared with embryos without PIMdba (G,H; CycAΔN), metaphase plates (arrows) in regions with stabilized Cyclin A are enriched in embryos that also express PIMdba (I,J; pimdba CycAΔN).

**Antibodies, immunoprecipitation and immunolabeling**

Mouse monoclonal antibodies against a myc epitope (9E10), against *Drosophila* Cyclin B (F2) and against α-tubulin (Neomarkers, Fremont, CA) were used. Rabbit polyclonal antibodies against *Drosophila* Cyclin B, Three rows (THR), PIM and Separase (SSE) have been described previously (Jäger et al., 2001). Double labeling with mouse (Promega, Madison, WI) or rabbit antibodies against β-galactosidase (Cappel, Aurora, OH) in combination with blue balancers was used for the identification of embryo genotypes. For co-immunoprecipitation experiments, extracts were prepared with eggs collected from either gpim-myc III.1, or UAS-Cdk1-myc II.2, armGAL4 flies, or from a cross of pim1/Cyo, P[w+, ftz-lacZ] females with pim1, g>stop>pimkenadba-myc II.1/Cyo, P[w+, ftz-lacZ]; P[bTub85D-FLP]/+ males during 2 hours on apple-juice agar plates followed by aging for 6 hours at 25°C. Immunoprecipitations were performed as described previously (Leismann et al., 2000). Fixation of embryos and immunolabeling was performed essentially as described previously (Leismann et al., 2000). Eggs were collected for 2 hours and aged at 25°C from the following crosses: – pim1/Cyo, P[w+, ftz-lacZ] females with pim1, gpim100 II.1/Cyo, P[w+, ftz-lacZ] males (aging 4.5 hours) or with pim1, gpim100-myc II.5/Cyo, P[w+, ftz-lacZ] males (aging 12 hours).
– pim1/CyO, P[w+, ftz-lacZ]; prd-GAL4/+ females with pim1, UAS-pimkema-myc 2.4/CyO, P[w+, ftz-lacZ] males (aging 4.5 hours)
– polo10/TM3, Sb, P[w+, Ubx-lacZ] females with gpimdba II.1, pim1/CyO, P[w+, ftz-lacZ]; polo10/TM3, Sb, P[w+, Ubx-lacZ] males (aging 12 hours)
– UAS-CycAΔ1-53 III.2 females with gpimdba II.1/CyO, P[w+, ftz-lacZ]; prd-GAL4/TM3, Sb, P[w+, Ubx-lacZ] males (aging 6 hours)
– UAS-CycAΔ1-70; gpim-myc III.5, gpim-myc II.6, prd-GAL4/TM3, Sb, P[w+, Ubx-lacZ] males (aging 6 hours)
– nos-GAL4-GCN4-bcd3′UTR females with either UAS-pim-myc III.3 or UAS-pimkema-myc III.3 males (aging 2 hours)

Results

Embryos homozygous for pim null mutations but equipped with a maternal pim+ contribution from pim heterozygous mothers progress normally through the initial embryonic cycles. Entry into mitosis 15 and progression to metaphase are still normal. Moreover, the transition from metaphase to anaphase is triggered as well, as evidenced by the degradation of the mitotic Cyclins A, B and B3. However, sister chromatid separation during mitosis 15 is completely inhibited (Stratmann and Lehner, 1996). This block of sister chromatid separation after the exhaustion of the maternal pim+ contribution is almost completely prevented when pim embryos inherit a gpimdba-myc transgene (Leismann et al., 2000). gpimdba-myc drives expression of PIM with C-terminal myc epitopes and a mutant D-box (AKPAGNLDA instead of KKPLGNDLN). PIMdba-myc is stable during mitosis according to confocal immunofluorescence microscopy, in contrast to PIM-myc with the wild-type D-box. gpimdba-myc expression is controlled by the normal pim regulatory region, and the resulting level of PIMdba-myc before mitosis 15 was found to be comparable to wild-type PIM levels. Because stabilized PIMdba-myc protein promoted sister chromatid separation in pim mutants, it appeared that sister chromatid separation is not dependent on degradation of the Drosophila securin PIM (Leismann et al., 2000). Analogous experiments with a gpimdba transgene driving expression of a D-box mutant of PIM version without myc epitopes also revealed rescue of mitosis 15 in pim mutants (data not shown), excluding the possibility that sister chromatid separation in the presence of stabilized PIMdba-myc occurs simply because C-terminal myc epitopes specifically abolish the inhibitory PIM function.

