Drosophila Separase is required for sister chromatid separation and binds to PIM and THR

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Drosophila PIM and THR are required for sister chromatid separation in mitosis and associate in vivo. Neither of these two proteins shares significant sequence similarity with known proteins. However, PIM has functional similarities with securin proteins. Like securin, PIM is degraded at the metaphase-to-anaphase transition and this degradation is required for sister chromatid separation. Securin binds and inhibits separase, a conserved cysteine endoprotease. Proteolysis of securin at the metaphase-to-anaphase transition activates separase, which degrades a conserved cohesin subunit, thereby allowing sister chromatid separation. To address whether PIM regulates separase activity or functions with THR in a distinct pathway, we have characterized a *Drosophila* separase homolog (SSE). SSE is an unusual member of the separase family. SSE is only about one-third the size of other separases and has a diverged endoprotease domain. However, our genetic analyses show that SSE is essential and required for sister chromatid separation during mitosis. Moreover, we show that SSE associates with both PIM and THR. Although our work shows that separase is required for sister chromatid separation in higher eukaryotes, in addition, it also indicates that the regulatory proteins have diverged to a surprising degree, particularly in *Drosophila*.

[Key Words: Mitosis; sister chromatid separation; securin; separase; *pimples*; *three rows*]

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A distinct hallmark of eukaryotes is their use of a microtubule-based spindle to segregate their genetic information onto two daughter cells during cell division. This mechanism necessitates regulated sister chromatid cohesion. Sister chromatids have to remain in association after DNA replication so that they can be recognized as such and oriented in the mitotic spindle during prometaphase. However, after their correct bipolar orientation in the mitotic spindle, cohesion has to be resolved so that sister chromatids can be segregated to opposite poles during anaphase.

Because regulated sister chromatid cohesion is an essential element of eukaryotic cell divisions, its molecular basis is expected to be conserved. Most of our current mechanistic understanding of how sister chromatid cohesion is established during S phase, maintained until the end of metaphase, and resolved at the onset of anaphase, has been obtained with yeast (for recent reviews, see Dej and Orr-Weaver 2000; Hirano 2000; Koshland and Guacci 2000; Nasmyth et al. 2000; Yanagida 2000).

In budding yeast, the cohesin protein complex is assembled on chromatin during S phase and is required for

holding sister chromatids together until the end of metaphase (Guacci et al. 1997; Michaelis et al. 1997; Uhlmann and Nasmyth 1998; Skibbens et al. 1999; Uhlmann et al. 2000). At the metaphase-to-anaphase transition, the Scc1p/Mcd1p subunit of the cohesin complex is proteolytically cleaved, which allows sister chromatid segregation during anaphase. The Esp1p protease, which is responsible for Scc1p cleavage, is kept inactive until metaphase by an inhibitory subunit, the Pds1p anaphase inhibitor. The timely activation of Esp1p at the onset of anaphase results from degradation of Pds1p by the anaphase-promoting complex/cyclosome (APC/C)-dependent pathway (Ciosk et al. 1998). The APC/C acts as an ubiquitin ligase that is regulated by the spindle assembly checkpoint (for review, see Zachariae and Nasmyth 1999).

Observations in other species support the notion that the mechanisms controlling sister chromatid cohesion are evolutionarily conserved. In particular, analyses in fission yeast and initial studies in vertebrates have given analogous results as described above for budding yeast. Proteins homologous to Scc1p have been shown to become cleaved at the metaphase-to-anaphase transition (Tomonaga et al. 2000; Waizenegger et al. 2000). Moreover, the Esp1p-like proteases (named separases) are all regulated by inhibitory protein subunits (named securins), which are degraded by the APC/C pathway at

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the end of metaphase (Funabiki et al. 1996; Zou et al. 1999).

Beyond these similarities, however, higher eukaryotes have evolved specific regulatory variations and additions. The majority of the cohesin complexes is dissociated from vertebrate chromosomes already during prophase and independent of separase activity (Losada et al. 1998; Sumara et al. 2000). This early dissociation of cohesin during prophase might be required to allow chromosome condensation, which is far more extensive in higher eukaryotes than in budding yeast. The minor amount of cohesin, which remains on chromosomes until the onset of anaphase, appears to be concentrated in the centromeric region (Waizenegger et al. 2000; Warren et al. 2000). In *Drosophila*, the protein MEI-S332 has been suggested to mediate this maintenance of cohesin specifically in the centromeric region (Tang et al. 1998). Whereas the dissociation of these remaining cohesin complexes from HeLa chromosomes has been shown to be accompanied by Scc1p cleavage that can be induced in vitro by immunoprecipitated activated separase (Waizenegger et al. 2000), a separase requirement for sister chromatid separation in higher eukaryotes has not yet been demonstrated directly. Moreover, the securin proteins that have been identified in budding yeast (Pds1p), fission yeast (Cut2p), and vertebrates (PTTG) do not share significant sequence similarity except for the presence of D-boxes, which target the proteins for APC/C-dependent mitotic degradation. This mitotic destruction appears to be required for sister separation in vertebrates also (Zou et al. 1999). However, it remains a possibility that securins have evolved to regulate proteins in addition to separase.

Our analysis of the two *Drosophila* genes, *three rows* (*thr*) and *pimples* (*pim*), which do not share significant similarity with known genes, has indicated that at least in *Drosophila*, sister chromatid separation involves distinct, nonconserved components also. Loss of *pim* and *thr* function completely blocks the separation of sister chromatids, primarily within the centromeric region, but it does not inhibit cell cycle progression (D'Andrea et al. 1993; Philp et al. 1993; Stratmann and Lehner 1996). After each cell cycle, therefore, a doubled number of chromosome arms emanating from a common centromeric region is displayed in these mutants during mitosis. The indistinguishable mutant phenotypes argued for a common function. Consistently, PIM and THR have been found to form a complex in vivo (Leismann et al. 2000).

Despite the lack of significant sequence similarities with known proteins, PIM has been shown to have clear functional similarities with securin proteins. PIM is degraded during mitosis via the APC/C pathway, and a nondegradable PIM mutant as well as high levels of wild-type PIM inhibit sister chromatid separation during mitosis (Leismann et al. 2000). Therefore, PIM might also bind and regulate a *Drosophila* separase. However, PIM is known to bind to THR, which clearly does not have the structural features of separases. PIM and THR, therefore, might either both regulate a *Drosophila* separase or

function in a distinct pathway. To address this issue, we have identified and characterized a *Drosophila* separase.

