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A role for Drosophila SMC4 in the resolution of sister chromatids in mitosis

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Background: Faithful segregation of the genome during mitosis requires interphase chromatin to be condensed into well-defined chromosomes. Chromosome condensation involves a multiprotein complex known as condensin that associates with chromatin early in prophase. Until now, genetic analysis of SMC subunits of the condensin complex in higher eukaryotic cells has not been performed, and consequently the detailed contribution of different subunits to the formation of mitotic chromosome morphology is poorly understood.

Results: We show that the SMC4 subunit of condensin is encoded by the essential gluon locus in Drosophila. DmSMC4 contains all the conserved domains present in other members of the structural-maintenance-of-chromosomes protein family. DmSMC4 is both nuclear and cytoplasmic during interphase, concentrates on chromatin during prophase, and localizes to the axial chromosome core at metaphase and anaphase. During decondensation in telophase, most of the DmSMC4 leaves the chromosomes. An examination of gluon mutations indicates that SMC4 is required for chromosome condensation and segregation during different developmental stages. A detailed analysis of mitotic chromosome structure in mutant cells indicates that although the longitudinal axis can be shortened normally, sister chromatid resolution is strikingly disrupted. This phenotype then leads to severe chromosome segregation defects, chromosome breakage, and apoptosis.

Conclusions: Our results demonstrate that SMC4 is critically important for the resolution of sister chromatids during mitosis prior to anaphase onset.

Background

Chromosome condensation serves not only to package the long DNA molecules present in eukaryotic cells but also to organize the sister strands into chromatids that can be resolved from one another and from other chromosomes [1, 2]. Early work indicated that DNA topoisomerase II (topo II) was a structural component of mitotic chromosomes [3, 4]. While topo II activity was also shown to be essential for establishing mitotic chromosome condensation both in vivo [5] and in vitro [6, 7], it may not be required to maintain the condensed state [8].

New insights into chromosome dynamics came with the identification of a novel family of conserved chromosomal ATPases, the SMC proteins, reviewed in [9, 10]. SMCs were initially identified in S. cerevisiae [11] and in the analysis of the ScII protein that, like topo II, is a component of the chromosome scaffold fraction [12]. SMCs have been identified in all eukaryotic organisms examined to date and fall into six clearly discernible subfamilies (SMC1–4, Rad18, and Rad18 related). All SMC proteins are large polypeptides that share partial sequence conservation and similar structural organization [9]. The N-terminal end contains a putative Walker A motif (ATP binding domain), and at the C-terminal end is located a characteristic “DA” box (Walker B motif) involved in ATP hydrolysis [12]. These domains are separated by a coiled-coil region, interrupted by a central globular hinge region. A complex containing a heterodimer of SMC2 and SMC4 has been shown to function in chromosome condensation [13–16], while an analogous complex containing an SMC1/SMC3 heterodimer plays a role in sister chromatid cohesion [17–19].

The “condensin” complex, first identified in Xenopus egg extracts, is essential for mitosis-specific chromatin condensation in vitro [13]. XCAP-C and XCAP-E, the Xenopus SMC2/SMC4 homologs, along with three non-SMC proteins (XCAP-D2, XCAP-G, and XCAP-H) comprise a 13S condensin complex, while an 8S complex consists of
The mechanism of protein-laden chromosomes is unknown. Genomic organization and molecular characterization of DmSMC4 suggests that SMC4 plays a crucial role in the final stages of chromosome condensation, leading to chromosome transheterozygotes died as late larvae, and the longitudinal axis of a chromosome can be shortened, while width-wise chromatid compaction and sister chromatid resolution are severely disrupted.

Results

Molecular cloning and characterization of DmSMC4

We have cloned a cDNA molecule containing an open reading frame (ORF) encoding a protein of 1409 amino acids with a predicted molecular weight of 160 kDa. The predicted structure of the protein closely resembles that described for other SMC proteins. Phylogenetic analysis of the protein sequence showed that it belonged to the SMC4-type protein family (see the Supplementary material available with this article on the internet). The Drosophila protein is closely related to other SMC4-type proteins, including Xenopus XCAP-C (41% identity), human HCAP-C (39% identity), S. cerevisiae SMC4 (35% identity), and S. pombe CUT3 (34% identity) orthologs, and it is more distantly related to other SMC-type proteins. Accordingly, we have named the gene DmSMC4.

