Condensin-dependent localisation of topoisomerase II to an axial chromosomal structure is required for sister chromatid resolution during mitosis

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Summary

Assembly of compact mitotic chromosomes and resolution of sister chromatids are two essential processes for the correct segregation of the genome during mitosis. Condensin, a five-subunit protein complex, is thought to be required for chromosome condensation. However, recent genetic analysis suggests that condensin is only essential to resolve sister chromatids. To study further the function of condensin we have depleted DmSMC4, a subunit of the complex, from Drosophila S2 cells by dsRNA-mediated interference. Cells lacking DmSMC4 assemble short mitotic chromosomes with unresolved sister chromatids where Barren, a non-SMC subunit of the complex is unable to localise. Topoisomerase II, however, binds mitotic chromatin after depletion of DmSMC4 but it is no longer confined to a central axial structure and becomes diffusely distributed all over the chromatin. Furthermore, cell extracts from DmSMC4 dsRNA-treated cells show significantly reduced topoisomerase II-dependent DNA decatenation activity in vitro. Nevertheless, DmSMC4-depleted chromosomes have centromeres and kinetochores that are able to segregate, although sister chromatid arms form extensive chromatin bridges during anaphase. These chromatin bridges do not result from inappropriate maintenance of sister chromatid cohesion by DRAD21, a subunit of the cohesin complex. Moreover, depletion of DmSMC4 prevents premature sister chromatid separation, caused by removal of DRAD21, allowing cells to exit mitosis with chromatin bridges. Our results suggest that condensin is required so that an axial chromatin structure can be organised where topoisomerase II can effectively promote sister chromatid resolution.

Key words: SMC4, DRAD21, Topoisomerase II, Condensin, Cohesin, Chromosomes, Mitosis, Drosophila

Introduction

During each cell division the genome of proliferating cells undergoes significant structural changes to ensure proper segregation during mitosis. Chromosome condensation and resolution of sister chromatids is thought to be required to resolve entangled chromatin fibres and to reduce chromosome arm length, as well as to increase the mechanical resistance of chromatids for segregation. Condensation of chromatin into compact mitotic chromosomes is a highly complex process that has only recently begun to be unravelled. A major breakthrough was the identification of condensin, a five-subunit protein complex (Hirano et al., 1997) that localises throughout the axis of sister chromatids (Hirano et al., 1997; Steffensen et al., 2001). Condensin is essential for cell viability in Saccharomyces cerevisiae, S. pombe, Caenorhabditis elegans, Drosophila melanogaster and humans (Biggins et al., 2001; Freeman et al., 2000; Hagstrom et al., 2002; Lavoie et al., 2000; Saka et al., 1994; Steffensen et al., 2001; Strunnikov et al., 1995; Sutani et al., 1999). Two of these subunits are SMC2 and SMC4 (DmSMC2 and DmSMC4 or gluon in Drosophila) that are members of the ubiquitous SMC (structural maintenance of chromosomes) protein family. The complex also contains three non-SMC proteins (Brn1p/Ycs3p/cnd2/XCAP-H/Barren/BRRN1, Ycg1/Yc5p/cnd3/XCAP-G/hCAP-G and Ycs4p/cnd1/XCAP-D2/hCAP-D2 in S. cerevisiae, S. pombe, C. elegans, D. melanogaster, Xenopus laevis and humans respectively), that are essential for its activity both in vivo and in vitro (Cubizolles et al., 1998; Hirano et al., 1997; Kimura et al., 2001; Kimura and Hirano, 2000; Lavoie et al., 2002; Lavoie et al., 2000; Ouspenski et al., 2000; Sutani et al., 1999). Biochemical characterisation of the complete condensin complex isolated from Xenopus extracts (13S) showed that it is composed by two subcomplexes: an 8S core subcomplex (8S), consisting of the SMC subunits XCAP-C/SMC4 and XCAP-E/SMC2 and an 11S regulatory subcomplex (11S) containing the three non-SMC subunits XCAP-D2, XCAP-G, XCAP-H. The 11S complex is thought to play a regulatory role since it facilitates the ATP-dependent positive supercoiling activity of the complex in vitro (Kimura et al., 2001). Even though both 8S and 13S can bind to DNA in vitro, only the 13S shows DNA-stimulated ATPase function and supports ATP-dependent chromatin condensation in Xenopus extracts (Kimura et al., 2001). Recently, in vitro studies in budding yeast showed that only the 13S complex is able to bind chromatin (Lavoie et al., 2002). Chromosome condensation and sister chromatid segregation...
also require topoisomerase II (Topo II) both in vivo (Uemura et al., 1987) and in vitro (Adachi et al., 1991; Hirano and Mitchison, 1991). Topo II has been shown to be a major component of mitotic chromosomes where it localises to centromeres and throughout the axis of sister chromatids (Earnshaw et al., 1985; Gasser et al., 1986). More recent results show that in HeLa cells Topo II accumulates at discrete sites along prometaphase chromatids alternating with sites where Barren localises (Maeshima and Laemmli, 2003). Since condensins have the ability to modify DNA Topology in vitro, it is of great interest to determine how they interact with Topo II. Previously, it was shown that Barren interacts physically with Topo II and directly modulates its activity in vitro (Bhat et al., 1996). barren mutants exhibit chromosome segregation defects, in which the centromeres separate but chromosome arms do not resolve. These mitotic phenotypes are very similar to those exhibited by DNA Topo II mutants of <i>S. cerevisiae</i> (Holm et al., 1985) with the exception that they do not accumulate catenated DNA molecules or show chromosome breakage (Bhat et al., 1996). However, we have recently shown that <i>Drosophila</i> cells mutant for <i>dmSmc4</i> show a phenotype reminiscent of yeast Topo II mutants (Steffensen et al., 2001). Loss of DmSMC4 in <i>Drosophila</i> allows normal longitudinal shortening of the chromosome but sister chromatids are not resolved resulting in the formation of anaphase chromatin bridges and DNA breakage. Since Topo II catalytic activity is required for the contraction of chromosomes (Gimenez-Abian et al., 1995; Gorbsky, 1994), it is likely that in the absence of DmSMC4, Topo II remains functional. However, Topo II also mediates decatenation of DNA early in mitosis (Gimenez-Abian et al., 1995), which might be affected in the <i>Drosophila DmSmc4</i> mutant resulting in the formation of chromatin bridges during anaphase.