Here, we report that PIM and THR both bind to the *Drosophila* separase homolog SSE, which is required for sister chromatid separation. Interestingly, the *Drosophila* SSE sequence is highly diverged, lacking some features conserved in homologs from trypanosomatids to vertebrates. Our results show, therefore, that the decisive role of separase in the control of sister chromatid separation has been conserved during evolution of higher eukaryotes. Nevertheless, the surprising degree of divergence of separase and regulatory proteins indicates that regulation is highly evolved, particularly in *Drosophila*.

Results

Drosophila SSE is a distant separase family member

To evaluate whether PIM binds to a separase, we first searched in the genome sequence for a *Drosophila* homolog. We identified a single gene (*CG10583*) with significant similarity to the known separase genes. Comparison of our cDNA and genomic sequences revealed the structure of this gene, which will be designated as *Separase* (*Sse*) (Fig. 1A). The predicted protein product (SSE) has 634 amino acids and a calculated molecular mass of 72.9 kD.

Thus, SSE appears to be much smaller than separase homologs from other organisms, which range in size between 150 and 230 kD. Several findings support our size prediction. The *Sse* upstream region has virtually no coding potential and three stop codons are present in frame upstream of and close to the presumptive translational start in several independent cDNAs. One of these short cDNAs prevents the phenotype resulting from a complete loss of *Sse* function when expressed in *Sse* mutants (see below). Moreover, antibodies against SSE detect a protein with an apparent molecular mass of ~75 kD (see below).

The sequence similarity of separase homologs is restricted to the C-terminal part. This domain includes two invariant residues, an histidine and a cysteine, surrounded by regions typically found in cysteine proteases of the CD clan (Uhlmann et al. 2000). This presumptive catalytic dyad is also present in SSE. However, two additional sequence blocks within this C-terminal domain, which are highly conserved among separase family members, are divergent in SSE (Fig. 1B). As a consequence, SSE is the most distant member in the separase family tree (Fig. 1C).

SSE is required for sister chromatid separation

To assess *Sse* function, we generated mutant alleles. Starting with a P-element insertion within a neighboring gene (*CG17334*), we isolated a small deficiency [*Df(3L)SseA*] by male recombination (Fig. 1A). A molecular breakpoint analysis indicated that this deficiency deletes *Sse* and parts of the neighboring genes, *CG17334*

Figure 1. The separase homolog of *D. melanogaster*. (A) Genomic organization of the *Sse* locus with the adjacent genes, *still life* (*sif*) and *CG17334*. Boxes indicate exon sequences. Open and solid boxes denote untranslated and translated regions, respectively. Arrows indicate the orientation of transcription. A triangle marks the insertion site of the P-element EP(3)0915. The extent of the deficiency *Df* (3*L*)*SseA* isolated as male recombinant after mobilizing EP(3)0915 is shown by a black bar. The genomic *Sse* rescue fragment (*gSse*) is indicated by a hatched bar. An asterisk marks the position of the 4-bp deletion in *Sse*^{13m}. (B) Sequence alignment of the C-terminal regions of various separase homologs. *C. elegans* 1 and *C. elegans* 2 extend for 116 and 104 amino acids beyond the shown region, respectively. Arrows indicate the invariant histidine and cysteine residues representing the catalytic dyad of CD-clan proteases. Hatched rectangles denote regions poorly conserved in *D. melanogaster* SSE. The arrowhead points to the position of the 4-bp deletion in the allele *Sse*^{13m}. Asterisks indicate cases in which only partial sequences were available. (C) Dendrogram of clustering relationships among the sequences shown in B.

and *sif*. Interestingly, this deficiency failed to complement the recessive lethal mutation *l(3)13m-281*. This mutation has been shown to affect mitotically proliferating cells in a way that might be expected for a mutation in *Sse* (Gatti and Baker 1989; see below). Sequence analysis of the *Sse* region isolated from the *l(3)13m-281* chromosome revealed a deletion of four bases between the positions encoding the invariant histidine and cysteine residues, resulting in a frame shift followed by a premature translational stop (Fig. 1B). The product encoded by *l(3)13m-281*, therefore, is expected to lack part of the conserved C-terminal separase domain, including the invariant cysteine residue believed to be involved in catalysis. Thus, *l(3)13m-281* is presumably a null allele of *Sse* and will be designated as *Sse*^{13m} in the following.

Previous phenotypic analyses (Gatti and Baker 1989) had indicated that *Sse*^{13m} homozygotes die at the larval-pupal boundary. In *Sse*^{13m} larvae at third instar wandering stage, imaginal discs were found to be abnormally small and the mitotic index in the brains to be strongly reduced. Moreover, the few mitotic figures observed in larval brain squashes were reported to contain endoreduplicated chromosomes with bundles of two, four, or eight times the normal number of sister chromatid arms. Our phenotypic analyses with *Sse*^{13m}/*Df(3L)SseA* and *Df(3L)SseA/Df(3L)SseA* larvae confirmed these findings. These larvae showed identical phenotypes to *Sse*^{13m} homozygotes.

In embryos, we were unable to detect mitotic abnormalities in *Sse* mutants, suggesting that the maternal *Sse* contribution is sufficient to allow normal cell divisions throughout embryogenesis. However, in brains of *Sse*^{13m}/*Df(3L)SseA* early second instar larvae, the number of phosphorylated histone H3 (PH3)-positive cells (234 in 7 brains) was reduced to ~40% of the number of PH3-positive cells in sibling brains (238 in 3 brains). PH3 is an excellent marker for cells during the early mitotic stages until anaphase. Whereas mutants and siblings showed very similar fractions of prophase and metaphase figures in these PH3-positive cells, anaphase figures were almost completely absent from mutant brains (1 anaphase figure in 234 PH3-positive cells compared with 20 anaphase figures in 238 PH3-positive cells of sibling brains). We conclude, therefore, that the maternal *Sse* contribution is no longer sufficient to support normal anaphase when mitotic proliferation of postembryonic brain neuroblasts resumes during the second instar stage, although entry into mitosis still occurs. If *Sse* mutants specifically fail in separating sister chromatids, a rapid accumulation of chromosomes with twice the number of normal arms (diplo-chromosomes), as well as more extensively endoreduplicated chromosomes, is expected. Analysis of squash preparations of second instar larval brains fully confirmed this expectation. A total of 95% of the mitotic cells contained either diplo-chromosomes (Fig. 2K) or more extensively endoreduplicated chromosomes (Fig. 2L), whereas none of the mitotic cells of siblings contained endoreduplicated chromosomes (Fig. 2M). In addition to the presence of endoreduplicated chromosomes, mitotic figures in mutants frequently

contained aneuploid numbers of chromosomes. However, we never observed polyploid figures with normal chromosomes, indicating that *Sse* mutants have no primary defect in cytokinesis.