Genomic organization and molecular characterization of mutant alleles

An examination of the corresponding genomic sequence (AC005119) allowed us to determine the genomic organization of the locus (Figure 1a). Database searches identified one embryonic-lethal P element insertion allele, l(2)k08819, that had been previously named gluon [28]. Subsequently, we identified a late larval-lethal P element insertion allele, l(2)k06821. We have named the alleles glu'/ glu', respectively. The localization of the P elements in glu' and glu' are indicated in Figure 1a. Molecular characterization of the glu' excision allele glu[glu88±82] was carried out by the sequence analysis of PCR products. In glu[glu88±82], a large internal region of the P element is deleted.

Developmental expression of DmSMC4 in wild-type and mutant cells

Polyclonal antibodies against an internal fragment of the DmSMC4 protein were generated and shown to specifically recognize the bacterially expressed polypeptide and a single band of approximately 190 kDa in extracts from third-instar larval brains (Figure 1b). We used the antibodies to determine the levels of expression during different developmental stages and in different tissues (Figure 1c). Although DmSMC4 was present throughout development, it was most abundant in embryos, third-instar larval brains, and adult ovaries and testes.

Genetic analysis of the mutant alleles showed that glu'/ glu' and glu[glu88±82] were embryonic lethal, glu'/glu' transheterozygotes died as late larvae, and glu'/glu' homozygotes died as early pupa. These results suggested a gradual decrease in the level of the DmSMC4 protein in the different mutant alleles. Probing third-instar larval brain extracts with the affinity-purified DmSMC4 antibody confirmed these results (Figure 1d). As compared...
to the wild-type control, DmSMC4 expression was moderately reduced in glu1/glut and significantly reduced in glu2/glut. Thus, the P element insertion in glu1 probably corresponds to a severe hypomorph allele, while glu2 is a weak hypomorph. To confirm that the P insertions were responsible for the mitotic phenotypes and lethality, we cloned the complete cDNA into a p-UAS transformation vector [29] and obtained several stable transformants. Driven by a GAL4 insertion (M1277), cDNA expression in neuroblasts produced viable [pUAS-Glon]; glu1/glut; M1277 individuals showing no mitotic defects.

Immunolocalization of DmSMC4 and Barren during the cell cycle
We determined the cell cycle distribution of DmSMC4 by simultaneously immunostaining fixed S2 cells with the DmSMC4 antibody (see Materials and methods) and with antibodies against Barren, one of the non-SMC condensin subunits (Figure 2). In interphase, both proteins were present in the cytoplasm (but were not apparently colocalized) and showed low levels of diffuse nuclear staining, excluding the nucleolus (Figure 2a). In prophase, increased nuclear labeling of both proteins showed accumulation with the condensing chromatin (Figure 2b). In metaphase, both proteins showed discrete colocalization along the condensed chromosome arms, with more intense labeling of the kinetochore regions and the nucleolar organizing region (NOR) of the X chromosome (Figure 2c). To visualize this more clearly, we also show a prometaphase cell stained for DmSMC4 and MPM2 (Figure 2g). In metaphase (data not shown) and early anaphase (Figure 2d) when chromosomes are fully condensed, the labeling was more uniform throughout the core of the chromatids. During later stages of anaphase, both proteins were still associated with the distal ends of segregating chromatids but were excluded from the decondensing centromere-proximal regions (Figure 2e). In telophase, both proteins showed low levels of diffuse nuclear and cytoplasmic staining (Figure 2f). These results show that a major pool of DmSMC4 and Barren undergoes significant recolocalization from the cytoplasm to the nucleus during chromosome condensation and back to the cytoplasm as cells exit mitosis. A minor pool appeared to be bound to chromatin independently of cell cycle stage. Similar results were obtained with third-instar larval neuroblasts. To confirm the specificity of the immunofluorescence, we preincubated the antibody with the purified antigen, which abolished all staining patterns (data not shown).
Figure 2