However, anaphase bridges could also result from inappropriate maintenance of cohesion between sister chromatid arms. Cohesion of sister chromatids during most of the cell cycle is mediated by cohesin, a protein complex made up of at least four subunits including SMC1 and SMC3, two members of the SMC protein family, and two non-SMC subunits, Scc1p/Mcd1p (Rad21p in fission yeast and DRAD21 members of the SMC protein family, and two non-SMC subunits, Scc1p/Mcd1p (Rad21p in fission yeast and DRAD213p (Guacci et al., 1997; Michaelis et al., 1997; Toth et al., 1999)). In yeast, cohesion is established during S phase and cohesins remain tightly associated with chromosomes until the metaphase-anaphase transition, when Scc1p is cleaved in a reaction dependent on a cysteine protease called Separase (Nasmyth et al., 2000; Uhlmann et al., 1999). This transition is triggered by the activation of the anaphase-promoting complex/cyclosome (APC/C) (Ciosk et al., 1998; Cohen-Fix et al., 1996; Funabiki et al., 1996; Glotzer, 1999; Uhlmann et al., 1999), which targets a protein called Securin for degradation by ubiquitin-dependent proteolysis. Destruction of Securin releases Separase, which can now cleave Scc1p. In Xenopus egg extracts (Losada et al., 1998), human (Sumara et al., 2000) and mouse cells (Darwiche et al., 1999) cohesins bind to chromatin during interphase but dissociates from chromosome arms early in mitosis, while centromere-associated cohesin remains bound until the metaphase-anaphase transition where it holds sister chromatids together until anaphase onset (Hoque and Ishikawa, 2001; Losada et al., 2000; Waizenegger et al., 2000; Warren et al., 2000). These observations have led to a model in which dissolution of sister chromatid cohesion in higher eukaryotes occurs as a two-step process (for a review, see Losada and Hirano, 2001). During prophase, as chromosomes condense, arm cohesion is lost through an APC/C-independent process. Subsequently, at anaphase onset, centromere-associated cohesion is lost through an APC/C-dependent pathway involving the Separase-mediated cleavage of the SCC1/Rad21 subunit.

Since loss of arm cohesion occurs at the time when chromosome condensation is taking place, it is possible that the two processes are mechanistically linked. Indeed, it has been shown in yeast that mutations in cohesion factors not only affect sister chromatid cohesion but also perturb the establishment and maintenance of chromosome condensation (Castano et al., 1996). More recently, it has been demonstrated that proper function of condensins requires previous binding of cohesins to chromatin (Lavoie et al., 2002). However, in higher eukaryotes the loss of cohesins does not affect chromosome condensation (Losada et al., 1998; Sonoda et al., 2001; Vass et al., 2003).

In this study we analysed the role of condensin in the organisation and segregation of mitotic chromosomes by depleting DmSMC4 in <i>Drosophila</i> S2 cells. We show that DmSMC4 can be depleted in S2 cells by dsRNAi resulting in abnormal chromosome condensation and segregation. Depletion of DmSMC4 also affects the localisation of Barren, a non-SMC subunit of the condensin complex, to mitotic chromatin, suggesting that only the complete condensin complex binds chromatin in vivo. Furthermore, after depletion of DmSMC4 Topo II fails to localise to a clearly defined chromatin axis structure and protein extracts from DmSMC4-depleted cells exhibit a significant decrease in Topo II DNA decatenation activity. Surprisingly, abnormally condensed chromosomes in DmSMC4-depleted cells appear to organise functional kinetochores that bind microtubules and undergo segregation. We also show that depletion of DmSMC4 does not affect the localisation or removal of the cohesin complex during mitosis suggesting that the chromatin bridges observed after depletion of condensin are not due to inappropriate localisation of cohesin. However, depletion of DmSMC4 prevents premature sister chromatid separation caused by loss of DRAD21, and allows cells to exit mitosis. Our results suggest that condensin is required for the organisation of a central axial chromatin structure where Topo II can localise and promote sister chromatid resolution.