In third instar wandering stage larvae, *Sse* mutant brains contained very few PH3-positive cells (<5% of the number of PH3-positive cells in sibling brains; Fig. 2; data not shown). The PH3-positive cells were abnormally large and had very high levels of DNA (Fig. 2C,D). We could not detect any normal anaphase and telophase figures in mutants, and squashes revealed highly abnormal and severely endoreduplicated chromosomes, as described previously (Gatti and Baker 1989). The progressive depletion of mitotic cells with increasing age suggests that most of the mitotically proliferating neuroblasts eventually die in *Sse* mutants, presumably as a consequence of hyper- and aneuploidy.

To show that the endoreduplicated unseparated chromosomes in *Sse*^{13m} larvae result from the loss of *Sse*⁺ function and not from linked second-site mutations, we performed rescue experiments. One copy of the *gSse* transgene with a 10-kb genomic DNA fragment encompassing only the *Sse* gene (Fig. 1A) rescued the phenotype of *Sse*^{13m} homozygotes to full viability and fertility. Moreover, the cytological defects of *Sse*^{13m} mutants could be prevented by a combination of *da-GAL4* and *UAS-HA-Sse* (Fig. 2B,E–J). All our findings show that *Sse* is an essential gene required for sister chromatid separation during mitosis. Moreover, the *Sse* gene product is likely to function as a cysteine protease, as we found that *da-GAL4* mediated expression of *UAS-HA-Sse*^{C497S}, in which the codon for the putative catalytic cysteine residue was mutated into a serine codon was unable to rescue *Sse* mutants.

Sse forms complexes with PIM and THR

To investigate whether the putative *Drosophila* securin PIM can bind to SSE, we used the yeast two-hybrid system. We observed a strong interaction of PIM and SSE (Fig. 3). Interestingly, we found that a mutant PIM protein with a small internal deletion (amino acids 110–114) failed to bind to SSE. This deletion was identified in *pim*², a *Drosophila* allele that results in an amorphic phenotype (Stratmann and Lehner 1996). Whereas PIM² failed to interact with SSE, it bound to a THR fragment (amino acids 1–933) just like wild-type PIM. The deletion of amino acids 110–114 therefore abolishes specifically the binding to SSE and does not result in destabilization or complete misfolding of the mutant PIM² protein. We conclude that the interaction of PIM and SSE is likely to be functionally significant.

The behavior of PIM² suggested that different domains of PIM mediate the binding to SSE and THR. To evaluate this notion, we analyzed PIM fragments in additional two-hybrid experiments. Like other securin proteins, PIM is composed of a basic N-terminal and an acidic C-terminal domain. The N-terminal domain was found to interact with SSE, whereas an interaction with THR 1–933 was barely detectable (Fig. 3). Conversely, the C-

Figure 2. Mutations in *Sse* affect mitotic cells. Whole brains (A,B) from third instar larvae with the genotype *Sse*^{13m}/*Df*(3L)*SseA* (A) or *UAS-HA-Sse* I.1/+; *Sse*^{13m}/*Df*(3L)*SseA*, *da-GAL4* (B) were immunostained for DNA (D,F,H,I) and PH3 (A–C,E,G,I). In mutant brains of third instar larvae, the PH3-positive cells contained large masses of condensed DNA (C,D) and anaphase figures could not be observed, whereas in the brains of larvae rescued by the HA-tagged *Sse*^{*} transgene prophase cells (E,F), metaphase cells (G,H) and cells in anaphase (I,J) could be detected. Squashes of brains from second instar larvae with the genotype *Sse*^{13m}/*Df*(3L)*SseA* (K,L) or of brains from balanced siblings (M) were stained with Hoechst 33258 to visualize DNA. Diplo-chromosomes (K) and a quadruple-chromosome (L, arrowhead) are visible. The asterisk indicates the balancer chromosome *TM3, Ser, Act5c-GFP* in M. (br) Brain; (vg) ventral ganglion; (ol) optic lobe.

terminal domain interacted with THR 1–933, but not with SSE. These results strongly indicate that SSE and THR bind to different PIM domains.

Experiments with SSE fragments indicated that PIM binds to the N-terminal regions of SSE (Fig. 3). The interaction of PIM with full-length SSE or with a SSE fragment comprising amino acids 1–467 appeared to be stronger than with a shorter SSE fragment (amino acids 1–247). We assume that region 1–247 of SSE is sufficient for PIM binding, but the region 248–467 of SSE further strengthens this interaction. Clearly, the conserved C-terminal region of SSE is not required for the interaction with PIM.

Experiments with THR fragments indicated that PIM binds to the N-terminal region of THR. Region 1–476 of THR was found to be sufficient for PIM binding (Fig. 3). We note that we were unable to observe an interaction between PIM and the full-length THR protein. The considerable size of full-length THR 1–1379 might preclude expression of sufficiently high levels and/or entry into the nucleus.

Finally, we tested for a direct interaction between THR and SSE. The THR fragment 1–933 was found to interact with full-length SSE, SSE 1–247, and SSE 1–467. These results therefore raise the possibility that THR and PIM bind to the same region of SSE and thus might be competing in vivo. Moreover, the interactions between THR and SSE appeared to be weaker than the interactions between PIM and SSE, as in the former case, only one of the two reporter genes was activated. We were not able to define the region in THR that mediates SSE binding in more detail, because the shorter constructs THR 1–476, THR 208–933, and THR 477–933 failed to bind to SSE (data not shown).

Taken together, the results of our two-hybrid experiments show that all three proteins, PIM, THR, and SSE can interact independently with each other.

To confirm that PIM, THR, and SSE interact in vivo, we performed coimmunoprecipitation experiments. Two antibodies against SSE were raised and affinity purified. In embryo extracts, these antibodies detected a prominent band at 75 kD, which comigrated with SSE

Figure 3. Interactions of PIM, THR, and SSE in the yeast two-hybrid system. Various deletion constructs of PIM (open bars), THR (light gray bars), and SSE (dark gray bars) were constructed as translational fusions with the GAL4 activation domain (AD-fusions) or with the GAL4 DNA-binding domain (BD-fusions). Interactions were scored as ++, if both GAL4 responsive reporter genes were activated by the respective combination and as - if no marker gene was activated. Activation of one reporter gene was scored as +.

translated in vitro (data not shown). This band was also detected in immunoprecipitates that were isolated with an anti-myc antibody from extracts of embryos expressing PIM or THR fused with myc epitope tags (Fig. 4A). Control experiments indicated that coimmunoprecipitation of SSE with PIM-myc and THR-myc is specific. This specific association was also observed when the antibodies against SSE were used for immunoprecipitation followed by immunoblotting with anti-myc (Fig. 4B). Taken together, these results clearly show that SSE associates in vivo with PIM and THR.

