Genetic interactions of separase regulatory subunits reveal the diverged Drosophila Cenp-C homolog

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Faithful transmission of genetic information during mitotic divisions depends on bipolar attachment of sister kinetochores to the mitotic spindle and on complete resolution of sister-chromatid cohesion immediately before the metaphase-to-anaphase transition. Separase is thought to be responsible for sister-chromatid separation, but its regulation is not completely understood. Therefore, we have screened for genetic loci that modify the aberrant phenotypes caused by overexpression of the regulatory separase complex subunits Pimples/securin and Three rows in Drosophila. An interacting gene was found to encode a constitutive centromere protein. Characterization of its centromere localization domain revealed the presence of a diverged CENPC motif. While direct evidence for an involvement of this Drosophila Cenp-C homolog in separase activation at centromeres could not be obtained, in vivo imaging clearly demonstrated that it is required for normal attachment of kinetochores to the spindle.

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Separase functions as a protease at the metaphase-to-anaphase transition of mitosis (for review, see Nasmyth 2002). At this crucial cell cycle transition, separase cleaves the α-kleisin subunit [Scc1/Mcd1.Rad21] of the cohesin complex and thereby promotes the final release of sister-chromatid cohesion. The careful control of separase activity during the cell division cycle involves regulatory subunits. Securin is a subunit that accumulates and associates with separase during interphase. It acts as an inhibitor of separase activity. Thus, the rapid degradation of securin at the metaphase-to-anaphase transition via the anaphase-promoting complex/cyclosome (APC/C) pathway of ubiquitin-dependent proteolysis results in separase activation. In Drosophila, securin is encoded by the pimples (pim) gene (Leismann et al. 2000) and the catalytic protease subunit by the Separse (Sse) gene [Jäger et al. 2001]. Drosophila Sse lacks the extensive N-terminal regulatory domain that is present in separases outside the dipterans because the corresponding gene region appears to have evolved into an independent gene, three rows (thr) [Herzig et al. 2002; Jäger et al. 2004]. Drosophila Thr binds to Sse and is required for sister-chromatid separation during mitosis [Jäger et al. 2001].

The precise role of Thr and the corresponding N-terminal domains in nondipteran separases is not understood. Moreover, Pim and other securins are not just separase inhibitors but also contribute in an unknown positive manner to sister-chromatid separation. In fission yeast, securin recruits separase to the mitotic spindle, and similar observations have been described in other organisms [Funabiki et al. 1996; Ciosk et al. 1998; Kumada et al. 1998; Jensen et al. 2001; Herzig et al. 2002; Chestukhin et al. 2003]. Separase activation and transport on spindle microtubules might confine its action to the congressed chromosomes in metaphase plates and in particular to the pericentromeric region. This hypothetical scenario might explain why only a minute and preferentially pericentromeric pool of Scc1 appears to be cleaved by separase during mitosis of higher eukaryotic cells, while the large majority of Scc1 remains intact.

To identify additional genes that might contribute to separase regulation and function, we have screened for chromosomal regions that act as genetic modifiers of the aberrant phenotypes resulting from overexpression of Pim or a dominant-negative Thr fragment during Drosophila eye development. Molecular characterization of an interacting locus revealed that it encodes a constitutive centromere protein. Mapping of its centromere localization domain in combination with sequence com-

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parisons among Drosophilid orthologs allowed its identification as the most diverged Cenp-C homolog. Cenp-C was originally identified as a human autoantigen localized to centromeres [Earnshaw and Rothfield 1985; Saitoh et al. 1992] and found to display limited sequence similarity to budding yeast Mif2 [Brown 1995; Meluh and Koshland 1995], which was identified by mutations affecting the fidelity of chromosome transmission during mitosis [Meeks-Wagner et al. 1986]. Homologs have also been described in nematodes [HCP-4] and plants [Dawe et al. 1999; Moore and Roth 2001; Oegema et al. 2001; Shibata and Murata 2004; Talbert et al. 2004]. For simplicity, we use Cenp-C as a designation for all these homologs. Interestingly, recent analyses have demonstrated that Cenp-C, as well as Cenp-A, a histone H3 variant present in centromeric nucleosomes, evolve rapidly and adaptively in many lineages, perhaps driven by the rapid evolution of centromeric satellite sequences, and in Drosophila, Cenp-C was supposed to be absent [Talbert et al. 2004]. Apart from providing further support for the striking sequence divergence of ubiquitous eukaryotic centromere components, our findings also raise the possibility that separase activity might be enhanced by such components.

**Results**

*Mutations in Cenp-C modify phenotypic consequences resulting from separase dysregulation*

Overexpression of pim during Drosophila eye development results in an aberrant rough eye phenotype in adults (Fig. 1A, GMR > pim). Although we have not characterized this phenotype at a cellular level, we assume that it results primarily from inhibition of sister-chromatid separation during mitotic divisions of eye imaginal disc cells, because pim overexpression during embryogenesis is known to have this effect [Leismann et al. 2000]. Moreover, analyses in salivary glands indicated that pim overexpression does not have obvious effects in cells progressing through endoreduplication cycles that lack mitotic divisions [data not shown].

When expressed during eye development, a mutant thr version lacking C-terminal sequences [thrΔC] resulted in a phenotype very similar to that caused by eye-specific pim overexpression (Fig. 1A, GMR-thrΔC). The effect of thrΔC was suppressed by concomitant expression of wild-type thr, suggesting that thrΔC acts in a dominant-negative manner. The severity of the aberrant phenotypes resulting from pim and thrΔC overexpression during eye development was correlated with transgene copy numbers [data not shown].