**Materials and Methods**

**RNA interference**

RNAi was performed in <i>Drosophila</i> S2 tissue culture cells as previously described (Clemens et al., 2000). A 950 bp <i>EcoRI-SalI</i> or an 800 bp <i>EcoRI-KpnI</i> fragment from the 5’ end of DmSMC4 or DRAD21 cDNAs, respectively, were cloned into both pSPT18 and pSPT19 expression vectors (Roche). The recombinant plasmids were used as templates for RNA synthesis using the T7 Megascript kit (Ambion) and 15 μg of dsRNA were added to 10<sup>6</sup> cells in all RNAi experiments. At each time point, cells were collected and processed for immunoblotting or immunofluorescence. For immunoblotting, 10<sup>6</sup> cells were collected by centrifugation, resuspended in 20 μl of sample buffer, sonicated, and boiled for 3 minutes before loading on 5-20% gradient SDS-polyacrylamide gels.
Immunofluorescence in S2 cells
S2 cells were fixed in 3.7% formaldehyde, 0.5% Triton X-100 in PBS for 10 minutes and then washed three times for 5 minutes in PBS-T (PBS with 0.05% Tween 20). Blocking was performed in PBS-TF (PBS-T with 10% fetal calf serum) for 1 hour at room temperature. Incubations with primary antibody were performed in PBS-TF overnight at 4°C or for 2 hours at room temperature. Slides were washed again three times for 5 minutes in PBS-T. Incubation of fluorescently labelled secondary antibodies was done according to manufacturer’s instructions (Jackson ImmunoResearch Laboratories and Vector Labs, UK). Slides were washed with PBS-T (3 times 5 minutes each), and then mounted in Vectashield (Vector, UK) containing either 1 μg/ml of DAPI, for observation in the Zeiss Axiolab 200M microscope (Zeiss, Germany), or 1.0 μg/ml propidium iodide for confocal microscopy, using a Biorad MRC600 microscope (Biorad, UK). Image stacks were recorded with an Axioacam (Carl Zeiss, Germany). Data stacks were deconvolved, using the microscope Axiovision 3.1 Software (Carl Zeiss, Germany).

Antibodies
The primary antibodies were anti-α-tubulin (mouse mAb B512), anti-phosphorylated H3 rabbit polyclonal, used at 1:500 (Upstate Biotechnology); anti-Cdc protein polyclonal, used at 1:200 (Blower and Karpen, 2001); anti-DmSMC4 antibodies (rabbit, 1:500 or sheep, 1:200) as described previously (Steifens et al., 2001); anti-DRAD21 (Warren et al., 2000) rabbit polyclonal (1:500); anti-Barren rabbit polyclonal, used at 1:1000 (Bhat et al., 1996), anti-Polo mouse mAb MA294, used at 1:50 (Llamazares et al., 1991) and anti-Topo II mouse monoclonal antibody P2G3 used at 1:20 (Swedlow et al., 1993).

Mitotic chromatin immunoprecipitation assay
Mitotic chromatin immunoprecipitation assays were carried out as described previously (Mirkovitch et al., 1988). S2 cells were grown exponentially to 2×10^6 cell/ml, blocked in mitosis with 30 μM colchicine for 8 hours, incubated on ice for 45 minutes and centrifuged at 1500 g for 15 minutes at 4°C. Cells were resuspended in 1 ml of cold PBS in the presence of protease inhibitors, kept on ice for 45 minutes and lysed in 1 ml of lysis buffer (15 mM Tris-HCl, pH 7.4, 0.2 mM spermine, 0.5 mM spermidine, 2 M K-EDTA, 1 mM EGTA, 0.15 mM KCl, 15 mM NaCl, 1 mM DTT, 0.05% Triton X-100) with 0.1% (v/v) digitonin. Cells were lysed with a B-type pestle in a Dounce homogeniser. Dyna-beads coated with sheep anti-rabbit IgG (Dynal Biotech) were washed with lysis buffer. 5 μl of Dyna-beads were used to clear 1 ml of cell protein extract. Mitotic chromatin was labelled by incubating cell extracts with anti-phosphohistone H3 (Upstate Biotechnology) overnight at 4°C. Immunoprecipitates were captured by incubation with 10 μl of Dyna-beads (6-7×10^5 beads/μl) according to the manufacturer’s instructions. Complexes were washed 3 times with lysis buffer and resuspended in 50 μl of 2× Laemmli sample buffer.