Cell cycle localization of DmSMC4 and Barren in Drosophila S2 tissue culture cells. In panels (a–f) all cells are stained for DNA, DmSMC4 and Barren, and in the corresponding merged images DNA is in blue, DmSMC4 is in green, and Barren in red. (a) Interphase cell showing mostly cytoplasmic staining of both DmSMC4 and Barren, although low-level nuclear staining is also observed. The merged image shows that the two antigens do not colocalize in the cytoplasm. (b) Cell in prophase showing increased nuclear staining of both DmSMC4 and Barren. (c) Prometaphase cell showing condensed chromosomes with DmSMC4 and Barren colocalizing to the sister chromatid cores. Note the strong labeling of the NOR of the X chromosome (arrow). (d) Early anaphase cell showing tight colocalization of DmSMC4 and Barren to a coiled axial structure of individual chromatids. (e) Cell in late anaphase showing simultaneous loss of DmSMC4 and Barren from the centromere proximal end of the segregating sister chromatids. (f) Telophase cell showing strong cytoplasmic and low-level nuclear staining of both proteins. We extracted preparations in (a) and (f) with Triton for 10 min after fixation and those in (b–e) during fixation to allow better chromosome staining (see Supplementary materials and methods). (g) Cell in prometaphase stained for DNA (not shown), DmSMC4 (green), and MPM2 (red). The DmSMC4 panel shows staining of the sister chromatid cores and some accumulation of the antigen at the centromeres of several chromosomes. Double immunostaining with MPM2 shows that DmSMC4 at the centromere colocalizes with kinetochores. The scale bar represents 5 μm.
Mitotic progression of DmSMC4 mutant cells

We then isolated brains from glu2/glu2 and glu1/glu2 third-instar larvae to determine mitotic parameters and phenotype. The mitotic index in wild-type neuroblasts was higher (1.11%) than that in either glu1/glu2 (0.87%) or glu2/glu2 (0.83%). Mitotic cells from mutant individuals showed several defects (Figure 3d–i) as compared to wild-type controls (Figure 3a–c). At prophase, when homologous chromosomes in Drosophila are known to pair, condensing chromatin appeared fuzzy (Figure 3d). This diffuse appearance was observed from prophase to anaphase and with different DNA dyes (data not shown). In addition, many prometaphase and metaphase figures did not show well-defined sister chromatids (Figure 3e). Most cells in anaphase showed abnormally decondensed chromatin and contained chromatin bridges and some chromosome fragmentation (Figure 3f–h). Chromatin bridges were also observed during telophase (Figure 3i). These chromosome phenotypes are unlikely to be a secondary consequence of dying cells because immunofluorescence analysis with various antibodies against a number of cellular antigens, including checkpoint proteins and mitotic regulators, shows normal distribution (data not shown).

Quantification of mitotic parameters in mutant cells showed that, of the cells in mitosis, the percentage of cells at different stages was similar to that for wild-type controls (Figure 3j). These data indicate that the mutations do not cause a delay in mitotic progression. Most cells in prophase and metaphase had defects in condensation and/or segregation (Figure 3k). These results indicate that the effects of the mutations manifest early in chromosome condensation and that they persist throughout mitosis. Also, a large proportion of the anaphase figures in glu1/glu2 (58%) and glu2/glu2 (82%) had chromatin bridges, indicating a direct correlation between condensation defects and the ability to segregate properly in anaphase. Additionally, the presence of chromatin bridges during telophase (54% and 81% respectively) showed that the abnormal chromatin does not resolve during segregation.

To study abnormal chromosome condensation in anaphases and telophases in more detail, we immunostained wild-type and mutant cells with an anti-phosphohistone H3 antibody (Figure 4a–h). Histone H3 phosphorylation at Ser-10 is highly correlated with the state of chromosome condensation. For comparison, (a–c) wild-type mitotic figures are shown above representative images from (d–i) glu2/glu2 cells. (a) Cell in prophase showing paired homologous chromosomes. (b) Prometaphase cell showing tightly condensed chromosomes and well-resolved sister chromatids. (c) Early anaphase cell showing migrating sister chromatids. (d) Mutant cell in prophase showing hypocondensed pairs of homologous chromosomes. (e) Prometaphase cell showing diffuse chromatin staining and only partial sister chromatid resolution. (f) Early anaphase cell showing diffuse chromatin staining of segregating chromatids. (g.h) Cells in anaphase showing abnormal chromatin condensation, chromatin bridges, and some chromosome fragmentation. (i) Cell in telophase showing extensive chromatin bridges. The scale bar represents 5 μm.