The interaction between THR and SSE observed in the yeast two-hybrid experiments was weak, raising the possibility that it might not be sufficiently strong to allow formation of THR-SSE complexes in vivo. PIM might therefore be required to bring THR and SSE together. To evaluate whether THR-SSE complexes can be formed in the absence of PIM in vivo, we expressed *gUAS-thr-myc* and *UAS-HA-Sse* in late embryos when endogenous PIM and THR levels are very low (D'Andrea et al. 1993; Stratmann and Lehner 1996; H. Jäger, A. Herzig, C.F. Lehner, and S. Heidmann, unpubl.). After precipitation of HA-SSE with an anti-HA antibody, we detected THR-myc as well as minor amounts of PIM in the immuno-

precipitates (Fig. 5A). Quantitative immunoblotting revealed that THR-myc was present in an at least fivefold molar excess over PIM in these immunoprecipitates (Fig. 5A). Because THR does not form oligomers (Leismann et al. 2000), we conclude that THR and SSE can associate in the absence of PIM.

To investigate whether PIM is able to bind to SSE in the absence of THR in vivo, we performed a complementary experiment by expressing *UAS-HA-Sse* and *UAS-pim-myc* in late embryos (Fig. 5B). Surprisingly, in contrast to the results of the yeast two-hybrid experiments, only minor amounts of PIM-myc could be coprecipitated with SSE (Fig. 5B, lanes SP). However, simultaneous co-expression of *gUAS-thr-myc*, *UAS-HA-Sse*, and *UAS-pim-myc* resulted in the recovery of significant PIM levels (Fig. 5B, lanes SPT). We estimate that <10% of PIM is precipitated in the absence of THR when compared with the amount precipitated in the presence of THR. Significantly, PIM²-myc does not form a stable complex with HA-SSE, even in the presence of THR (Fig. 5B, lanes SP2T). These results indicate that PIM, THR, and SSE form a trimeric complex in vivo, and that SSE is not sufficient to recruit PIM in the absence of THR. This notion was supported by the behavior of a THR deletion

Figure 4. SSE forms complexes with PIM and THR in vivo. Extracts (extract) were prepared from embryos expressing either no transgene (*w¹*), *gpim-myc* (*pim-myc*), *gthr-myc* (*thr-myc*), or *Cdk1-myc* (*Cdk1-myc*). These extracts, as well as anti-myc (IP anti-myc) (A) or anti-SSE (IP anti-SSE) and rabbit nonimmune IgG (IP ni-IgG) (B) immunoprecipitates isolated from these extracts, were analyzed by immunoblotting with antibodies against SSE (top), the myc-epitope (middle), or Cyclin B (bottom). (*) Signal caused by IgG heavy chains of the anti-myc antibodies.

mutant (THR 445–1379-myc), which was lacking the region required for the two-hybrid interaction with PIM. When this mutant protein was expressed from a transgene under the control of the normal *thr* regulatory region and immunoprecipitated from embryo extracts, we detected almost no coimmunoprecipitation of PIM (Fig. 5C, lane 6). However, SSE was readily detected in the immunoprecipitates, confirming that THR and SSE can form a complex without PIM. Quantitative immunoblotting experiments indicated that SSE associates with THR 445–1379-myc with at least 50% efficiency when compared with its binding to full-length THR, whereas binding of PIM to THR 445–1379-myc was reduced to <5% (data not shown). On the basis of the results of our two-hybrid experiments, in which SSE interacts strongly with PIM, the SSE present in the THR 445–1379-myc immunoprecipitates would be expected to result in coimmunoprecipitation of PIM as well. However, the absence of PIM in the immunoprecipitates suggests that PIM cannot join SSE and THR in a trimeric complex when it is not bound by THR. Control experiments with THR-myc versions that contained the N-terminal PIM-binding region showed that coimmunoprecipitation of PIM along with SSE can be readily detected in these cases (Fig. 5C, lanes 3,4).

THR 1–478-myc did not form complexes with PIM in vivo, whereas THR 1–476 and PIM associate in our yeast two-hybrid experiments (Fig. 3). This discrepancy might result from different positions of the fused tags. Whereas in the two-hybrid experiments, the GAL4-binding domain was fused to the N terminus, the 10 myc epitope tags were fused to the C terminus of the THR fragment analyzed in *Drosophila* embryos. The C-terminal myc

tags, therefore, might interfere with PIM binding to THR 1–478-myc.

The C-terminal fragment THR 932–1379-myc did not bind PIM or SSE, as expected from the yeast two-hybrid analysis. This fragment was present at very low levels in the embryo extracts as determined for four independent transgenic lines. This low abundance is presumably due to protein instability, as all genomic THR constructs contained identical 5'- and 3'-noncoding regulatory sequences. We also did not detect coimmunoprecipitation of PIM or SSE when loading was adjusted to compensate for the low abundance of THR 932–1379-myc (data not shown).

In summary, the behavior of THR fragments expressed in embryos during the proliferative stages extended our findings resulting from yeast two-hybrid experiments. We observe that the THR–SSE interaction is not necessarily mediated by PIM. Furthermore, our results show that the binding of PIM to SSE requires THR, strongly suggesting the existence of trimeric PIM–THR–SSE complexes in vivo.

Discussion

Our study shows that *Drosophila* Separase (SSE) is required for sister chromatid separation during mitosis, as shown previously in lower eukaryotes. However, we also show that this essential protease and its regulatory subunits have evolved rapidly, particularly in *Drosophila*, presumably to allow for more sophisticated regulation.