To identify loci interacting with pim and thr, we crossed a collection of chromosomal deficiencies into the backgrounds with the transgenes resulting in pim or thrΔC overexpression during eye development. Heterozygosity for the deficiency Df[3R]p-XT103, which deletes the chromosomal interval 8SA–8SC, was found to enhance the rough eye phenotypes caused by pim and thrΔC overexpression. Based on analyses with additional deficiencies, the interacting region could be narrowed down [Fig. 1B]. Moreover, heterozygosity for the EMS-induced, recessive lethal mutation II(3)85Aa109c1, which had previously been mapped to this chromosomal region [Jones and Rawls 1988], was also found to enhance the aberrant eye phenotype resulting from pim and thrΔC overexpression [Fig. 1A, GMRpim prl41 and GMR-thrΔC].
Cep-C variants fused either to the enhanced yellow fluorescent protein [EYFP] or to myc epitope copies at the N or C terminus. In addition, affinity-purified rabbit antibodies were generated. Both immunolabeling of wild-type Cep-C or tagged variants as well as in vivo imaging of EYFP fusions in Drosophila embryos [Fig. 2A, D] indicated that Cep-C is a constitutive centromere protein. This notion was confirmed by comparing the localization of Cep-C with that of the centromere protein Cid, the Drosophila Cep-A homolog. The comparison revealed colocalization during interphase (Fig. 2D) and mitosis [data not shown].

Analysis of Cep-C expression during development by immunoblotting (Fig. 2B) and immunofluorescence [Fig. 2C] revealed a correlation with mitotic proliferation. Anti-Cep-C signals declined rapidly in the embryonic epidermis after the final mitotic division, while they were still intense in central nervous system cells that proliferate mitotically. In late embryos, where mitotic proliferation is restricted to only very few cells mainly in the brain, Cep-C levels were found to be minimal. Moreover, neither immunoblotting [Fig. 2B] nor immunofluorescence and EYFP-Cep-C fluorescence [data not shown] revealed Cep-C in the endoreduplicating salivary glands from third instar larvae.

Consistent with the shared intracellular localization of Cid/Cep-A and Cep-C, we observed genetic interactions between cid and Cep-C. The aberrant rough eye phenotype caused by cid overexpression during eye development [Jäger et al. 2005] was found to be enhanced by lowering the number of functional Cep-C gene copies from two to one (Fig. 2F). A noncentromeric excess of overexpressed Cid/Cep-A, therefore, might titrate limiting amounts of Cep-C away from the centromere.

Consistent with the idea that Cid/Cep-A and Cep-C are in close association, immunolabeling of cid mutant embryos with antibodies against Cep-C revealed that Cid/Cep-A is required for normal centromeric Cep-C localization. In cid mutant embryos, the presence of a maternal contribution prevents mitotic abnormalities until late in embryogenesis. However, already at earlier developmental stages when maternally contributed Cid/Cep-A was still detectable [data not shown] and mitotic defects were not yet apparent, anti-Cep-C signals were clearly weaker in cid mutants compared with sibling control embryos [Fig. 2E]. Immunolabeling of Cep-C mutant or deficient embryos with anti-Cid/Cep-A did not reveal a reciprocal dependence of Cid/Cep-A localization on normal Cep-C expression and function [see below].

To define centromere localization domains in Cep-C, we expressed various Cep-C fragments fused to enhanced green fluorescent protein [EYFP] in Drosophila embryos from appropriate transgenes [Fig. 3A]. The N-terminal third of Cep-C [amino acids 1–575] and the central third [amino acids 558–1038] failed to localize the fused EYFP to the centromere (Fig. 3B, N+ and M+). In contrast, the C-terminal third [amino acids 1009–1411] was sufficient to direct efficient centromere localization [Fig. 3B, C+]. Further analysis of this region...
Figure 2. *Drosophila* Cenp-C is a constitutive centromere protein colocalizing and interacting with Cid/Cenp-A. (A) In vivo imaging of syncytial embryos, which express EYFP-Cenp-C (green) and histone H2Av-mRFP (red), indicates that Cenp-C is centromeric during interphase (inter), prophase (pro), metaphase (meta), anaphase (ana), and telophase (telo). (B) Immunoblotting with embryo extracts prepared at different developmental ages (0–1, 1–2, 2–3, 3–8, 8–13, and 13–18 h after egg deposition) revealed high levels of Cenp-C during stages with intense mitotic cell proliferation (0–8 h) and decreasing levels during late embryogenesis when few cells proliferate mitotically (8–18 h). Thirty embryos were loaded per lane except in the first two where three (10%) and 10 (33%) 0–1-h embryos were loaded. Moreover, while Cenp-C was clearly detected in an extract prepared from 20 6–8-h embryos (e), it could not be detected in a comparable amount of protein in an extract prepared from 30 salivary glands of third instar larvae (s). (C) Immunofluorescence experiments with embryos homozygous for *Df(3R)Exel149*, which deletes Cenp-C (*Cenp-C*), and sibling control embryos (*Cenp-C*) revealed strong anti-Cenp-C signals in the epidermis of *Cenp-C* embryos at the stage of mitosis 16 (epi 6.5 h) and in the central nervous system, where mitotic proliferation occurs also at 9 h (cns 9 h). In contrast, weak signals were observed in the post-mitotic epidermis of *Cenp-C* embryos at 9 h (e p i 9 h) and already at the stage of mitosis 16 in *Cenp-C* embryos (epi 6.5 h). (D) An interphase nucleus from a syncytial embryo expressing Cenp-C-myc is shown at high magnification after labeling with anti-myc (top panel and green in bottom panel), anti-Cid/Cenp-A (middle panel and red in bottom panel), and a DNA stain (blue in bottom panel). (E) Labeling of *cid* mutant embryos (*cid*-) and sibling control embryos (*cid*+) with anti-Cenp-C (green) and a DNA stain (red) indicates that Cid/Cenp-A is required for normal Cenp-C localization at the centromere. High-magnification views of epidermal regions at the stage of mitosis 16 show reduced centromeric anti-Cenp-C signals in *cid* mutants. (F) Overexpression of *cid*/*Cenp-A* results in an aberrant eye phenotype that is enhanced by mutations in *Cenp-C*. The eyes shown are from flies with the following genotypes: *Cenp-C*ΔR35+/+, GMR-GAL4 UAS-cid IL3B/+ (+/+, GMR > cid), GMR-GAL4 UAS-cid IL3B/+; *Cenp-C*ΔR35+/+; GMR > cid, GMR-GAL4 UAS-cid IL3B/UAS-Cenp-C II.1; *Cenp-C*ΔR35+/+; GMR > cid GMR > *Cenp-C*, GMR-GAL4 UAS-cid IL3B/+; *Cenp-C*ΔR35*/UAS-EGFP-Cenp-C III.2; *Cenp-C*ΔR35+/+ GMR > cid GMR > E/Cenp-C.

indicated that the subregion from amino acids 1009–1205 was also sufficient to confer centromere localization [Fig. 3B, CN+], while two other subregions [amino acids 1094–1306 and 1204–1411] were not sufficient [Fig. 3B, CM+ and CC+].