Instead of being required during each mitosis, PIM degradation might be important to keep protein levels below a critical threshold. We have previously shown that already moderate overexpression of wild-type pim (about fivefold) is sufficient to block sister chromatid separation. Moreover, although gpimdba rescued sister chromatid separation during mitosis 15 and 16 in pim mutants, it did not allow later divisions (data not shown), perhaps because the levels of stabilized PIMdba had built up beyond the critical threshold.
If degradation of the securin PIM was not an obligatory process required during each mitosis, separase bound to securin would be expected to have sufficient basal activity to allow sister chromatid separation. In this case, premature sister chromatid separation during interphase and early mitosis would have to be prevented by securin-independent regulation. As securin-independent regulation at the level of Sec1 phosphorylation by Cdc5/Polo kinase has been described in yeast, we analyzed whether a reduction in polo function enhances the effects of stabilized PIM\textsubscript{dba}. Within the CNS of polo-mutant embryos, we observed many abnormal cells with very large polyploid nuclei, when these embryos also carried gpim\textsubscript{dba} (Fig. 1E,F). Similar abnormal cells were almost never observed in either polo\textsuperscript{+} sibling embryos with gpim\textsubscript{dba} (Fig. 1A,B) or in polo\textsuperscript{-} sibling embryos without gpim\textsubscript{dba} (Fig. 1C,D). In the presence of stabilized PIM\textsubscript{dba}, therefore, the remaining level of maternal polo\textsuperscript{+} contribution is no longer sufficient to mask phenotypic abnormalities in polo-mutant embryos. Moreover, reduced polo\textsuperscript{+} function enhances the effects of stabilized PIM\textsubscript{dba}.

In addition to Sec1 regulation by Cdc5/Polo kinase, vertebrate Cdk1 has been shown to regulate separase independently of securin (Stemm et al., 2001). The effects of stabilized Cyclin A in Drosophila embryos (Sigrist et al., 1995; Jacobs et al., 2001) are consistent with the finding that vertebrate Cdk1 phosphorylates and thereby inhibits separase. Mutant Cyclin A versions that cannot be degraded during mitosis delay progression through the embryonic cell divisions during metaphase before sister chromatid separation. Therefore, Drosophila Cyclin A-Cdk1 complexes might inhibit separase activity. Accordingly, the effects of stabilized Cyclin AA1-53 are expected to be enhanced by expression of stabilized PIM\textsubscript{dba}. Labeling with antibodies against tubulin (data not shown) and a DNA stain clearly revealed an increased number of metaphase figures in epidermal regions of embryos expressing both Cyclin AA1-53 and PIM\textsubscript{dba} (Fig. 1J), compared with embryos expressing only Cyclin AA1-53 (Fig. 1G,H). The stabilized Cyclin AA1-53 therefore results in a more pronounced metaphase delay in the presence of the stabilized PIM\textsubscript{dba}.

In principle, stabilized Cyclin A might delay cells in metaphase because it results in an inhibition of PIM degradation during mitosis. However, cells delayed in...
metaphase by stabilized Cyclin AΔ1-170 no longer contained PIM-myc according to immunolabeling experiments, whereas metaphase cells that do not express Cyclin AΔ1-170 were always positive for PIM-myc (Fig. 2). We conclude, therefore, that the metaphase delay induced by stabilized Cyclin A does not result from delayed PIM degradation.

The phenotypic interactions between stabilized PIMdba and Polo or Cyclin A are consistent with the notion that separase complexed with non-degradable securin might have sufficient activity to allow sister chromatid separation and that the timing of this process is controlled by pathways other than securin degradation. However, the observed sister chromatid separation in PIMdba-expressing cells might also be supported by residual mitotic PIMdba degradation. A KEN motif, which is found close to the N-terminus in all of the securins (Fig. 3A), might allow some limited mitotic PIMdba degradation, escaping detection by confocal microscopy as applied in our previous experiments.

To determine whether the KEN motif of PIM functions as a degradation signal, we analyzed the mitotic stability of a myc-tagged PIM version with a mutant KEN-box (PIMkena-myc with AAA instead of KEN). PIMkena-myc, and PIM-myc for control, were expressed in the anterior region of embryos during cycle 14, as described previously (Leismann et al., 2000). Immunolabeling at the stage of mitosis 14 indicated that PIMkena-myc is largely stable throughout mitosis (Fig. 3F-I), in contrast to PIM-myc, which was detected before but not after the metaphase-to-anaphase transition (Fig. 3B-E). Progression beyond the metaphase-to-anaphase transition was monitored by the labeling of DNA and Cyclin B, which is rapidly degraded when cells enter anaphase. Our results show that the KEN-box is required and that the variant D-box (KKPLGNLDN), which is still present in PIMkena-myc, is not sufficient for normal mitotic PIM degradation.