Preparation of cell extracts
The preparation of cell extracts for Topo II assays was done using 30 ml of exponentially growing cells at 2×10^6 cell/ml as previously described (Mohamed et al., 1987). Cells were incubated on ice for 45 minutes, collected by centrifugation at 1500 g for 15 minutes at 4°C and resuspended in 1 ml of cold PBS. Cells were then centrifuged for 10 minutes at 4°C and washed in 5 ml of extraction buffer (50 mM Tris-HCl pH 7.5, 25 mM KCl, 3 mM MgCl2, 0.25 M sucrose, 2 mM DTT, 1 mM EDTA) in the presence of protease inhibitors. Pellets were resuspended in 2 ml of the same buffer and homogenised in a Dounce homogeniser at 4°C. The extracts were adjusted to 400 mM KCl and centrifuged at 50,000 rpm for 60 minutes at 4°C. The supernatant was adjusted to 70% (NH4)2SO4 with a saturated solution and left at 4°C for 30 minutes. The precipitated proteins were collected by centrifugation at 10,000 g for 15 minutes at 4°C and resuspended in 0.5 ml dialysis buffer (40 mM Tris-HCl, pH 7.5, 2 mM DTT, 1 mM EDTA, 0.25 M sucrose).

Topoisomerase II assays
Topo II assays were performed with kinetoplast DNA (kDNA) extracted from cultures of Leishmania infantum clone MHOM/MA67ITMAP263 as previously described (Kelly, 1993) in the presence of 1 mM ATP. kDNA decatenation assays were performed for 30 minutes at 37°C and stopped by addition of 4 μl of 2.5% SDS; 25 mM EDTA, 30% sucrose, 0.25% Bromophenol Blue.

Results
Depletion of SMC4 by dsRNA interference in S2 cells
In order to deplete DmSMC4, Drosophila S2 cells were treated with dsRNA and the levels of the protein was monitored at different times by immunoblotting (Fig. 1A). DmSMC4 protein decreased significantly at 48 hours and was barely detectable 120 hours after treatment. Titration of the antibody confirmed that at these cell densities the protein should have been easily detected (Fig. 1B). Cell viability was not affected as determined by Trypan Blue staining (Fig. 1C) and the doubling time of control and DmSMC4 dsRNA-treated cells was similar throughout the experiment (Fig. 1D). The percentage of mitotic cells and overall phenotype of DmSMC4 dsRNA-treated cells was analysed after immunostaining with an anti-phosphohistone H3 antibody to identify mitotic cells (Fig. 1E-G; Table 1). The mitotic index in DmSMC4-depleted cells was similar to that of controls (Fig. 1E) and mitotic progression was not significantly affected (Fig. 1F). However, soon after DmSMC4 RNAi treatment (24 hours) mitotic cells had abnormally condensed chromosomes that did not show well-defined sister chromatids, and chromatin bridges were observed in anaphase cells (Fig. 1G and Table 1; see also Fig. 2). At later times virtually all mitotic cells showed abnormal chromosome condensation during prometaphase and chromatin bridges during anaphase and telophase (Table 1). Incubation of DmSMC4-depleted cells with colchicine causes mitotic arrest with unseparated sister chromatids indicating that the spindle

| Time (h) | Control | RNAi |
|---------|---------|------|
| 24      | 0       | 28.6 |
| 48      | 0       | 54.5 |
| 72      | 0       | 73.7 |
| 96      | 0       | 97.2 |
| 120     | 0       | 100  |
| 144     | 0       | 100  |

| *Unresolved sister chromatids (%) | Chromatin bridges |
|----------------------------------|-------------------|
| Control | RNAi | Control | RNAi |
| 0       | 0    | 0       | 0    |
| 0       | 0    | 0       | 0    |
| 0       | 0    | 0       | 0    |
| 0       | 0    | 0       | 0    |
| 0       | 0    | 0       | 0    |

*Cells in prometaphase. Total number of cells counted for controls: 24 hours n=1768, 48 hours n=2041, 72 hours n=2304, 96 hours n=2724, 120 hours n=2066 and 144 hours n=1146. Total number of cells counted for RNAi-treated cells: 24 hours n=1792, 48 hours n=1958, 72 hours n=2270, 96 hours n=2888, 120 hours n=1902 and 144 hours n=2091.
checkpoint is active in these cells (data not shown; see also Fig. 3B). These results indicate that depletion of DmSMC4 does not compromise the length-wise compaction of mitotic chromosomes but causes abnormalities in sister chromatid resolution and segregation in Drosophila S2 cells.

Immunolocalisation of Barren after depletion of DmSMC4

In order to evaluate whether DmSMC4 is required for the localisation of non-SMC proteins of the condensin complex, we analysed the distribution of Barren on mitotic chromatin after depletion of DmSMC4 (Fig. 2). In control prometaphase and anaphase cells, DmSMC4 and Barren showed co-localisation to a well-defined chromosomal axial structure. By contrast, soon after the addition of DmSMC4 dsRNA, when cells showed a significant reduction in the level of DmSMC4, chromosomes displayed abnormal condensation and most did not resolve sister chromatids. More significantly, the lower levels of DmSMC4 or Barren detected in these chromosomes no longer localised to a well-defined chromatin axis but appeared diffusely distributed all over the chromatin. After
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Depletion of DmSMC4 for longer periods the protein was not detected by immunofluorescence and Barren was completely absent from the abnormally condensed chromosomes. All prometaphase cells contained chromosomes with unresolved sister chromatids and most cells in anaphase/telophase showed chromatin bridges (Fig. 2). Since Barren was detected in DmSMC4-depleted cell extracts by immunoblotting (see below and data not shown), these results indicate that its localisation to mitotic chromatin requires DmSMC4. These findings suggest that only an intact condensin complex is able to associate with chromatin and that normal levels of DmSMC4 are needed to maintain its localisation to a clearly defined chromatid axis.