To identify amino acid positions crucial for centromere localization, we randomly mutagenized the sequences encoding the region sufficient for centromere localization in vitro by error-prone PCR and used these mutated sequences to replace the corresponding wild-type sequence in a construct allowing expression of the C-terminal third of Cenp-C fused to EGFP. Three-hundred-ninety-four mutagenized clones were analyzed for their ability to localize to the centromere after transfection of *Drosophila* SR+ cultured cells. All 50 mutants that had lost centromere localization were subjected to DNA sequence analysis. In addition, 25 randomly selected mutants that localized to the centromere were sequenced as well. These sequences revealed that mutant constructs contained on average 1.9 mutations and that every amino acid position within the region sufficient for centromere binding should have been covered by about two missense mutations in our mutagenesis experiment. Yet only a single-point mutation [R1101G] that abolished centromere localization in the transfection assay was identified [Fig. 3C,D]. R1101, therefore, appears to be of special importance for centromere localization. All other mutants that failed to localize to the centromere were characterized by the presence of premature stop codons. The location of these stop codons in combination with the location of a premature stop codon [1192stop] that did not abolish centromere localization in the transfection assay indicated that the region between amino acids 1084 and 1192 is important for centromere localization [Fig. 3C,D].

*Drosophilid Cenp-C homologs contain a diverged CENPC motif*

The deposition of *Drosophilid* genome sequences in the public internet domain allowed the identification of Cenp-C orthologs from *Drosophila simulans*, *Drosophila yakuba*, *Drosophila erecta*, *Drosophila ananassae*, *Drosophila pseudoobscura*, and *Drosophila virilis*. Amino acid comparisons between the predicted proteins revealed the presence of conserved blocks [Fig. 4A]. Sev-
Drosophila melanogaster

The Drosophila Cenp-C proteins share some regions of high similarity. As discussed above, the C-terminal region and the CENPC motif within the region sufficient for centromere localization are well conserved [Fig. 4A, C and C-term]. However, the most extensive conservation is present within the N-terminal third in an arginine-rich region [Fig. 4A, R] and in another region unique to the Drosophilid Cenp-C proteins [Fig. 4A, DH, for Drosophilid homology region]. Fragments of human Cenp-C encompassing the equivalent positions can function as a second centromere localization domain, independent of the previously mentioned C-terminal centromere localization domain (Yang et al. 1996; Song et al. 2002). In the case of Drosophila melanogaster Cenp-C, the highly conserved N-terminal block does not confer normal centromere localization. Fused to EGFP, the N-terminal region including DH is not sufficient for centromere localization [Fig. 3B, N+], nor does Cenp-C lack-

### Figure 3
Identification of the Cenp-C centromere localization domain. (A) The Cenp-C regions present in UAS-EGFP transgene variants [TG] are indicated by black horizontal lines with numbers indicating amino acid positions. The intracellular localization of the EGFP-Cenp-C variants expressed from these transgenes is listed [Loc] and was found to be either cytoplasmic [(Cy)], nuclear [(Nu)], centromeric [(Cen)], or all over the cell [(Ce)]. Brackets indicate results that were observed only after strong expression. The ability of these transgenes to prevent phenotypic abnormalities after expression in Cenp-C mutant embryos is also listed [Res] with + or − indicating those that did or did not rescue, respectively. [nd] Not done. The two black horizontal lines at the bottom represent the Cenp-C fragments expressed from the Cenp-C trailer and Cenp-C trailer alleles, which both carry premature stop codons after codons 1106 and 857, respectively. Black boxes on top indicate the following Cenp-C regions: arginine-rich region [R], a region highly conserved in the Drosophilid Cenp-C homologs [DH], predicted AT hooks [AT1 and AT2] and nuclear localization signal [NLS], the CENPC motif [C], and the metazoan-like C-terminal region [C-term]. (B) High-magnification views from the epidermis of embryos with a heat-inducible GAL4 transgene and UAS-EGFP-Cenp-C transgene variants [CN, CM, CC, C, M, N] [see A]. The embryos were either heat-treated (+) or not (−), fixed, and labeled with anti-GFP [GFP, green in bottom panel], anti-Cid/Cenp-A [Cid, red in bottom panel], and a DNA stain [DNA, blue in bottom panel]. (C) Variants of EGFP-Cenp-C-C obtained after random mutagenesis of the region from amino acids 1009–1235 of Cenp-C were expressed after transfection of Drosophila SR+ cells and analyzed for their ability to localize to the centromere. The arrowhead pointing down to position 1192 indicates a premature stop codon that does not interfere with centromere localization. The filled arrowheads pointing up indicate premature stop codons that abolish centromere localization. The open arrowhead pointing up indicates the single identified point mutation (R1101G) that prevents centromere localization. (D) GFP signals in representative transfected SR+ cells expressing EGFP-Cenp-C variants. The following variants were expressed: EGFP-Cenp-C-C (+), EGFP-Cenp-C-C with G instead of R at position 1101 [R1101G], EGFP-Cenp-C-C with a premature stop codon at position 1192 [1192stop].