Drosophila SSE contains a C-terminal region with significant similarity to a cysteine endoprotease domain,

Figure 5. PIM, THR, and SSE form a trimeric complex. (A) An extract was prepared from embryos 12–15 h after egg deposition expressing *UAS-thr-myc* and *UAS-HA-Sse* under the control of *da-GAL4*, and was used for anti-HA immunoprecipitation. The precipitate was analyzed by immunoblotting with anti-HA (HA-SSE), anti-THR (THR-myc), and anti-PIM (PIM) antibodies (left). For quantitation, a dilution series of a mixture containing bacterially expressed full-length PIM and a THR fragment was loaded on the same gels (right). The corresponding left and right panels show identical exposures of the same blot. The amount of the proteins is given in fmole. (B) Extracts were prepared and analyzed as in A from embryos coexpressing either *UAS-HA-Sse*, *UAS-pim-myc* and *gUAS-thr-myc* (SPT), *UAS-HA-Sse* and *UAS-pim-myc* (SP), or *UAS-HA-Sse*, *UAS-pim²-myc* and *gUAS-thr-myc* (SP2T). For quantification of precipitated proteins, a dilution series of the anti-HA immunoprecipitate from the SPT extract was loaded onto the same gel. The numbers represent percentage values. (C) Extracts were prepared from embryos expressing either no transgene (*w¹*), *Cdk1-myc* (*Cdk1-myc*), *gthr-myc* (*thr 1-1379-myc*), *thr 1-930-myc* (*thr 1-930-myc*), *gthr 1-478-myc* (*thr 1-478-myc*), *gthr 445-1379-myc* (*thr 445-1379-myc*), or *gthr 932-1379-myc* (*thr 932-1379-myc*), and were used for the isolation of anti-myc immunoprecipitates, which were analyzed by immunoblotting with antibodies against the myc-epitope (top), PIM (middle), or SSE (bottom).

which is found in the C-terminal region of all separases. Whereas this SSE region includes an invariant histidine as well as the putative catalytic cysteine residue that we show to be required for function, it diverges significantly from the other separases in two additional conserved sequence blocks. One of these blocks is functionally important in budding yeast and has been proposed to represent a Ca^{2+} -binding motif (Uzawa et al. 1990; Jensen et al. 2001). The *Drosophila* SSE sequence does not contain this Ca^{2+} -binding motif. If separase activity is regulated by binding of Ca^{2+} to the conserved region in the C terminus, then *Drosophila* SSE activity may be regulated by Ca^{2+} binding to (an) accessory protein(s). Another striking difference is the smaller size of SSE when compared with other separases. However, our genetic analysis clearly shows that SSE is required for sister chromatid separation.

Mutants, which cannot express functional *Sse* zygotically, complete embryogenesis presumably using the

maternal *Sse* contribution. During the larval stages, however, the mitotically proliferating imaginal cells are specifically affected in these mutants. Our cytological analysis of larval brains confirmed the findings first described by Gatti and Baker (1989) for the mutant *l(3)13m-281*, which we show here to reflect a complete loss of zygotic *Sse* function. Mitotic cells in *Sse* mutant larvae contain endoreduplicated chromosomes with supernumerary arms all connected primarily in a centromeric region. Such chromosomes are also observed in embryos with mutations in the genes *pim* or *thr*, which are required for sister chromatid separation (D'Andrea et al. 1993; Stratmann and Lehner 1996), and which encode proteins that bind to SSE.

It is conceivable that in *pim* and *thr* mutants, SSE is destabilized, resulting in the failure to separate sister chromatids, which would explain the requirement of PIM and THR function for sister chromatid separation. However, Western blot analyses of extracts prepared

from *pim* and *thr* mutant embryos show that SSE is still present in these mutants. We favor the possibility that in these mutants a regulatory function is affected, resulting in the absence of SSE activity.

The *pim*, *thr*, and *Sse* mutant phenotypes argue that SSE activity is required primarily for sister chromatid separation within the centromeric region. In contrast, entry into mitosis, including assembly of a mitotic spindle, chromosome condensation, and congression into a metaphase plate do not appear to depend on SSE activity. Moreover, the degradation of mitotic cyclins and exit from mitosis (chromosome decondensation, spindle disassembly, nuclear envelope formation) appear to occur with normal kinetics, even though sister chromatids fail to be separated and segregated to the spindle poles (D'Andrea et al. 1993; Philp et al. 1993; Stratmann and Lehner 1996). The defects in these mutants, therefore, do not appear to be detected by an efficient checkpoint mechanism comparable with the mitotic exit network of budding yeast (Cohen-Fix and Koshland 1999; Tinker-Kulberg and Morgan 1999). Cytokinesis is also attempted in *pim* and *thr* mutants, but cannot be completed. Whether the failure to complete cytokinesis is simply a consequence of the presence of nonseparated chromosomes within the equatorial plane or whether SSE activity is directly involved in cytokinesis, is not known and difficult to resolve. Another unresolved and difficult issue at present is the potential involvement of SSE activity in the control of anaphase spindle dynamics, which is suggested by analyses in yeast (Kumada et al. 1998; Uhlmann et al. 2000; Jensen et al. 2001).

The early onset of phenotypic abnormalities in *pim* and *thr* mutants reflects the rapid disappearance of maternally contributed wild-type products. This disappearance is rapid because PIM and THR are both partially degraded during exit from mitosis (Stratmann and Lehner 1996; Leismann et al. 2000; A. Herzig, C.F. Lehner, and S. Heidmann, unpubl.). In contrast, the late onset of phenotypic abnormalities in *Sse* mutants indicates that SSE is a stable protein. In fact, we have been unable to detect SSE degradation during mitosis by immunoblotting experiments. Unfortunately, our antibodies do not allow SSE detection by immunofluorescence, which might be more sensitive and could also provide information on subcellular localization. Nevertheless, our present evidence strongly argues against the idea that SSE

activity is regulated by SSE degradation. Partial cleavage of human separase has been observed during exit from mitosis, but its significance is not yet known (Waizenegger et al. 2000). This mitotic cleavage of human separase occurs upstream of the conserved endoprotease domain and might therefore represent a mode of regulation that is not conserved.

During the divisions of *Drosophila* embryogenesis, we have not only failed to detect cleavage of SSE but also of the *Drosophila* homolog of the yeast cohesin subunit Scc1p (A. Herzig, C.F. Lehner, and S. Heidmann, unpubl.). As in vertebrate cells, most of the *Drosophila* Scc1p homolog has also been shown to dissociate from chromosomes already during prophase (Warren et al. 2000). However, some can be visualized in the centromeric region of metaphase chromosomes until the onset of anaphase. We assume that cleavage by SSE is responsible for the subsequent disappearance of this centromeric pool and that the sensitivity of our immunoblotting experiments is insufficient to detect the cleavage of this minor fraction. So far, it has been impossible to show SSE protease activity directly.