**Organisation of centromeres and kinetochores in the absence of DmSMC4**

DmSMC4-depleted cells show abnormally condensed chromosomes, however these cells progress into anaphase and attempt to segregate their chromosomes but display extensive chromatin bridges. To determine whether centromere and kinetochore organisation is normal, cells lacking DmSMC4 were immunostained for a centromere marker CID, the *Drosophila* homologue of CENP-A (Henikoff et al., 2000) and for Polo, a protein that localises to the kinetochore (Logarinho and Sunkel, 1998). In control cells CID localised to the centromeres of mitotic chromosomes and lead sister chromatids during segregation (Fig. 3A). Depletion of DmSMC4 did not alter the localisation of CID, which appeared as discrete dots over the abnormally condensed chromatin throughout mitosis (Fig. 3B). However, in early anaphase cells, centromeres as shown by CID staining, appeared to stretch poleward sometimes well beyond the chromatin and during later stages centromeres were clearly leading sister chromatids to the poles. Dots of CID staining were only rarely observed associated with chromatin bridges during anaphase. If cells were incubated with colchicine to depolymerise microtubules and thus prevent exit from mitosis, CID staining was consistently observed as two dots on each side of prometaphase chromosomes as in control cells (Fig. 3B).

To analyse kinetochrome organisation DmSMC4-depleted cells were immunostained for Polo (Fig. 3C,D). In control cells Polo localised to the centromeres from late G2 to cytokinesis and to kinetochores from early prometaphase to late anaphase.
In DmSMC4-depleted cells (Fig. 3D), Polo localisation at kinetochores showed a very similar pattern to that described for CID. During early anaphase, kinetochores stained for Polo also appeared to stretch poleward outside the chromatin. This phenotype was not observed after incubation with colchicine, CID-stained centromeres localise over the chromosomes. (C) Control cell immunostained with anti-POLO antibodies show localisation to kinetochores and to centrosomes located at either side of the metaphase plate. (D) After depletion of DmSMC4, POLO localises to kinetochores that during anaphase can also be seen to stretch towards the poles. (E) Control cells stained for CID and tubulin show centromeres associated with microtubule bundles. A higher magnification image is shown in the left panel. (F) In DmSMC4-depleted cells centromeres stretched beyond the chromatin associated with microtubule bundles. Higher magnification images are shown in the left panels. Scale bars: 5 µm.

(Fig. 3C). In DmSMC4-depleted cells (Fig. 3D), Polo localisation at kinetochores showed a very similar pattern to that described for CID. During early anaphase, kinetochores stained for Polo also appeared to stretch poleward outside the chromatin. This phenotype was not observed after incubation with colchicine (data not shown). To determine whether these kinetochores were able to bind to microtubules, DmSMC4-depleted cells were immunostained for CID, tubulin and DNA (Fig. 3E,F). In control cells, microtubule bundles can be clearly seen to run from the spindle poles and terminate at CID-positive centromeres. In DmSMC4-depleted cells, microtubule bundles were also observed to contact CID-positive centromeres that appear to be located outside chromatin (Fig. 3F). These results suggest that in the absence of DmSMC4 centromere and kinetochore components localise properly, and that kinetochores bind microtubules and are mostly segregated to the poles of the spindle. Furthermore, our data also shows that during early stages of anaphase centromeres and kinetochores are significantly stretched towards the poles suggesting that centromeric cohesion is likely to be resolved but sister chromatids arms are unable to segregate properly.

**Immunolocalisation of Topo II in the absence of DmSMC4**

Absence of DmSMC4 in *Drosophila* results in the formation...
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of anaphase chromatin bridges, a phenotype that was also described for temperature-sensitive top2 mutations in yeast (DiNardo et al., 1984; Uemura et al., 1987). It has been suggested that condensin might regulate Topo II-mediated decatenation of sister chromatids in vivo (Hirano, 2000; Koshland and Strunnikov, 1996). Therefore, we next asked whether DmSMC4 was required for the proper localisation of Topo II (Fig. 4). In control mitotic cells, Topo II mostly co-localised with DmSMC4 throughout the axis of sister chromatids and stronger accumulation at the centromeric region was sometimes observed (Fig. 4A). However, detailed analysis of prometaphase chromosomes stained for both DmSMC4 and Topo II showed that there is only partial co-localisation (Fig. 4B). The localisation of DmSMC4 and Topo II appears to alternate along the axis of sister chromatids with some regions of overlapping staining. After depletion of DmSMC4, the localisation of Topo II was significantly different (Fig. 4C). In all prometaphase and metaphase cells analysed, Topo II was now found diffusely distributed all over the chromosomes and a defined central axis was never observed. In cells undergoing anaphase, Topo II was also diffusely distributed on the chromatin bridges (Fig. 4C).