(j.k) Quantification of mitotic figures in Or-R, glu2/glu2, and glu1/glu2. (j) Distribution of mitotic cells in prophase, metaphase, and anaphase as a percentage of the total number of mitotic cells (telophases not included). (k) Quantification of abnormal mitotic figures as a percentage of the total number of figures in each phase. For wild-type, glu2/glu2, and glu1/glu2 cells, 45,700 nuclei (4 brains), 63,000 nuclei (8 brains), and 95,500 nuclei (11 brains) were counted, respectively.
Figure 4

Analysis of chromatin bridges in mutant cells at different developmental stages. We used immunofluorescence to detect the distribution of phosphohistone H3 epitopes (green) during mitosis in (a–h) wild-type and (e–h) glu1/glul2 neuroblasts. Note that phosphorylated histone H3 persists in mutant cells up to telophase. The scale bar represents 5 μm. (i) Images of mitotic chromosomes from the ventral nerve chord of wild-type (top half) and homozygous gluon88–82 (bottom half) embryos, stained with DAPI to show DNA and antibodies to α-tubulin and the phosphorylated form of histone H3. Similar anaphase and telophase bridges were also common in embryos in which gluon88–82 was combined with deficiencies spanning the SMC4 locus. The scale bar represents 1 μm. (j) Quantification of mitotic figures. The proportion of wild-type and glu88–82 cells in metaphase, anaphase, or telophase as well as the proportion of cells with chromatin bridges are shown. A total of 361 wild-type and 271 glu88–82 cells were counted.
condensation [30]. Consistently, anaphase bridges that persisted until telophase were always highly stained, and this result suggests that decondensation had not been completed.

An analysis of mitotic domains was performed in the \( \text{glu}^{88-82}/\text{glu}^{88-82} \) excision derivative since the phenotype appeared to be more severe than either \( \text{glu}^{88-82}/\text{glu}^{88-82} \) or \( \text{glu}^{88-82}/\text{glu}^{88-82} \). Labeling of wild-type cells for DNA, phosphohistone H3, and tubulin showed the predicted patterns during mitosis (Figure 4i). However, in mutant embryos, anaphases and telophases showed chromatin bridges that were stained by the anti-phosphohistone H3 antibody (Figure 4i). Quantification of the mitotic phenotypes in mutant embryos indicated that a number of cells arrest in metaphase and that the majority of cells reaching anaphase show chromatin bridges (Figure 4j). The fact that embryos show metaphase arrest while larval neuroblasts do not may be because the embryonic alleles are more severe than the larval lethal allele, or it may imply different outcomes when the function of DmSMC4 is compromised. Thus, the mitotic phenotype caused by mutations in gluon showed that DmSMC4 was required for proper chromosome segregation in both embryonic and larval tissues.

Chromatin bridges, chromosome fragmentation, and apoptosis in DmSMC4 mutant neuroblasts
Cytological analysis of mutant neuroblasts showed that many mutant cells contained anaphase bridges as well as DNA fragments. In order to determine the type of chromosomal aberrations present in these cells, we performed in situ hybridization with centromeric and telomeric probes (Figure 5). The results indicate that, of the anaphase or telophase cells containing chromatin bridges, only 27% showed centromeric signals in the bridges (Figure 5a), while 43% showed a telomeric signal (Figure 5c) and the remaining 30% contained neither (Figure 5b). These results indicate that in most cases centromeres segregated normally and that chromatin bridges were most frequently associated with telomeres or euchromatin.

We observed prophase and metaphase cells with very irregular chromosome structure and condensation. These included chromosomes with abnormal condensation patterns (Figure 5d,f) and others that did not contain telomeric sequences (Figure 5f,g,h,i). Other alterations/rearrangements included circular Y chromosomes (Figure 5e), pericentromeric/centromeric fragments, and telomeric fragments (Figure 5g). We also observed metaphase chromosomes that appeared to be connected to chromatin masses (Figure 5i). These observations suggest that chromosome fragmentation and chromosome rearrangements may involve different chromosome regions.