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### Table 1
| Cenp-C Variants | Location | Localization |
|-----------------|----------|-------------|
| CN              | 1009–1235| cytoplasmic |
| CM              | 1009–1235| cytoplasmic |
| CC              | 1009–1235| cytoplasmic |
| C               | 1009–1235| cytoplasmic |
| M               | 1009–1235| cytoplasmic |
| N               | 1009–1235| cytoplasmic |

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**Independent functional domains in Cenp-C**

The Human Cenp-C region encompassing the equivalent positions can function as a second centromere localization domain, independent of the previously mentioned C-terminal centromere localization domain (Yang et al. 1996; Song et al. 2002). In the case of Drosophila melanogaster Cenp-C, the highly conserved N-terminal block does not confer normal centromere localization. Fused to EGFP, the N-terminal region including DH is not sufficient for centromere localization [Fig. 3B, N+], nor does Cenp-C lack-
ing this region [deletion of amino acids 36–546] fail to localize to the centromere [Fig. 3A; data not shown].

Three other small regions of D. melanogaster Cenp-C displayed similarities to nuclear localization signals [amino acids 1058–1075] [Fig. 3A, NLS] and to AT hooks, which can mediate binding to the minor groove of DNA [amino acids 593–612 and amino acids 1207–1219] [Fig. 3A, AT1 and AT2]. These regions showed limited conservation among Drosophilids and were found to deviate from the consensus sequence derived from fungal, plant, and other metazoan Cenp-C proteins [Talbert et al. 2004], in particular toward the C-terminal end.

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Is Cenp-C involved in sister-chromatid separation?

To define the Cenp-C function in further detail, we analyzed the consequences of loss-of-function mutations. In particular, since our identification of Cenp-C by genetic interactions argued for a functional cooperation between separase regulatory subunits and this centromere protein, we were interested in whether the Cenp-C mutant phenotype provides evidence for a direct interaction.

The two available Cenp-C alleles [prl41 and IR35] both behaved as recessive lethal mutations. Homozygous and trans-heterozygous embryos, as well as embryos carrying either of the two alleles over a deficiency [Df(3R)p25] did not hatch. Immunolabeling with antibodies against Cyclin B and a DNA stain indicated striking abnormalities within the central nervous system (CNS) during the second half of embryogenesis [Fig. 6A, Cenp-C]. The CNS of Cenp-C mutant embryos included many oversized cells with abnormally high levels of Cyclin B. In addition, pycnotic nuclei characteristic of apoptotic cells were more frequent within the Cenp-C mutant CNS, which overall contained far fewer cells than the CNS of wild-type embryos. In contrast, other tissues of Cenp-C mutant embryos appeared to be relatively normal. These findings indicated that initial development of Cenp-C mutant embryos is normal, presumably reflecting the presence of the maternal Cenp-C contribution, which can be demonstrated by immunofluorescence [Fig. 2C], RT-PCR, and immunoblotting experiments [data not shown]. However, this maternal Cenp-C contribution evidently is no longer sufficient to support the extended cell proliferation known to occur during CNS development, where continued cell cycle progression despite chromosome segregation defects might result in oversize cells and apoptosis in the Cenp-C mutants. Accordingly, the observation that the CNS is most severely affected in the Cenp-C mutants suggests that Cenp-C is only required in dividing cells. This notion is also supported by the observed correlation between Cenp-C expression and mitotic proliferation [Fig. 2B,C].

To characterize the Cenp-C mutant phenotype in fur-
ther detail at a cellular level, we focused on the onset of phenotypic abnormalities. Analyses with the two alleles in homo-, hemi-, or trans-heterozygous embryos gave similar results. Starting during embryonic cycle 15, immunolabeling revealed declining Cenp-C levels in Cenp-C-deficient embryos compared with sibling controls (data not shown). Based on double-labeling with a DNA stain and scoring of mitotic figures, the division completing cycle 15 appeared still largely normal in the mutants. At the stage of mitosis 16, residual maternally derived Cenp-C levels were further reduced, but still above background at least in some of the mutant embryos (Fig. 2C). However, mitosis 16 was severely affected. Metaphase figures appeared relatively normal, but anaphase and telophase figures frequently displayed abnormal chromatin bridges [Fig. 6B]. Labeling with antibodies against Cid/Cenp-A revealed centromere signals of normal intensity in the Cenp-C mutants. Interestingly, in 70% of the chromatin bridges, Cid/Cenp-A signals were observed in the middle [Fig. 6C, Cenp-C−]. Chromatin bridges appearing during mitosis 15 in Cap-G mutant embryos almost never displayed comparable central centromere signals [Fig. 6C, Cap-G−]. Cap-G is a subunit of the condensin complex and provides a major function required for sister-chromatid resolution during mitosis. Interestingly, Cap-G has recently been shown to interact genetically and physically with the centromere component Cid/Cenp-A [Jäger et al. 2005]. However, the

Figure 5. Distinct Cenp-C regions cause defects after overexpression. MS1096 and GMR-GAL4 were used to drive overexpression of various UAS-Cenp-C transgene variants during wing [top row] and eye [bottom row] development, respectively. Wild-type wings and eyes are present in control flies (+) with a GAL4 transgene but without an UAS transgene. Aberrant wing and eye phenotypes were observed to variable degrees in flies with either UAS-Cenp-C II.2 (FL), UAS-EGFP-Cenp-C II.2 (E-FL), UAS-EGFP-Cenp-C-N II.1 (E-N), UAS-EGFP-Cenp-C-M III.2 (E-M), UAS-EGFP-Cenp-C-C II.1 (E-C), or UAS-EGFP-Cenp-CN II.2 (E-CN) in combination with a GAL4 transgene. The strongest defects were observed after overexpression of N-terminally truncated Cenp-C fragments containing the centromere localization domain (E-C and E-CN). However, aberrant wings and eyes were also caused by the N-terminal region, which does not localize to the centromere [E-N], while the middle region did not have an effect [E-M]. Several independent insertions of each transgene variant were analyzed and found to behave similarly to those shown.