In order to determine whether loss of DmSMC4 caused a reduction in the level of Topo II associated with mitotic chromatin we carried out chromatin immunoprecipitation assays followed by immunoblotting (Fig. 5). In total protein extracts we found that the level of Topo II was reduced (19.1%) as compared to controls (Fig. 5A). This reduction in the level of Topo II appears to be associated only with the non-mitotic chromosome fraction since immunoblotting of mitotic chromatin immunoprecipitated by anti-phosphohistone H3 antibodies showed significant Topo II levels (Fig. 5B). These results suggest that DmSMC4 is not required for the association of Topo II to mitotic chromatin but is required for
the localisation of Topo II to a defined central axial structure where it is normally confined. Furthermore, the data suggest that depletion of DmSMC4 causes a reduction in the levels of non-chromosomal associated Topo II.

Topo II decatination activity in the absence of DmSMC4
The results presented above indicated that after depletion of DmSMC4, Topo II can associate with chromatin but does not accumulate at a defined axial structure within sister chromatids. To determine whether depletion of DmSMC4 might have an effect upon the DNA decatination activity of Topo II, we devised an in vitro assay. Protein extracts from either control or DmSMC4 dsRNAi-treated cells were prepared and kDNA decatination activity of Topo II (Marini et al., 1980) was tested (Fig. 6). The results showed that extracts from control cells (Fig. 6A) were able to induce decatination of the kDNA and addition of the specific inhibitor ICRF-187 (Roca et al., 1994) completely abolished this activity. However, when DmSMC4-depleted cell extracts were used (Fig. 6B), no significant amount of decatinated DNA was detected. If exogenous Topo II was added in excess, DNA decatination was observed, indicating that the cell extracts are capable of supporting this activity. These results suggest that the endogenous Topo II appears to be dependent upon the presence of DmSMC4 for its decatination activity.
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between Topo II and SMC4 is unlikely to be direct since we were unable to detect a specific interaction by immunoprecipitation (data not shown).

Immunolocalisation of cohesin after depletion of DmSMC4

DmSMC4-depleted mitotic chromosomes are unable to localise Topo II to a defined axial structure and its in vitro activity appears to be compromised, suggesting that sister chromatid resolution requires the concerted activity of condensin and Topo II. However, since an interaction between condensins and cohesins has been suggested, it is also possible that loss of DmSMC4 could lead to inappropriate maintenance of cohesin at chromosome arms during mitotic exit resulting in the formation of chromatin bridges. Therefore, we analysed the distribution of DRAD21 (the Drosophila homologue of the SCC1 cohesin subunit) in DmSMC4-depleted cells (Fig. 7). In control cells (Fig. 7A) DmSMC4 was confined to the centromeric region between the axial structure stained by DmSMC4. During anaphase while DmSMC4 localises throughout sister chromatids, DRAD21 is not detected. (B) After depletion of DmSMC4, DRAD21 is still highly abundant in prophase chromosomes but during prometaphase it is restricted to defined regions of the condensed chromosomes. In anaphase, DRAD21 is never detected either on the chromatids or the chromatin bridges. Scale bars: 5 μm. (C) Western blot of total protein extracts from control and DmSMC4-depleted cells after 96 hours show that depletion of DmSMC4 does not affect the overall levels of DRAD21 but Barren levels are reduced compared to control extracts. (D) Analysis of protein extracts from immunoprecipitated mitotic chromatin of either control or DmSMC4-depleted cells and the corresponding supernatants. In chromatin extracts from control cells DmSMC4, DRAD21 and Barren can be easily detected while in extracts from dsRNAi-treated cells neither DmSMC4 nor Barren are detected and the level of DRAD21 is reduced compared to controls. Phosphohistone H3 was used as a loading control. The corresponding supernatants are shown below. DmSMC4 is not detected by immunoblotting and the levels of DRAD21 are not different from controls. However, note that depletion of DmSMC4 causes a significant decrease in the level of Barren present in the supernatant fraction. α-tubulin was used as loading control.

Fig. 7. Localisation of DRAD21 in DmSMC4-depleted Drosophila S2 cells.

(A) Immunolocalisation of DmSMC4 and DRAD21 in control cells. In prometaphase cells, DmSMC4 and DRAD21 do not appear to co-localise. DRAD21 is always confined to the centromeric region between the axial structure stained by DmSMC4. During anaphase while DmSMC4 localises throughout sister chromatids, DRAD21 is not detected. (B) After depletion of DmSMC4, DRAD21 is still highly abundant in prophase chromosomes but during prometaphase it is restricted to defined regions of the condensed chromosomes. In anaphase, DRAD21 is never detected either on the chromatids or the chromatin bridges. Scale bars: 5 μm. (C) Western blot of total protein extracts from control and DmSMC4-depleted cells after 96 hours show that depletion of DmSMC4 does not affect the overall levels of DRAD21 but Barren levels are reduced compared to control extracts. (D) Analysis of protein extracts from immunoprecipitated mitotic chromatin of either control or DmSMC4-depleted cells and the corresponding supernatants. In chromatin extracts from control cells DmSMC4, DRAD21 and Barren can be easily detected while in extracts from dsRNAi-treated cells neither DmSMC4 nor Barren are detected and the level of DRAD21 is reduced compared to controls. Phosphohistone H3 was used as a loading control. The corresponding supernatants are shown below. DmSMC4 is not detected by immunoblotting and the levels of DRAD21 are not different from controls. However, note that depletion of DmSMC4 causes a significant decrease in the level of Barren present in the supernatant fraction. α-tubulin was used as loading control.
axis of sister chromatids while DRAD21 localised to the centromeric and pericentromeric regions between the axis of the two sister chromatid, showing that DmSMC4 and DRAD21 never co-localise. During anaphase, DmSMC4 was detected along sister chromatid arms and DRAD21 was no longer present. In DmSMC4-depleted cells (Fig. 7B) DRAD21 localised to chromatin at prophase but during prometaphase was present only at centromeric and centromeric regions of the abnormally condensed chromosomes. More significantly, during anaphase or telophase, DRAD21 was never found localised to the chromatin bridges. These results suggest that cohesin does not appear to be responsible for the formation of chromatin bridges after anaphase onset since its release from mitotic chromatin in DmSMC4-depleted cells follows normal kinetics.