Overexpression of PIMkena-myc resulted in mitotic defects. Normal anaphase and telophase figures were not observed in PIMkena-myc-positive cells that had progressed beyond the metaphase-to-anaphase transition according to the absence of anti-Cyclin-B labeling. Instead of pairs of well-

![Image]

**Fig. 4.** PIMkena does not rescue sister chromatid separation in *pim* mutants. (A-P) Embryos were labeled with a DNA stain (A,D,E,H,I,L,M,P; DNA) and anti-Cyclin B (B,F,J,N; CYCB) at the stage of mitosis 15 (A-C,E-G,I-K, M-O) or after mitosis 15 (D,H,L,P). High-magnification views from epidermal regions, including merged views (C,G,K,O; DNA in red and anti-CycB in green), are shown. Mitosis 15 proceeds normally in *pim*+ sibling embryos (A-D; *pim*+) as well as in *pim*-mutant embryos with a recombined *g>stop>pim* transgene lacking the stop cassette (E-H; *pim*->*pim*), as evidenced by normal telophase figures (arrows) during mitosis 15 and normal nuclear counts after mitosis 15 (white numbers in D,H,L,P). By contrast, sister chromatid separation does not occur during mitosis 15 in *pim*-mutant embryos with either an unrecombined *g>stop>pimkena* transgene (I-K; *pim*->*pimkena*) or the recombined transgene lacking the stop cassette (M-O; *pim*->*pimkena*). Instead of normal late mitotic figures, these embryos contained decondensing metaphase plates (arrowheads) during mitosis 15 and a twofold lower nuclear count after mitosis. (Q-T) Expression of *g>pimkena* in *pim*+ embryos allows normal proliferation during the early mitotic divisions but not during the late divisions in the CNS. DNA staining at stage 14 reveals the presence of many large polyploid abnormal nuclei (arrowheads) in the CNS of *g>pimkena* embryos (R,T; *g>pimkena*), which are absent in control siblings (Q,S; *pim*+). S and T show high-magnification views with the CNS from the embryos displayed in Q and R, respectively. (U) Co-immunoprecipitation experiments show that PIMkena-myc associates normally with SSE and THR. Anti-myc immunoprecipitates (IP anti-myc) isolated from extracts (extract) of embryos expressing Cdk1-myc (Cdk1-myc), PIMkena-myc (*pimkena*-myc) or PIM-myc (*pim*-myc) were probed by immunoblotting for the presence of SSE (SSE), THR (THR), Cyclin B (CYCB) and tubulin (TUB).
separated telophase daughter nuclei, which were readily observed in Cyclin-B-negative regions in the PIM-myc control experiments (Fig. 3D,E, arrowheads). Cyclin-B-negative regions of PIMKenadba-myc-expressing embryos displayed decondensing metaphase plates or chromatin bridges between partially separated nuclei (Fig. 3H,I arrows). These abnormalities caused by PIMKenadba-myc were indistinguishable from those previously observed with PIMDbamyc which has been shown to inhibit sister chromatid separation (Leismann et al., 2000).

Sister chromatid separation is also inhibited by strong overexpression of wild-type PIM-myc (Leismann et al., 2000). By contrast, at low physiological expression levels, PIM-myc and, remarkably, also the stabilized versions PIMDbamyc (Leismann et al., 2000) and PIMKenadba-myc (Fig. 3J-L), can promote sister chromatid separation in pim mutants.

To analyze the function of PIM with mutations in both D- and KEN-box, we constructed additional transgenes (g>stop>pimKenadba and g>stop>pimKenadba-myc), allowing the expression of PIMKenadba or PIMKenadba-myc under the control of the normal pim regulatory region. To establish chromosomal insertions of these potentially detrimental transgenes, we inserted a stop cassette flanked by FLP recombinase target sites (>stop>) into the 5’ untranslated region. This stop cassette was eventually excised by transmitting the established insertions via males expressing FLP recombinase specifically in spermatocytes. Expression of the paternally recombined transgenes (g>pimKenadba and g>pimKenadba-myc) started at the onset of zygotic expression during cycle 14 of embryogenesis. Expression of g>pimKenadba and g>pimKenadba-myc in pim-mutant embryos did not allow sister chromatid separation during mitosis 15 (Fig. 4M-O and data not shown). Instead of normal mitotic figures, which were readily apparent in pim+ sibling embryos (Fig. 4A, arrows), only decondensing metaphase plates were observed during exit from mitosis (Fig. 4M, arrowheads). Thus, pim-mutant embryos expressing g>pimKenadba and g>pimKenadba-myc displayed the same phenotype as pim mutants without transgene (Leismann et al., 2000) (and data not shown) or with the non-recombined g>stop>pimKenadba transgene (Fig. 4I-K).