Mutant brains were generally smaller than wild-type brains of similar stages. Since the mitotic progression of mutant neuroblasts was not delayed and the mitotic indices were only moderately lower than those for wild-type controls, this difference could only be explained by the elevated loss of cells during development. Accordingly, glu+/glu− and glu+/glu− third-instar larval brains were stained for apoptotic cells. Wild-type brains had a low frequency of apoptotic cells (0.5%), while mutant brains contained a much higher number of apoptotic cells (2.5% in glu+/glu− and 3.0% in glu+/glu−). Since chromosome breakage was detected in mutant cells, it appeared most likely that
the increased level of apoptosis was a consequence of irreversible chromosome damage.

**Chromosome condensation in DmSMC4 mutant neuroblasts**

The cytological analysis of mutant cells suggested that chromosomes had abnormal condensation patterns (see Figure 3a). Two specific parameters of chromosome morphology were analyzed in detail. We measured the length of chromosome arms to assess whether mutant cells were able to shorten the longitudinal axis of chromosomes, and we also quantified the total number of chromosomes that had clearly defined sister chromatids in order to assess whether proper sister chromatid resolution takes place. We arrested mutant cells in prometaphase with colchicine for different periods of time, and we hybridized them with subtelomeric- and pericentromeric-specific probes to avoid measuring chromosomes that had lost distal fragments (Figure 6a). Only chromosomes that had both pericentromeric and subtelomeric signals were used for the quantification (Figure 6b,c and Table 1). Representative samples of wild-type, *glu1*/*glu2*, or *glu1*/*glu2* cells that were hybridized with probes to chromosome II are shown (Figure 6a). Chromosome length was measured as the number of pixels (0.091 μm/pixel) from the center of the subtelomeric hybridization signal to the center of the pericentromeric signal for each arm, and the two values were added (Figure 6b and Table 1). Overall, the results showed that mean chromosome length (chromosome II and III) for either mutant combination (*glu1*/*glu2* and *glu1*/*glu2*) that had been incubated for different times in colchicine (0, 30, and 60 min) did not differ significantly from that of the wild-type controls. Accordingly, the dynamics of chromosome shortening in these experimental conditions were similar in both wild-type and mutant cells (Table 1).

In order to evaluate if sister chromatid resolution was affected, we counted the number of complete chromosomes that showed clearly resolved sister chromatids (Figure 6c). We found that, in the absence of colchicine incubation, the percentage of mitotic chromosomes in mutant cells showing sister chromatid resolution, *glu1*/*glu2* (46.8%) and *glu1*/*glu2* (32%), was significantly lower than in wild-type controls (75%). As expected from the severity of the alleles, this phenotype was stronger in *glu1*/*glu2* or *glu1*/*glu2* cells that had been incubated for different times in colchicine (0, 30, and 60 min) did not differ significantly from that of the wild-type controls. Incubation of either wild-type or mutant cells in colchicine for 30 min did not have any effect in terms of resolution. However, incubation for 60 min led to a reduction in the percentage of chromosomes exhibiting visible sister chromatid resolution in both wild-type and mutant cells. This effect was probably a consequence of overshortening the longitudinal axis of chromosomes during mitotic arrest, which thus made it difficult to distinguish sister chromatids. Nevertheless, the differences observed between mutant and wild-type chromosomes could never be abolished, and this finding suggests that chromatin of mutant cells was not able to compact properly to allow adequate sister chromatid resolution.