The fact that the majority of Scc1 is clearly not cleaved during mitosis in higher eukaryotes indicates that the regulation of SSE activity within the cell is presumably complex and targeted to the centromeric region. Although much further work remains to be done to understand SSE regulation in detail, some insights can be derived from our analysis of the interactions of SSE with PIM and THR (Fig. 6). We show that PIM associates with SSE in vivo. This result further supports the role of PIM as a *Drosophila* securin. According to two-hybrid experiments, PIM binds to the N-terminal region of SSE. The yeast securins also interact with the N-terminal regions of the separases (Kumada et al. 1998; Jensen et al. 2001). Surprisingly, however, neither the securins nor the N-terminal separase regions display sequence conservation (Uzawa et al. 1990; Zou et al. 1999). Equally surprising is the finding that THR is required for the association of PIM with SSE. Moreover, for efficient complex formation, PIM also requires to contact SSE, because PIM² is not efficiently incorporated in a trimeric complex, despite its ability to bind to THR. We assume therefore that a trimeric PIM–THR–SSE complex, as schematically illustrated in Figure 6, is formed during interphase and present during entry into mitosis.

Figure 6. A model for the PIM–THR–SSE complex. In interphase cells, a trimeric complex composed of PIM (white), THR (light gray), and SSE (dark gray) is assembled, in which SSE is kept inactive. At the end of metaphase, PIM is degraded in an APC-dependent manner. This enables an activating contact between THR and SSE, allowing SSE to cleave its target, the *Drosophila* Scc1p cohesin subunit homolog, resulting in sister chromatid separation. The C terminus of THR is shown in association with the N terminus of SSE (broken lines) to illustrate the possibility that THR corresponds to the N terminus of separases from other organisms.

Although the analysis of the binding site for PIM in SSE is difficult in vivo because of the dependency on THR, our two-hybrid experiments indicate that PIM and THR might bind to the same region within SSE. Our model of the trimeric complex takes this into account by showing that PIM, and not THR, contacts SSE at its N terminus, and THR can only bind to this region of SSE after PIM has been degraded. However, PIM and THR interact with each other by use of binding sites that are distinct from those that contact SSE. SSE activity might be inhibited in the trimeric complexes because PIM prevents THR from providing an activating contact to SSE by competitive binding within the same SSE region. The mitotic degradation of PIM might then give way to the activating THR–SSE interaction, resulting in SSE activity at the onset of anaphase (Fig. 6).

We note some discrepancies between our two-hybrid results and those obtained in vivo by coimmunoprecipitation. Particularly, the strong interaction between PIM and SSE observed in yeast contrasts with the low abundance of PIM in SSE immunoprecipitates when almost no THR is present. We speculate that additional levels of regulation of complex formation may be present in the *Drosophila* embryo, which are lacking in yeast. Nevertheless, the PIM–SSE complex formation observed in yeast is likely to reflect a significant interaction, as PIM² does not associate with SSE in yeast, as it fails to do in the embryo.

We have speculated previously that an ancient separase gene might have broken into two genes during the evolution of *Drosophila* (Leisemann et al. 2000). Accordingly, THR might correspond to the nonconserved N terminus and SSE to the conserved C-terminal endoprotease domain of the other separase proteins. This hypothesis predicts that the longer separase proteins might have two binding sites for securin. The published interaction studies do not rigorously exclude this possibility. The fact that the C-terminal acidic region of the *Drosophila* securin PIM interacts with THR, whereas the C-terminal acidic regions of yeast securins interact with the N-terminal nonconserved separase regions (Kumada et al. 1998; Jensen et al. 2001) is consistent with this hypothesis.

We emphasize that our suggestions (Fig. 6) are speculative. Moreover, important issues are not resolved by these hypotheses. For instance, they do not address why PIM is required for sister chromatid separation. PIM might be responsible for targeting SSE to specific subcellular locations, just like the yeast securins are required for spindle association or nuclear localization of the associated separases (Kumada et al. 1998; Jensen et al. 2001). Furthermore, if in fact a complex pathway controls SSE activation, the inactivation process is also an important issue that needs to be addressed. The requirement for an especially efficient SSE inactivation during the extremely rapid syncytial division cycles at the onset of embryogenesis might explain the particular high divergence of SSE and its regulators in *Drosophila*. This mechanism of regulation might be typical for insects and SSE might therefore be an interesting target for insecticide compounds.

Materials and methods

Fly stocks

For expression of UAS transgenes, we used *da-GAL4* G32 (Wodarz et al. 1995) and *arm-GAL4* (Sanson et al. 1996). *UAS-Cdk1-myc* and *UAS-pim-myc*, as well as *gpim-myc* and *gthr-myc* lines, which allow expression of myc-tagged products under the control of the normal genomic regulatory regions, have been described previously (Stratmann and Lehner 1996; Leisemann et al. 2000). All of the myc-tagged products are capable of rescuing complete loss-of-function mutations in the corresponding genes.

UAS-HA-Sse lines were obtained after P-element-mediated germ-line transformation with a pUASP (Rørth 1998) construct following standard procedures. A fragment encoding six HA epitopes was amplified by PCR from the plasmid pWZV90 (Knop et al. 1999) and inserted into an *AgeI* site downstream of the translational start codon of an *Sse* cDNA. Several *Sse* cDNAs were isolated from a *Drosophila* embryo cDNA library (Brown and Kafatos 1988) by use of radioactively labeled primers derived from genomic sequences determined by the Berkeley *Drosophila* Genome Project (BDGP) and identified by BLAST searches using separase sequences from other species. The sequence of these cDNAs confirmed the exon/intron structure predicted by the sequence of a single expressed sequence tag (LD08709) identified by the BDGP and the sequence (CT29682) predicted by the BDGP from the genomic sequence for the *Sse* gene (CG10583). For the generation of *UAS-HA-Sse*^{C497S} lines, we used an analogous construct in which a single codon was mutated using the QuikChange site-directed mutagenesis kit (Stratagene). The details of these and of the following plasmid constructions are available on request. PCR products for generation of expression constructs (bacterial, yeast, and fly) were obtained by use of *Pfu* polymerase (Stratagene), and sequenced.

Lines carrying *gSse* were generated by use of the germ-line transformation vector pP[w⁺myc, 3×P3-EYFPaf] (Horn and Wimmer 2000), a 10-kb genomic *Sse*⁺ fragment assembled from a PCR fragment containing 5.4 kb of upstream regulatory sequences amplified from genomic DNA, a restriction fragment containing the complete coding sequences, and 2.3 kb of downstream sequences derived from a clone isolated from a *Drosophila* genomic lambda DASH library.