Figure 6. Cenp-C mutant phenotype. (A) Embryos after germ-band retraction, when mitotic cell proliferation is restricted to the nervous system, were labeled with antibodies against Cyclin B [CyclB] and a DNA stain [DNA]. While the entire embryos are shown in the two top rows, high-magnification views of CNS regions are shown in the two bottom rows. In comparison to sibling control embryos [Cenp-C+], Cenp-C−/Df(3R)IR35 embryos [Cenp-C−] contain fewer but often much larger nuclei are present in these embryos [arrows]. Sca-GAL4-mediated expression of UAS-EGFP-Cenp-C [data not shown] or UAS-EGFP-Cenp-C-DAT1 in the Cenp-C mutant embryos [Cenp-C− Sca > ΔAT1] prevents these abnormalities almost completely. (B) Epidermal cells during mitosis 16 from either sibling control embryos [Cenp-C+] or Cenp-C−/Df(3R)IR35 mutant embryos [Cenp-C−] are presented after labeling with antibodies against tubulin [Tub] and a DNA stain [DNA]. While metaphase cells [meta] appear to be indistinguishable, anaphase [ana] and telophase [telo] cells frequently displayed chromatin bridges [arrowhead]. (C) Epidermal regions after labeling with antibodies against Cid/Cenp-A [Cid, green] and a DNA stain [DNA, red] contain late mitotic cells with chromatin bridges in both Cenp-C+/Cenp-C−, Cenp-C−/Df(3R)IR35 [Cenp-C−] and Cap-G−/Df(3R)IR35 [Cap-G−] mutant embryos. However, while the majority of the chromatin bridges in Cenp-C− embryos display anti-Cid/Cenp-A signals at the midpoint [arrowheads], bridges in Cap-G− embryos are devoid of such signals [arrows].

Drosophila Cenp-C
early phenotypic consequences of mutations in Cenp-C and Cap-G are evidently distinct.

Our observations in fixed mutant embryos indicated that Cenp-C is particularly important for separation of sister centromeres during mitosis and/or for segregation of sister chromatids to the poles. A distinct increase in the number of phospho-histone H3-positive cells was observed after incubation in colchicine, demonstrating that the mitotic spindle checkpoint is still functional in Cenp-C mutants, at least in most cells at the stage of mitosis 16. Nevertheless, immunolabeling with an antibody against the checkpoint component BubR1 already revealed less intense signals at the kinetochores during prometaphase 16 in Cenp-C mutants compared with sibling controls [data not shown]. Moreover, the relatively normal appearance and number of metaphase plates during mitosis 16 in the Cenp-C mutants also argued that most kinetochores are still quite functional, allowing for chromosome attachment to the mitotic spindle, chromosome congression into the metaphase plate, and spindle checkpoint silencing.

Figure 7. Mitotic chromosome segregation in Cenp-C mutant embryos. (A) Mitotic divisions in Cenp-C−/Cenp-C+ or Cenp-C−/Cenp-C− embryos expressing gcid-EGFP-cid (GFP-Cid/Cenp-A) and His2Av-mRFP1 [histone H2Av-mRFP] were analyzed by time-lapse in vivo imaging. The time (in seconds) relative to the last frame before the onset of anaphase, which was set to 0, is indicated in each frame. All frames in the top row are from a cell progressing through mitosis 16 in a Cenp-C+ sibling control embryo and all frames in the middle row from a Cenp-C− embryo at the same stage. Magnification in the top two rows is indicated by the white bar in the upper left panel, which corresponds to 3 µm. Various metaphase plates are shown in the bottom row always in the same orientation with the bar corresponding to 3 µm illustrating the magnification shown in the first four panels. Even higher magnifications are shown in the two right-most panels in which sister centromeres of lagging chromosomes in Cenp-C− embryos are indicated by white arrowheads and those of nonlagging chromosomes by white arrows. Chromosomes that were subsequently lagging behind during anaphase in Cenp-C− embryos did not only have more closely spaced sister centromeres than nonlagging chromosomes (see also C) but were also often not oriented in the axis of the spindle, as evident in the right-most panel in the bottom row. (B) The average time (in minutes) from the onset of mitosis 16 until the last frame before the onset of anaphase [pro-meta/anal], as well as from the onset of anaphase until telophase [meta/ana-telo], was determined after time-lapse analysis of embryos that were either Cenp-C−/Cenp-C+ [white bars] or Cenp-C−/Cenp-C− [black bars]. At least 20 mitoses from more than four embryos were analyzed. (C) The distance between sister centromeres (in micrometers) was determined during prophase [pro] and immediately before the onset of anaphase [meta] after time-lapse analysis of embryos at the stage of mitosis 16. Average values obtained with Cenp-C− embryos are represented by white bars. Values obtained for chromosomes that were subsequently lagging behind during anaphase in Cenp-C− embryos are indicated by the gray bar, while the values observed in Cenp-C− embryos during prophase and before the metaphase-to-anaphase transition in case of nonlagging chromosomes are given by black bars. At least 11 chromosomes from more than four embryos were analyzed.
segregation of one or two chromosomes [Fig. 7A]. Measuring the distance between the sister kinetochores just before the metaphase-to-anaphase transition indicated that these late or nonsegregating chromosomes were not attached to the spindle in a normal bipolar fashion. Bipolar attachment results in a significant increase in the intersister kinetochore distance, as confirmed by comparing the values in sibling control embryos during prophase and metaphase [Fig. 7C]. This increase was also observed in the Cenp-C mutants but only in the normally segregating chromosomes. In contrast, in the non-segregating chromosomes, the intersister kinetochore distance at the metaphase-to-anaphase transition was not greater than the average distance observed during prophase [Fig. 7C]. These findings indicate that Cenp-C is required for normal bipolar kinetochore attachment.