**Localization of Barren in DmSMC4 mutant neuroblasts**

Since, similar to wild-type cells, chromosomes in mutant neuroblasts retained the ability to shorten their arms with normal kinetics, we wanted to address whether these still contained detectable levels of other condensin subunits that might mediate this process. We chose to use the anti-Barren antibody to determine the distribution of this condensin subunit in mutant cells since it has a much higher titer than do DmSMC4 antibodies. This higher titer allows visualization of very low protein levels (Figure 7). Wild-type cells show Barren localized throughout the chromosome axis and some accumulation at the centromere (Figure 7a). Mutant cells that show chromosomes with largely resolved sister chromatids contained normal levels of Barren (Figure 7b). However, most mutant cells that displayed abnormal chromatin compaction and partly resolved sister chromatids had reduced amounts of Barren, and this finding suggests that DmSMC4 may be essential for its proper localization (Figure 7c, top panel). In these cells, the level of sister chromatid resolution was directly related to the intensity of Barren staining. This result suggests an inverse correlation between the level of condensin complex and the severity of the phenotype. Additionally, chromosomes in mutant cells frequently showed most intense labeling of the centromeric region, with labeling intensity decreasing gradually to the telomeres (Figure 7c). Finally, in mutant cells that contained metaphase chromosomes with clearly unresolved sister chromatids, the localization of Barren appeared diffuse (Figure 7c, lower panel). This suggests that the sister chromatid cores did not individualize and that sister chromatid resolution probably requires stoichiometric amounts of the Barren subunit but likely also of the entire condensin complex.

**Discussion**

The results presented in this study extend our understanding of the role of the SMC4 protein in mitotic chromosome dynamics. Until now, yeast genetic analyses and *Xenopus* biochemical data have been crucial for the identification and study of the condensin subunits. Here we have been able to exploit the genetics and well-defined cytology of the fruit fly to study the consequences of the loss of the core condensin subunit SMC4 on chromosome structure and mitotic progression in a metazoan organism.

**The gluon locus encodes the Drosophila SMC4 condensin subunit**

Our results show that *Dmsmc4* encodes a protein that clearly belongs to the SMC4 subfamily of SMC proteins; this protein is one of the two SMC subunits of the condensin complex. In *Xenopus* and yeast, the protein is found...
Figure 6

Characterization and quantification of chromosome condensation parameters. (a) Representative images of prometaphase chromosomes used for the quantification. Wild-type, glu2/glu2, and glu1/glu2 neuroblasts, were incubated in colchicine for different times (0, 30, and 60 min) and hybridized with a centromeric probe for chromosome 2 (RSP; shown in blue) and specific sub-telomeric probes for 2L and 2R (shown in green). DNA is stained in red. We also did similar preparations, not shown in this figure, for chromosome 3 by using different probes. (b) Frequency distribution \( f(l) \) of the length \( l \) of chromosomes II and III in wild-type, glu2/glu2, and glu1/glu2 neuroblasts after they were incubated for different times in colchicine. (c) Graphical representation of the percentage of chromosomes (shown in Table 1) showing resolved sister chromatids in wild-type, glu2/glu2, and glu1/glu2 neuroblasts after they were incubated for different times in colchicine. A total of 979 chromosomes from 52 brains were scored. The scale bar represents 5 μm.

in a multi-subunit complex consisting of SMC2/4 as well as CAP-G, -H, and -D2 proteins [16, 20]. DmSMC4 is likely to be found in a similar complex in Drosophila, as we have identified all subunit homologs by sequence homology [9], and DmSMC2/DmSMC4/Barren and additional proteins can be communoprecipitated from embryo extracts (Shen and Wu, personal communication).

DmSMC4 is an essential gene, as animals homozygous for either of two P insertion alleles die as embryos (glu*) or as larvac/pupae (glu*). The maternal contribution of DmSMC4 is presumably sufficient to sustain embryos through the early syncytial blastoderm cycles. DmSMC4 was found to be expressed in all mitotically active tissues and developmental stages. The highest levels of protein...
were found in tissues containing a high number of proliferating cells, and this observation is consistent with DmSMC4 being required for both mitotic and meiotic cell divisions.

Table 1
Quantification of chromatid arm length in wild-type and mutant cells after colchicine incubation (μm).

<table>
<thead>
<tr>
<th>Genotypes</th>
<th>Time in colchicine</th>
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<tbody>
<tr>
<td></td>
<td>0 min</td>
</tr>
<tr>
<td>Wild type</td>
<td>II</td>
</tr>
<tr>
<td></td>
<td>6.80 ± 3.23</td>
</tr>
<tr>
<td>Glu1/glu2</td>
<td>II</td>
</tr>
<tr>
<td></td>
<td>7.60 ± 2.45</td>
</tr>
<tr>
<td>Glu2/glu2</td>
<td>II</td>
</tr>
<tr>
<td></td>
<td>6.59 ± 1.32</td>
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Results for chromosome 2 (II) or chromosome 3 (III) are shown.