Control experiments with g>stop>pim transgenes encoding wild-type PIM showed that expression after stop-cassette removal was sufficient to promote normal sister chromatid separation in pim mutants (Fig. 4E-G). Moreover, additional control experiments showed that the recombined g>pimKenadba-myc transgene was expressed as expected. Anti-myc immunoblotting clearly showed expression (data not shown), and co-immunoprecipitation experiments (Fig. 4U) indicated that the PIMKenadba-myc protein associates efficiently with Separase (SSE) and Three rows (THR), a Drosophila protein known to form trimeric complexes with SSE and PIM (Jäger et al., 2001). In addition, although g>pimKenadba-myc expression in pim+ sibling embryos had little effect during the initial embryonic cell divisions (mitosis 14-16), it resulted in a severe mutant phenotype in the CNS where additional cell divisions occur (Fig. 4T). Wild-type PIM therefore appears to protect cells from the effects of PIMKenadba-myc but only as long as the latter has not yet accumulated to high levels. In summary, our experiments with g>pimKenadba and g>pimKenadba-myc in pim mutants show that sister chromatid separation does not occur in the presence of physiological levels of the double mutants PIMKenadba and PIMKenadba-myc, in contrast to our findings with the single mutants PIMDbamyc, PIMDbamyc and PIMKenadba-myc.

**Discussion**

Sister chromatid separation during the metaphase-to-anaphase transition in *Drosophila* is strictly dependent on accumulation of the securin PIM (Stratmann and Lehner, 1996). Because we do not understand why *Drosophila* PIM has to accumulate to allow sister chromatid separation, we cannot evaluate whether PIM versions with mutations in both the D- and the KEN-box (PIMKenadba and PIMKenadba-myc) are still capable of providing this positive function. However, we emphasize that these versions still bind normally to the known partners SSE and THR.

Mutations in either the D- or the KEN-box result in significant stabilization of PIM protein during mitosis. Neither the D- nor the KEN-box, therefore, are sufficient for normal degradation during the embryonic cell divisions in *Drosophila*. Similar observations have been described for human securin (Hagting et al., 2002; Zur and Brandeis, 2001). However, in contrast to *Drosophila*, mitotic degradation of human securin still occurs quite effectively when either only the D- or the KEN-box is intact. The D- and KEN-boxes of *Drosophila* PIM, therefore, might function less independently than the corresponding motifs in human securin. Eventually, the understanding of D- and KEN-box function will require structural analyses of their interactions with Fizzy/Cdc20 and Fizzy-related/Cdh1, which recruit proteins with these degradation signals to the APC/C (Burton and Solomon, 2001; Pfleger et al., 2001). Fizzy and Fizzy-related are clearly both involved in PIM degradation, at least indirectly, as PIM is stabilized in both fizzy and fizzy-related mutants (Leismann et al., 2000) (data not shown).

Under the assumption that PIMKenadba and PIMKenadba-myc are still capable of providing the positive PIM function, our results with these stabilized mutants suggest that PIM must be degraded during each and every mitosis to allow sister chromatid separation. Although not detectable by confocal microscopy, the single mutants PIMDbamyc and PIMKenadba might not be completely stable in mitosis. After low-level expression in pim-mutant embryos, residual mitotic degradation of single-mutant proteins might free some separase activity sufficient for sister chromatid separation. Similar results have been observed with the fission yeast securin Cut2, which is completely stabilized in a *Xenopus* extract destruction assay by mutations in either of the two D-boxes, and yet, low-level expression of single- but not double-mutant proteins is able to complement growth of cut2-ts strains at the restrictive temperature (Funabiki et al., 1997). We emphasize that even in wild-type cells, mitotic PIM degradation appears to be far from complete, and it can be speculated that it is the PIM protein of a special pool of separase complexes that is more efficiently degraded, perhaps on kinetochores or during transport on spindles towards kinetochores. At high expression levels of PIM with or without single mutations, free excess of this securin might rapidly re-associate and inhibit the activated separase, resulting in the observed block of sister chromatid separation.

Our results also point to alternative pathways that might regulate separase activity and sister chromatid separation.
independently of PIM degradation. As in yeast, the success of mitosis in cells with reduced separase function is dependent on Polo kinase in Drosophila embryos. Moreover, as expression of mitotically stabilized Cyclin A versions result in a metaphase delay without inhibiting PIM degradation, Cyclin A appears to contribute independently of PIM to the inhibition of premature sister chromatid separation. Even though it remains to be analyzed whether Polo kinase and Cyclin A-Cdk1 act during Drosophila divisions as proposed for Polo homologs (Alexandru et al., 2001) and vertebrate Cyclin B-Cdk1 (Stemmann et al., 2001), our results indicate that separase and sister chromatid separation are unlikely to be regulated exclusively by securin degradation.

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