Discussion

Our identification of Drosophila Cenp-C closes a prominent gap in the arguments for homologous centromere organization. Centromeric DNA sequences have evolved extremely rapidly and appear to have driven the coevolution of centromeric proteins during eukaryote evolution [Henikoff et al. 2001; Talbert et al. 2004]. The resulting low sequence similarity between centromeric proteins has effectively concealed the existence of a common set of constitutive eukaryotic centromere proteins until very recently. The first features demonstrated to be shared among fungal, plant, and animal centromeres were centromere-specific histone H3 variants (Sullivan et al. 1994; Stoler et al. 1995; Buchwitz et al. 1999, Henikoff et al. 2000, Takahashi et al. 2000; Talbert et al. 2002). In addition to these Cenp-A homologs, only one further constitutive centromere component, Cenp-C, has so far been shown to be present in each of the three main eukaryotic branches [Saitoh et al. 1992; Brown 1995; Meluh and Koshland 1995; Dawe et al. 1999; Moore and Roth 2001; Talbert et al. 2004]. Our findings now also establish the existence of Drosophila Cenp-C, which had previously escaped detection by careful bioinformatics genome analyses [Talbert et al. 2004] because of its very limited sequence similarity. In combination with the recent identification of related Cenp-H-, Cenp-I-, and Mis12-like proteins in both vertebrates and yeast [Nishihashi et al. 2002; Goshima et al. 2003; Westermann et al. 2003; Cheeseman et al. 2004; Obuse et al. 2004], our results provide strong support for the notion of a common set of constitutive centromere proteins. These proteins, which are centromeric throughout the cell cycle, appear to provide a foundation for kinetochore assembly and spindle attachment during mitosis by recruiting several distinct multisubunit complexes that also contain highly diverged proteins [Wigge and Kilmartin 2001; De Wulf et al. 2003; Westermann et al. 2003; Cheeseman et al. 2004].

The extensive sequence divergence characteristically observed among homologous eukaryotic centromere and kinetochore proteins is striking, especially in the light of their common fundamental cellular function. The average amino acid identity observed in a genome-wide comparison of D. melanogaster and D. pseudoobscura ortholog pairs is 77% [Richards et al. 2005] and only 38% in case of the Cenp-C pair. Moreover, based on the ratio between radical charge mutations and conservative substitutions in D. melanogaster and D. pseudoobscura ortholog pairs, Cenp-C is one of 44 genes likely to have evolved under positive selection [Richards et al. 2005]. Except for a few very restricted regions [data not shown], our comparison of D. melanogaster Cenp-C with the orthologs from D. erecta and D. yakuba, which are closer relatives than D. pseudoobscura and thus amenable to dN/dS analyses (Comeron 1999), did not reveal strong evidence for positive selection, in contrast to the recent findings in plant and mammalian lineages [Talbert et al. 2004]. However, we point out that these dN/dS analyses ignore insertions and deletions [indels], which have occurred considerably more often during Cenp-C evolution in Drosophilids than in the mammalian lineage. Most of the indels are observed within the minimally conserved central regions of Drosophilid Cenp-C. Similar variabilities resulting from recurrent exon duplications have been observed in the central region of the plant Cenp-C genes [Talbert et al. 2004].

The adaptively evolving regions of mammalian Cenp-C have been shown to bind to DNA in vitro, consistent with the proposed coevolution of centromeric DNA and protein sequences [Talbert et al. 2004]. However, this DNA binding in vitro is not sequence-specific (Sugimoto et al. 1994), suggesting that interactions with centromere-specific proteins contribute to centromere localization of Cenp-C. As in other organisms [Howman et al. 2000; Moore and Roth 2001; Oegema et al. 2001; Van Hooser et al. 2001; Westermann et al. 2003], Cid/Cenp-A is also required for centromere localization of Cenp-C in Drosophila. High-resolution light microscopy of mitotic chromosomes has indicated that human Cenp-C covers the poleward-oriented peripheral region of the Cenp-A-containing centromeric chromatin (Blower et al. 2002). Direct interactions between Cenp-A and Cenp-C have not yet been demonstrated in any organism. Our attempts with yeast two-hybrid experiments were also unsuccessful [R. Schittenhelm, S. Heeger, and C.F. Lehner, unpubl.].

The CENPC motif has recently been identified as the only region conserved among the Cenp-C orthologs from fungi, plant, and animals [Talbert et al. 2004]. In Drosophilids, even this short motif of ∼24 amino acids is not fully conserved in its C-terminal part. Our results suggest that this CENPC motif is crucial for centromere localization. We demonstrate that a single-point mutation affecting one of the invariant positions in the CENPC motif interferes with centromere localization of the C-terminal domain of Cenp-C in our transfection assay. This mutation was identified as the only missense mutation interfering with centromere localization after extensive random mutagenesis. Further experiments will reveal whether and how the CENPC motif contacts Cid/Cenp-A nucleosomes. We would like to emphasize, however, that also in Drosophila Cenp-C, other regions
than the CENPC motif clearly contribute to efficient centromere localization. Centromere localization of the CN subregion (1009–1205), for instance, is only detected in our transfection assay in live but not in fixed cells, while centromere localization of the larger C region (1009–1411) is resistant to fixation.

The highest conservation among Drosophilid Cenp-C proteins is observed within the N-terminal third, which is neither required nor sufficient for normal centromere localization. Nevertheless, prolonged overexpression of this domain in proliferating eye and wing imaginal disc cells results in severe defects. The conserved N-terminal Cenp-C domains (R and DH) might bind to kinetochore proteins and titate these away from the centromere when overexpressed. Biochemical and genetic characterizations in *Saccharomyces cerevisiae* and *Caenorhabditis elegans* have suggested that Cenp-C is not only associated with Cenp-A, but that it also recruits the next layer of kinetochore proteins, in particular the Mts12/MITw1 and Ndc80 complexes, which remain to be identified in *Drosophila* (De Wulf et al. 2003; Pinsky et al. 2003; Westermann et al. 2003; Cheesman et al. 2004).