In order to confirm these results, we analysed the levels of cytoplasmic or mitotic chromatin-associated DRAD21 from either control or DmSMC4 dsRNA-treated cells. Western blots of total protein extracts from control and DmSMC4-depleted cells show that DmSMC4 is significantly reduced, DRAD21 is not affected while and the level of Barren is also reduced as compared to α-tubulin, the loading control (Fig. 7C).

Mitotic chromatin was then immunoprecipitated with anti-phosphohistone H3 antibodies, separated by SDS-PAGE and immunoblotted against DmSMC4, DRAD21, Barren and Histone H3 or α-tubulin as loading controls (Fig. 7D).

Increasing amounts of the immunoprecipitates for either non-treated or DmSMC4 dsRNA-treated cells were used. DmSMC4, DRAD21 and Barren were easily detected in the immunoprecipitated chromatin fraction from non-treated cells. However, in mitotic chromatin immunoprecipitated from DmSMC4-depleted cells, neither DmSMC4 nor Barren could be detected. More significantly, DRAD21 not only did not accumulate to higher levels but was somewhat reduced as compared to controls. Analysis of the supernatant fraction showed that DmSMC4 was not present, as expected, while DRAD21 was present at significant levels. Also, we observed that although Barren was still detected in the extracts from DmSMC4-depleted cells, its levels in the non-chromatin fraction were consistently decreased. These results suggest that the distribution of cohesins and their release from chromosomes during mitotic progression is not affected by the depletion of DmSMC4.

Mitotic progression after simultaneous depletion of DRAD21 and DmSMC4

To exclude the possibility that chromatin bridges, observed after depletion of DmSMC4, might be due to some DRAD21-dependent mechanism we carried out simultaneous depletion of DmSMC4 and DRAD21. The results showed that chromatin bridges can form independently of DRAD21 (Fig. 8). Firstly, we depleted DRAD21 alone. Titration of the anti-DRAD21 antibody showed that the number of cells used at each time point was sufficient to easily detect the protein (Fig. 8A) and that the level the protein was not affected in control cultures (Fig. 8B). However, cells treated with DRAD21 dsRNA showed significant depletion of the protein by 96 hours (Fig. 8B). To determine whether depletion of DRAD21 causes mitotic abnormalities, cells were stained for DRAD21 and DNA (Fig. 8C). Cells treated with DRAD21 RNAi showed a slightly elevated mitotic index (Fig. 8E) with highly condensed and separated sister chromatids (82.6%). Analysis of mitotic progression (Fig. 8F) indicated that DRAD21-depleted cells accumulated mostly in prometaphase (68.9%) and some displayed an anaphase-like configuration (3.5%). Most importantly, no cells with chromatin bridges during anaphase or telophase were observed. These results are in agreement with previous reports (Sonoda et al., 2001; Vass et al., 2003). We then carried out simultaneous depletion of DmSMC4 and DRAD21, and after 96 hours the level of both proteins was significantly reduced (Fig. 8B). Surprisingly, mitotic cells showed a phenotype very different from that resulting from depletion of DRAD21 alone (Fig. 8D). Cells depleted of DRAD21 and DmSMC4 showed abnormal chromosome condensation with unresolved sister chromatids (86.4%), very low levels of premature sister chromatid separation (3.3%) and all anaphase or telophases had chromatin bridges. Quantification of mitotic progression (Fig. 8F) showed that cells depleted of both DRAD21 and DmSMC4 progress through prophase and prometaphase but accumulate in anaphase and telophase. These cells are clearly exiting mitosis since cyclin B levels are not different from those of control cells at similar mitotic stages (data not shown). These observations support the results of the previous section and show that chromatin bridges present in DmSMC4-depleted cells are formed independently of DRAD21. Furthermore, the data shows that depleting DmSMC4 prevents premature sister chromatid separation and overrides the prometaphase arrest caused by loss of DRAD21.