DmSMC4 localizes to mitotic chromosomes

DmSMC4 exhibits a cell cycle distribution expected of a protein involved in the condensation and maintenance of mitotic chromosome structure. It is loaded onto condensing chromosomes early in prophase and dissociates late in anaphase/telophase when the condensation process is reversed. During these stages, the protein shows a restricted distribution running along the longitudinal axis of chromatids, similar to what has been observed for topo II and ScII/SMC2 [12, 31]. A minor pool of DmSMC4 remains associated with chromatin in telophase and interphase, whereas the major fraction becomes cytoplasmic at this time. The initial increase in chromatin association occurred before the onset of nuclear-envelope breakdown, as we observed by costaining for the nuclear lamina (data not shown). While immunostaining for SMC4 has revealed interphase cytoplasmic localization in S. pombe [16], in Xenopus the protein appears to be nuclear during interphase [13]. The human and chicken CAP-E [SMC2] proteins have also been reported to be nuclear [12, 32]. The differences in interphase localization could be due to differential solubility during fixation procedures of nuclear versus cytoplasmic SMC4. In Xenopus, mitosis specific phosphorylation of condensin controls its activity, whereas in S. pombe activity appears to be controlled by the shuffling of condensin in and out of the nucleus.

We have additionally shown that Barren colocalizes with DmSMC4 on condensed chromosomes and that the localization of Barren is dependent on the presence of DmSMC4. These findings are consistent with participation in a common complex, as has been shown in Xenopus and yeast [13, 16]. This complex may only be formed late in interphase, however, as Barren and DmSMC4 do not appear to colocalize in the cytoplasm. This finding is consistent with the presence of a separate condensin “regulatory” complex containing the non-SMC subunits [33]. On condensed chromosomes, DmSMC4 and Barren are more concentrated in the centromeric region. This observation is consistent with chromosome condensation initiating at the centromere and then spreading distally, similarly to the way in which mitosis-specific phosphorylated forms of histone H3 accumulate [30]. A role for DmSMC4 and Barren in the condensation of the centromeric hetero-

Immunolocalization of Barren in metaphase chromosomes from wild-type and glu1/glu2 neuroblasts. (a) Wild-type neuroblasts in metaphase that show Barren staining throughout the chromosome arms and accumulation at the centromere region. (b) Mutant neuroblast showing well-condensed chromosomes with separated sister chromatids and high levels of axial staining. (c) Two neighboring cells showing abnormally condensed chromosomes with unresolved sister chromatids and diffuse labeling of the longitudinal axis of the chromosomes. Note the stronger accumulation of Barren at the primary constriction. The scale bar represents 5 μm.
chromatin underlying the kinetochore may be necessary for the proper assembly of the kinetochore. Indeed studies in *S. cerevisiae* have implicated BRN1 in normal kinetochore function [27]. Interestingly, topo II also shows a similar enrichment at centromeres in a number of mammalian cells [34].

**DmSMC4 is required for sister chromatid resolution**

To analyze in detail the effects of *DmSMC4* mutants on chromosome morphology, we quantified two aspects of chromosome condensation in neuroblasts. Our results unambiguously indicated that the shortening of the longitudinal axis of the chromosome was unaffected in *DmSMC4* mutants and occurred with the same kinetics as in wild-type cells. However, we observed a significant reduction in the ability of *DmSMC4* mutant cells to resolve sister chromatids during prometaphase. Arresting mutant neuroblasts with colchicine to allow more time for the condensation process did not rescue the defect in resolution, while chromosome shortening proceeded as in wild-type cells.

How is the chromosome axis shortened when DmSMC4 levels are reduced? The axial shortening of chromosomes observed in mutant cells suggests that either the residual level of DmSMC4 is sufficient to support this aspect of condensation or other factors are responsible for this process. The fact that the kinetics of length reduction appear to be independent of DmSMC4 levels would argue against DmSMC4 involvement in this process. Several results support the hypothesis that topo II might be the enzyme responsible in part for the shortening of the axes [35]. Firstly, topo II has been shown to be required for the hypercondensation of mitotic chromosomes after the treatment of yeast cells with microtubule-depolymerizing agents [5]. Secondly, blocking mammalian cells with the ICRF topo II inhibitors leads to hypocondensed mitotic chromosomes [36, 37]. Thirdly, topo II is targeted to chromosome independently of the condensin complex in vitro [20]. Alternatively, other members of the condensin complex may still mediate arm shortening.