As in yeast [Meluh and Koshland 1995], chicken [Fukagawa and Brown 1997; Fukagawa et al. 1999], and mice [Kalitsis et al. 1998], Cenp-C is also an essential gene in *Drosophila*. Antibody microinjection experiments in mammalian cells [Tomkiel et al. 1994]; RNA interference in *C. elegans* [Moore and Roth 2001; Oegema et al. 2001]; and phenotypic analysis in yeast [Brown et al. 1993], chicken cells [Fukagawa et al. 2001], and mutant *Drosophila* embryos demonstrate that Cenp-C is required for normal chromosome segregation during mitosis. Our in vivo imaging in Cenp-C mutant embryos discloses these defects in detail. Previously, in vivo imaging has also been applied in *C. elegans CENP-C(RNAi) embryos* [Moore and Roth 2001; Oegema et al. 2001]. The formation of holocentric chromosomes and transient Cenp-C recruitment only during mitosis differentiates *C. elegans* from other metazoans like *Drosophila* and mammalian cells. Moreover, in contrast to the findings in *C. elegans*, chromosome congression into a central plane is still observed in the *Drosophila* Cenp-C mutants. Presumably, this chromosome congression reflects the function of residual maternally provided Cenp-C, which is still detectable at the stage of our analyses. Moreover, our time-lapse analyses demonstrate that chromosome congression is not entirely normal in the Cenp-C mutant embryos. Metaphase plate formation is delayed and often does not lead to the highly ordered arrangement of all chromosomes characterized previously observed before anaphase onset in wild type. Occasional chromosomes fail to achieve bipolar attachment in the Cenp-C mutants. These chromosomes do not segregate normally during anaphase. Cenp-C is, therefore, clearly required for normal attachment of kinetochores to the mitotic spindle.

Evidently, the insufficiently attached chromosomes in Cenp-C mutant embryos are unable to inhibit the onset of anaphase, even though the mitotic spindle checkpoint appears to be at least partially functional in Cenp-C mutants at the analyzed stage. However, assembly of mitotic spindle checkpoint proteins might fail, particularly on the kinetochores of those chromosomes that do not attach correctly to the mitotic spindle.

In principle, the chromosome segregation defects observed in the Cenp-C mutants might not only reflect impaired interactions between kinetochores and spindle. Segregation of sister chromatids to the spindle poles also depends on complete resolution of sister-chromatid cohesion at the metaphase-to-anaphase transition. This final separation of sister chromatids is thought to be achieved by separase-mediated cleavage of the Sce1/Rad21 subunit of those cohesin complexes that perdure in the pericentromeric region until the metaphase-to-anaphase transition. Several observations are consistent with the idea that a localized full activation of separase might be assisted by centromeric proteins [see above]. Accordingly, mutations in Cenp-C might reduce separase activity and thereby explain the genetic interactions with the regulatory separase subunits Pim/securin and Thr that led to our identification of *Drosophila* Cenp-C. We have failed to observe coimmunoprecipitation of Cenp-C with separase complex proteins and have been unable to detect effects of Cenp-C mutations on Pim and Thr levels [O. Leismann and C.F. Lehner, unpubl.]. Our analysis has, therefore, not exposed clear evidence for separase activation at centromeres. On the other hand, Cenp-C is clearly required for normal chromosome attachment to the mitotic spindle, and thus the observed genetic interactions most likely reflect a summation of negative effects on the efficiency of sister-chromatid separation [by separase] and segregation [by the spindle]. However, we would like to emphasize that our results certainly do not rule out a local activation of separase within the centromeric region.

**Materials and methods**

**Fly stocks**

We used the GAL4 driver lines GMR-GAL4, MS1096, Sea-GAL4, 2sex-Hs-GAL4 332.5, UAS-pim-myc [Leismann et al. 2000], and UAS-cid [Jäger et al. 2005] have been described previously. For the generation of GMR-thrΔC lines, we used a pGMR construct (Hay et al. 1994) containing the same insert fragment including the myc-epitope region as present in UAS-thr1-930-myc [Jäger et al. 2001]. The deficiency kit, as well as Df(3R)Exel6149, was obtained from the Bloomington *Drosophila* Stock Center. Its composition at the time of our screen, as well as a list of the deficiencies that were identified as dominant modifiers of the adult rough eye phenotype resulting from GMR-thrΔC or GMR-GAL4 UAS-pim-myc expression is given in the Supplemental Material. I(3)85Aa1411 [Jones and Rawls 1988] was also obtained from the Bloomington *Drosophila* Stock Center. I(3)85Aa2252 was kindly provided by K. De and T. Orr-Weaver [The Whitehead Institute, Cambridge, MA]. cid112-1 and cid222-4 were generously provided by Thom Kaufman [Indiana University, Bloomington] and used for the analysis of Cenp-C localization in trans-heterozygous embryos. Cap-G0 [Jäger et al. 2005] was crossed over Df(2R)vg56 for the phenotypic comparison with Cenp-C mutants. The His2Av-mRFP1
and the gcid-EGFP-cid transgenic lines, which express functional fusion proteins as assessed by mutant rescue experiments, will be described in detail elsewhere.

Lines carrying genomic fragments from the SSA region were constructed for the identification of the locus interacting with eye-specific pim and thrAC overexpression, using insert DNA derived from the BAC clone R48A14 [Hoskins et al. 2000] and pcSpeR4 followed by standard P element germline transformation. Three olfactory receptor genes [Or85b, Or85c, Or85d] within the region delimited by deficiency breakpoints were excluded from further analysis. A transgene containing a 15-kb fragment with the predicted genes CG11737, CG31454, and CG31259 did not prevent the lethality of the I(3)S85Aagrid mutants. The abc transgene contained a 14.6-kb Ndel-Xbal fragment including CG31453, mRps18C, CG31460, and Cenp-C. The same fragment after filling and religation of a SalI site within exon 5 of Cenp-C was used in the abcd transgene. The proximal 6.4-kb Ndel-EcoR1 subfragment lacking Cenp-C was used in the abc transgene, and the d transgene contained the distal 8.7-kb Pml-I-Xbal subfragment including only Cenp-C.