Lines allowing Gal4-dependent expression of *thr* fused to 10 myc epitopes at the C terminus [*gUAS-thr-myc*] were obtained with a modified *gthr-myc* construct (Leisemann et al. 2000). Briefly, a PCR fragment encompassing the five *UAS*_{GAL4} sites and the *Hsp70* basal promoter was amplified from pUAST (Brand and Perrimon 1993) and cloned upstream of the *thr* translational initiation codon. Lines allowing Gal4-dependent expression of *pim* fused to six myc epitopes at the C terminus [*UAS-pim-myc*] have been described previously (Leisemann et al. 2000). For the construction of the *UAS-pim*²-*myc* transgene, we replaced a *SexAI/HindIII* restriction fragment in *pim-myc* with a corresponding fragment that was excised from plasmid *pim*²1 × 1, containing a 15-bp in-frame deletion coding for amino acids 110–114 of PIM.

Various lines [*gthr* 1–478-*myc*, *gthr* 1–930-*myc*, *gthr* 445–1379-*myc*, *gthr* 932–1379-*myc*] expressing truncated THR proteins fused C-terminally to 10 copies of the myc epitope were also generated with modified *gthr-myc* constructs. In the case of *gthr* 1–930-*myc*, we obtained only a single line, which did not express the transgene, presumably because of the toxicity of the transgene product. Therefore, using pUAST, we generated *UAS-thr* 1–930-*myc*, allowing conditional Gal4-dependent expression.

Deficiencies deleting *Sse* were isolated as male recombinants as described (Preston and Engels 1996; Preston et al. 1996) after mobilizing the P-element EP(3)0915 inserted in *CG17334*. This adjacent gene, downstream of *Sse*, encodes a potential cold-shock protein with similarity to the heterochronic *lin-28* gene product of *Caenorhabditis elegans*. EP(3)0915 homozygotes are viable and fertile without visible phenotypes, suggesting that *CG17334* is not an essential gene. Virgin EP(3)0915 females were crossed to +/CyO, $\Delta 2-3$; *ve st e*/TM3, *Ser* males. Of the progeny, 250 +/CyO, $\Delta 2-3$; EP(3)0915/*ve st e* male individuals were crossed to 500 *ve st e* virgin females. A total of 40,000 progeny were scored for recombination of the markers *ve* and *e* flanking EP(3)0915. A total of 71 out of 233 recombinants were *ve*⁺ *e*, indicating a recombination event at the right end of EP(3)0915 (Preston et al. 1996). Six of the seventy-one recombinant chromosomes were lethal when homozygous or hemizygous over *Df(3L)ZN47*, which deletes a large region on the left arm of the third chromosome, including *Sse*. Animals carrying any of the six recombinant chromosomes were viable when transheterozygous with the lethal P-element insertion *l(3)02331* located ~110 kb proximal to the EP(3)0915 insertion. Recombinants isolated after mobilization of P elements in the male germ line often have chromosomal deletions on one side of the P-element insertion, which is usually retained (Preston et al. 1996). For a molecular characterization of the recombinants by sequence analysis, therefore, we recovered DNA from five lines by plasmid rescue. Four of the lines were found to have deficiencies deleting more than 100 kb, and one line, *Df(3L)SseA*, carried a smaller deletion of 34 kb. This deletion encompasses part of *CG17334*, the complete *Sse* gene and part of *still life* (*sif*). Individuals homozygous for a *sif* null mutation are viable (Sone et al. 2000). The absence of the *Sse* coding region in *Df(3L)SseA* was further confirmed by PCR analysis of genomic DNA obtained from homozygous deficient larvae.

The EMS induced recessive lethal mutation *l(3)13m-281*, designated here as *Sse*^{13m}, was kindly provided by M. Gatti (University La Sapienza, Rome). Standard genetic complementation tests indicated that *Sse*^{13m} does not complement *Df(3L)SseA* and *Df(3L)ZN47*, whereas it did complement the deficiencies *Df(3L)h-i22* and *Df(3L)Scf-R6*, which delete regions in which *Sse*^{13m} originally had been mapped by meiotic recombination. To characterize the *Sse* sequence present on the *Sse*^{13m} chromosome, we amplified the coding region by PCR and sequenced the cloned products from three independent amplification reactions. In all three cases, a 4-bp deletion in exon 6 was detected 7 bp downstream of the triplet encoding the essential histidine residue of the presumptive separase catalytic dyad. The predicted C-terminal sequence encoded by *Sse*^{13m} starting from the essential histidine, is, therefore, HGSGSTSMVA.

Sequence comparison

TBLASTN searches of the nr and htgs databases of GenBank, of *Drosophila* sequences deposited at the BDGP Blast server, and of the assembled cDNA sequences available at The Institute of Genomic Research (TIGR; <http://www.tigr.org/tdb/tgi.shtml>) by using, as query, the C-terminal regions of human and fungal separase proteins. A multiple sequence alignment of the C-terminal regions of the various separase homologs was performed by using CLUSTALW (<http://www2.ebi.ac.uk/clustalw/>) with default parameters. A shaded display was obtained by using Boxshade version 3.21. A tree was drawn using the PILEUP program of the GCG program package with default parameters.

The sources for the sequence comparison were as follows: *C. elegans* 1 (AAF60651), *C. elegans* 2 (T27859), *Trypanosoma bru-*

cei (CAB95528), *Leishmania major* (conceptual translation of a region of AL499620, position 1003853–1007689), *Saccharomyces cerevisiae* Esp1 (S64403), *Schizosaccharomyces pombe* Cut1 (A35694), *Emericella nidulans* BimB (P33144), and *Homo sapiens* (BAA11482). The partial sequences of *Mus musculus*, *Rattus norvegicus* and *Xenopus laevis* separases are conceptual translations of assembled cDNAs obtained from the TIGR Gene Index website (see above) and have the accession numbers TC120478, TC143396, and TC5660, respectively. The *Arabidopsis thaliana* separase homolog (CAA19812) is a hypothetical protein sequence deduced from genomic sequence. However, this protein sequence lacks the highly conserved cysteine residue and surrounding invariant residues. Assuming an alternative splice acceptor of an intron located in this region, the sequence GAQYIPPREIEKLDNCSATFLMGCSGSLWLKGCYIP QGVPLSYLL can be inserted in frame at position 1635 of CAA19812, thus restoring the conserved region.

Yeast two-hybrid experiments

Protein-protein interactions were analyzed using the Matchmaker Two-Hybrid system and the yeast strain AH109 (Clontech). Interactions were scored by analysis of growth of transformants on medium selecting for the activation of the *HIS3* or *ADE2* genes. To evaluate the strength of interactions, we supplemented plates lacking histidine with varying amounts of 3-aminotriazole (3-AT). For control experiments, we used plasmids encoding the SV40 T-Antigen fused to the GAL4 activation domain (pGADT7-T) or p53 fused to the GAL4 DNA-binding domain (pGBKT7-p53).