The fact that *DmSMC4* mutant cells are able to shorten their longitudinal chromosome axes but are unable to properly resolve sister chromatids indicates that these two aspects of chromosome condensation can be separated by mutation. Current models suggest that one purpose of chromosome condensation (probably shortening of the axis) is to displace the topo II catenation/decatenation enzyme responsible in part for the shortening of the axes [35]. The fate of *Drosophila* cohesin components [40] in cells that carry mutations of condensin components will be an important question to address.

**Mutations in DmSMC4 cause abnormal chromosome segregation**

*DmSMC4* mutations affect not only chromosome condensation but also chromosome segregation. All alleles examined in this study cause chromosome segregation defects, manifest as chromatin bridges in anaphase and telophase. Chromosome segregation defects are likely to be the consequence of aberrant sister chromatid resolution. Studies in yeast of mutations for *top2*, *cut3*, and *cut14* strongly suggest that proper chromosome condensation is essential for normal sister chromatid separation [5, 14]. We believe that the chromatin bridges present in *DmSMC4* mutant cells are unlikely to be resolved, as significant chromosome breakage can be observed. The ultimate consequence of this state is probably the increased level of apoptosis observed in mutant brains.

**Materials and methods**

**Analysis and quantification of mitotic phenotypes**

All fly stocks were grown at 25°C in standard media. Oregon-R or Canton-S lines were used as wild-type stocks. To identify homozygous *glu2* or transheterozygous *glu1*/*glu2* third-instar larvae, we balanced the *glu1* and *glu2* bearing chromosomes over *T(2;3)SM5a-TM6B* (Cayetano González, personal communication). For the quantification of mitotic figures, we prepared third-instar larval brains by using standard protocols except that we chose timing of the hypotonic treatment (1 min) that would allow us to identify the condensation defects without disrupting anaphase figures. This protocol did not allow distinction between telophase and interphase nuclei in wild-type brains, but in the mutants, telophases were easily recognized due to their elongated morphology. We performed quantification by counting the total number of nuclei in brains. To identify embryos homozygous for *glu*, we balanced the mutant chromosomes over *CyO, P[w*¹⁸ = GAL4-Kr.C]DC3, P[w*¹⁸ = UAS-GFP.S65T]DC7* [41]. One hour collections of embryos were aged for 12 hr, dechorionated, and then sorted under a fluorescence dissecting microscope. The sorted embryos were fixed in 40% EM grade paraformaldehyde for 3 min and devitellinized in methanol prior to staining for α-tubulin, phosphohistone H3, and DNA.

**Generation of antigen and antibody**

We produced a polyhistidine (6×His)-tagged fragment of DmSMC4 corresponding to residues 80–303 by cloning a Ncol/BgIII fragment of the cDNA into the pET23d expression vector (Novagen) and expressing it in *E. coli* BL21(DE3). The protein was partially purified on Ni-agarose columns (Qiagen) before it was applied to SDS-PAGE. Purified protein was obtained from gel slices and injected into rabbits and sheep (Scottish Antibody Production Unit). The anti-DmSMC4 antibodies were used for Western blotting and immunofluorescence as whole serum (1:1000 and 1:500) respectively or were affinity purified on antigen-coated nitrocellulose strips (1:100 and 1:5 respectively), with similar outcomes.

**Quantification of chromosome condensation**

For quantitative studies of arm length and sister chromatid separation, brains were incubated in 10 µM colchicine for 0, 30, and 60 min. They were then treated with a hypotonic solution for 3 min before fixation with
acetic acid, as previously described [42]. Constant-size (59 × 40 mm) images were collected with a Confocal Microscope MRC600. Quantification analysis was performed with the University of Texas Health Science Centre Image Tool version 2.00 for Windows (http://ddsdx.uthscsa.edu/dig/download.html).

Supplementary material
Supplementary material including additional materials and methods is available with the electronic version of this article at http://current-biology.com/supmatin.htm.

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