For the expression of Cenp-C fused to EYFP or 10 copies of the myc epitope under control of the Cenp-C regulatory region, we modified the d transgene construct for the generation of the gEYFP-Cenp-C and gmyc-Cenp-C lines. A slightly larger subfragment (10.3-kb Stul-Xbal fragment) was used for the gCenp-C-EYFP and gCenp-C-myc lines. While gCenp-C-EYFP, gmyc-Cenp-C, and gCenp-C-myc insertions were unable to confer full rescue when crossed into Cenp-C mutant backgrounds, gEYFP-Cenp-C allowed Cenp-C mutant development to the adult stage.

The vector pUAST [Brand and Perrimon 1993] and the EST clone RE27988 [Berkeley Drosophila Genome Project], which contains a full-length Cenp-C cDNA insert according to our sequence analyses, were used for the generation of transgenic lines allowing GAL4-dependent expression of wild-type and mutant Cenp-C versions [Fig. 3]. Moreover, the EYFP coding sequence was introduced into these transgenes immediately after the start codon.

Mutagenesis and transfection

The GeneMorph II random mutagenesis kit (Stratagene) was used for the enzymatic amplification of a Cenp-C cDNA region under mutagenic conditions using the primers m1 [5'-AC-GAGCTCTACGATCTTGC-3'] and m2 [5'-GGAAGGGAAGTACATAGCC-3'] and the plasmid pRS1 as template. This pBluescript KS+ derivative contained the Gal4-binding sites and the basal Hsp70b promoter region isolated from pUAST upstream of the EGFp-Cenp-C-C [amino acids 1009–1411] fusion gene. The amplification products were digested with HindIII and EcoR1 and used to replace the corresponding 688-bp region in pRS1, resulting in a mutant clone library after electroporation of Escherichia coli DH10B cells. The QAprep 96 Turbo Miniprep kit (Qiagen) was used for the preparation of plasmid DNA from 394 random clones.

Expression of the mutant library was achieved by transfection of Drosophila SR cells [Yanagawa et al. 1998]. These cells were cultured at 26°C in Schneider’s Drosophila Medium (GIBCO) containing 10% fetal bovine serum, 1% penicillin, and 1% streptomycin. Before transfection, we plated 10^6 cells/well of a pClear 96 well plate (GIBCO) in 0.2 mL of medium. After 24 h, medium was exchanged with 0.16 mL of fresh medium and 0.04 mL of transfection mix containing 0.25 µg of a given mutant library plasmid, 0.75 µg of pBluescript KS+ as carrier DNA, and 3 µL of FuGene6 [Roche] in Dulbecco’s Modified Eagle’s Medium (GIBCO). EGFP signals were analyzed 24 h after transfection in live cells using an inverted confocal laser scanning microscope [Leica DM IRBE TCS SP1] with a 40× oil immersion objective. Initial experiments involving transfection of the same pUAST-EGFP-Cenp-C constructs that had previously been analyzed after Drosophila germline transformation indicated that the subcellular localization of these mutant Cenp-C versions [FL, N, M, C, CN, CC, AN, ΔAT1, ANLS, ΔAT2] [see Fig. 3] in SR cells was identical as in transgenic embryos. In initial experiments, cotransfection of pUAST-EGFP-Cenp-C constructs with an Act5c-βgal4 plasmid resulted in very high levels of expression, which obscured centromere localization in many cases. The Act5c-βgal4 plasmid was therefore omitted in subsequent experiments, and basal expression of the pUAST derivatives proved to be sufficient for detection of centromere localization.

In vivo imaging and immunofluorescence

Syncytial blastoderm-stage embryos of a gEYFP-Cenp-C II; His2Av-mRFP1 III.1 line were analyzed by in vivo imaging essentially as described previously [Pandey et al. 2005]. Moreover, embryos obtained from a cross of His2Av-mRFP1 II.2, gcid-EGFP-cid II.1; Cenp-C[prl41]TM3, Sb, Kr-GAL4, UAS-GFP females with Cenp-C[prl41]TM3, Sb, Kr-GAL4, UAS-GFP males were analyzed analogously at the stage when the epidermal cells progress through mitosis 16.

Immunofluorescence and DNA labeling of fixed embryos was performed essentially as described previously [Leismann et al. 2000]. Affinity-purified rabbit antibodies against Cid/Cenp-A [Jäger et al. 2005], Cyclin B [Jacobs et al. 1998], and GFP [Molecular Probes Inc.], and mouse monoclonal antibodies against tubulin DM1A [Sigma] were used at a dilution of 1:500, 1:500, 1:5000, and 1:8000, respectively. An affinity-purified rabbit antibody against Drosophila Cenp-C was generated with a bacterially expressed protein fragment (amino acids 502–939) with a His-tag and used at a dilution of 1:5000. For the analysis of the CNS phenotype, 12–14-h embryos were collected from a cross of scagal4A; Df(3R)p25/TM3, Sb, P[SSUbx-iacZ2/2] males and Cenp-C[prl41]TM3, Sb, P[SSUbx-iacZ2/2] females with or without a UAS-EGFP-Cenp-C transgene. Cenp-C[prl41]Df(3R)p25 embryos were identified after double-labeling with antibodies against β-galactosidase.

Sequence comparisons

The VISTA Browser [Cournou et al. 2003] and the Drosophila Species Genome Web pages [http://bugbase.bio.indiana.edu:7151] were used for the identification of Cenp-C genes in genome sequences of D. ananassae, D. erecta, D. pseudoobscura, D. simulans, D. viridis, and D. yakuba [prepublication data from the European Bioinformatics Institute CLUSTAL W Server [Thompson et al. 1994]]. Cenp-C-box consensus motifs were displayed using the Logos program on the Blocks WWW Server [http://blocks.fhcrc.org]. K-estimator [Comeron 1999] was used for dS/dD analyses that estimate and compare the rate of non-synonymous and synonymous exchanges in Cenp-C gene pairs from closely related species. Prior to analysis, insertions were removed from the coding sequences.

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