The initial THR deletion constructs were cloned as fusions with the Gal4p DNA-binding domain into pGBKT7 (Clontech). To obtain fusions of THR fragments with the Gal4 activation domain, the respective deletion constructs were excised as *NcoI*–*Bam*HI fragments from the individual pGBKT7–THR constructs and cloned into pGADT7.

The *pim* coding region was cloned in frame into the vector pGADT7. From the resulting construct pGADT7–PIM, we constructed pGADT7–PIM² using the QuikChange mutagenesis kit to delete the 15 nucleotides coding for amino acids 110–114 (FPNEK). pGADT7–PIM 1–114 was constructed by subcloning a PCR fragment. In pGAD–PIM 115–199, the C-terminal part of the PIM coding region was fused at its C terminus to the Gal4p activation domain, in contrast to all our other pGADT7 constructs in which the Gal4p activation domain is N-terminal. The *Sse* constructs were generated with pGBKT7 and appropriate PCR fragments.

Immunoprecipitation, immunoblotting, and immunolabeling

Antibodies against phospho-histone H3 (Upstate Biotechnology) and secondary antibodies (Jackson ImmunoResearch) were obtained commercially. The antibodies against the human c-myc epitope (mAb 9E10; Evan et al. 1985), the HA epitope (mAb 12CA5; Niman et al. 1983), *Drosophila* Cyclin B (Knoblich and Lehner 1993), PIM (Stratmann and Lehner 1996), and THR (Leismann et al. 2000) have been described previously. An additional rabbit antiserum against PIM was raised and used for immunoblotting in a dilution 1:3000 without further purification.

For the generation of antibodies against SSE, we expressed an N-terminal SSE region (amino acids 1–281) with a hexahistidine tag in bacteria using a pQE30 (QIAGEN) construct. The fusion protein was purified by Ni²⁺ affinity chromatography and used for the immunization of two rabbits. The immune sera were

affinity purified using antigen immobilized on BrCN-activated sepharose (Sigma).

For the coimmunoprecipitation experiments shown in Figure 4, we collected eggs from either *w¹*, or *UAS-Cdk1-myc II.2/CyO*; *arm-GAL4*, or *gpm-myc 3A*, or *gthr-myc III.1* flies for 3 h on apple juice agar plates and aged them for 3 h at 25°C before extract preparation. After dechorionization, the embryos were homogenized in 4 vol of lysis buffer (50 mM HEPES at pH 7.5, 60 mM NaCl, 3 mM MgCl₂, 1 mM CaCl₂, 0.2% Triton X-100, 0.2% Nonidet NP-40, 10% glycerol, 1 mM DTT, 2 mM Pefabloc, 2 mM Benzamidin, 10 µg/mL Aprotinin, 2 µg/mL Pepstatin A, 10 µg/mL Leupeptin). The extracts were cleared by centrifugation and the supernatants were used for immunoprecipitation with the anti-myc antibody, nonimmune IgG from rabbits (Jackson Immunoresearch), immunopurified anti-SSE antibody bound to Protein A-Sepharose 6MB beads (Amersham Pharmacia Biotech), or CL4B beads (Sigma). The immunoprecipitates were analyzed by immunoblotting by use of horseradish peroxidase-coupled secondary antibodies followed by ECL detection (Amersham Pharmacia Biotech).

For the coimmunoprecipitation experiment shown in Figure 5C, eggs were collected from either *w¹*, or *UAS-Cdk1-myc II.2/CyO*; *arm-GAL4*, or *gthr-myc III.1*, or *thr 1-478-myc III.1*, or *thr 445-1379-myc III.1*, or *thr 932-1379-myc III.1* flies, or from a cross of *UAS-thr 1-930-myc III.1* and *da-GAL4* flies. For the analysis of complex formation of THR, PIM, and SSE in late embryos (Fig. 5A,B), we prepared extracts from 12- to 15-hour-old embryos derived from crosses of *daGAL4 G32* males with *UAS-HA-Sse I.1;stg*, *gUAS-thr-myc III.1/TM3*, *Ser* or *UAS-HA-Sse I.1;stg*, *UAS-pim-myc III.2/TM3*, *Ser* or *UAS-HA-Sse I.1;stg*, *gUAS-thr-myc III.1*, *UAS-pim-myc III.2/TM3*, *Ser* or *UAS-HA-Sse I.1;stg*, *gUAS-thr-myc III.1*, *UAS-pim²-myc III.1/TM3*, *Ser* females. The embryos were treated and extracts were prepared and analyzed as detailed above. To allow an estimation of the amounts of coprecipitated THR-myc and PIM in the experiment shown in Figure 5A, we loaded onto the same gel a dilution series of a mixture of bacterially expressed full-length protein (PIM) and a C-terminal protein fragment (THR), both of which had been used to raise the antibodies (Leismann et al. 2000). The amounts of protein in this mixture was determined by comparison with protein standards in Coomassie-stained gels. After immunoblotting and detection with ECL, signal intensities of the precipitated protein were compared with the dilution series on nonsaturated exposures.

For the analysis of mitotic cells in larval brains, we dissected these organs from early second instar (48–50 h) and third instar wandering stage larvae. The following genotypes were analyzed: *Df(3L)SseA*, and *Sse^{13m}*, and *Df(3L)SseA/Sse^{13m}*, and *UAS-HA-Sse I.1/+*; *da-GAL4 G32*, *Df(3L)SseA/Df(3L)SseA*, and *UAS-HA-Sse I.1/+*; *da-GAL4 G32*, *Df(3L)SseA/Sse^{13m}*, and *UAS-HA-Sse^{C497S}/+*; *da-GAL4 G32*, *Df(3L)SseA/Sse^{13m}*. These larvae were derived from parents carrying *TM3*, *Ser*, *Act5c-GFP*, which allowed for an identification of individuals lacking endogenous *Sse⁺* gene function as well as *Sse⁺* siblings by analyzing GFP fluorescence. Brains were fixed and immunostained as described (Gonzalez and Glover 1993). Double labeling of DNA was achieved with Hoechst 33258. For squash preparations to visualize individual chromosomes, brains from second instar larvae of the genotype *Df(3L)SseA/Sse^{13m}*, and *Df(3L)SseA/TM3*, *Ser*, *Act5c-GFP*, or *Sse^{13m}/TM3*, *Ser*, *Act5c-GFP* were prepared. The dissection of brains and subsequent treatment with a hypotonic shock were essentially performed according to Pimpinelli et al. (2000). Fixation and squashing of the brains and staining of DNA with Hoechst 33258 was done according to Gonzales and Glover (1993).

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