Discussion

We have shown that dsRNAi can be used to severely deplete DmSMC4 in tissue culture cells resulting in mitotic phenotypes that are very similar to those previously described for dmSmc4 mutant Drosophila cells (Steffensen et al., 2001). Already 24 hours after RNAi treatment some mitotic cells show abnormal resolution of sister chromatids and later, cells in anaphase or telophase begin to show chromatin bridges indicating that the frequency with which these phenotypes are observed depends on the level of depletion of DmSMC4. Loss of DmSMC4 causes the formation of short mitotic chromosomes with poorly defined sister chromatids. These chromosomes are unlikely to contain other proteins of the condensin complex since immunofluorescence and mitotic chromatin immunoprecipitation shows that binding of Barren to condensing chromosomes is dependent on DmSMC4. These observations are in agreement with previous findings indicating that non-SMC condensins can only bind DNA in the presence of the entire condensin complex (Cubizolles et al., 1998; Hirano et al., 1997; Kimura et al., 2001; Kimura and Hirano, 2000; Lavoie et al., 2000; Ouspenski et al., 2000; Sutani et al., 1999). Also, it was shown in S. pombe and S. cerevisiae that all members of the regulatory subcomplex are essential for chromatin association of yeast condensin in vivo (Freeman et al., 2000; Lavoie et al., 2002). Together, these results strongly suggest that, in Drosophila, the assembly of the condensin complex to mitotic chromatin requires all protein subunits. Moreover, our results demonstrate that certain aspects of chromosome condensation, namely shortening of the longitudinal axis of sister chromatids, can occur in the absence
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of condensins, fully supporting our previous results (Steffensen et al., 2001).

Although, chromosomes do not condense normally in DmSMC4-depleted cells, genetic studies in Drosophila showed that loss of DmSMC4 (Steffensen et al., 2001) or Barren (Bhat et al., 1996) does not prevent cells from entering anaphase and attempting sister chromatid segregation. However, recently it has been suggested that the condensin complex might contribute to ensure proper function of the centromere. In S. cerevisiae, BRN1 the homologue of Barren, has been implicated in the formation of functional mitotic kinetochores (Ouspenski et al., 2000) and in C. elegans condensin activity is required for the normal orientation of the centromere towards the mitotic spindle (Hagstrom et al., 2002). Here we show that depletion of DmSMC4 does not affect the localisation of centromere or kinetochore proteins and that microtubules associate with kinetochores. Furthermore, we observed that at early stages of mitosis, kinetochores associated with spindle microtubules appear to stretch poleward, sometimes well beyond the chromatin. However, when microtubules are depolymerised by colchicine, kinetochores localise only over mitotic chromatin, suggesting that stretching of kinetochores is microtubule dependent. Similar observations were reported after expressing GFP-tagged centromeres in a BRN1 mutant background (Ouspenski et al., 2000). These results suggest that condensin is not required for the formation of functional kinetochores and that at metaphase-anaphase transition sister centromeres disjoin normally and segregate but sister chromatid arms remain attached causing stretching of the centromeres.

Immunofluorescence and biochemical studies have suggested that condensed chromatids contain a central axial structure (Paulson and Laemmli, 1977). Topoisomerase II (Earnshaw et al., 1985; Gasser et al., 1986) and condensin...
(Hirano and Mitchison, 1994; Steffensen et al., 2001) have been identified at this elusive structure. Previously we showed that in *Drosophila S2* cells condensins associate with chromatin at prophase localising to the axis of sister chromatids throughout mitosis (Steffensen et al., 2001). In this study we show that Topo II also localises to the axis of sister chromatids throughout mitosis. However, in prometaphase chromosomes it is clear that condensin and Topo II only show partial co-localisation. DmSMC4 and Topo II localise to discrete sites that alternate along the chromatid axis. Although similar patterns of localisation has been recently described for hBarren and Topo II in *HeLa* cells, hBarren appears to bind to chromosomes and centromeres (Christensen et al., 2002; Tavormina et al., 2002). In vivo analysis of condensin subunits bind chromatin independently (see above). Furthermore, we show here that depletion of DmSMC4 abolishes the localisation of Topo II to a well-defined axial structure even though there is no significant reduction in the level of chromatin-associated Topo II. Similarly, in yeast, localisation of Topo II to mitotic chromatin has been shown to depend upon condensin function (Bhalla et al., 2002). However, more recent data has suggested that in chromatin assembled in *Xenopus* extracts, Topo II localisation to an axial structure of chromatids occurs independently of condensin (Cuvier and Hirano, 2003). These apparently contradictory results could be explained if condensin was not completely depleted in the *Xenopus* extracts, allowing partial accumulation of Topo II to an axial chromatin structure. Since in our RNAi depletion studies we did not find DmSMC4 either by immunofluorescence or western blotting, our results suggest that condensin plays an essential role in the organisation of the chromatin so that Topo II can localise to the chromatid axis. This structure is likely to be highly dynamic since recent live imaging and FRAP analysis in mammalian cells shows that Topo II exchanges rapidly between a cytoplasmic pool and that bound to chromosomes and centromeres (Christensen et al., 2002; Tavormina et al., 2002). In vivo analysis of condensin accumulation to the axis of sister chromatids should provide valuable insights on the dynamics of this ‘ill-defined’ structure.

The abnormal distribution of Topo II to mitotic chromatin resulting from depletion of DmSMC4 prompted us to ask whether its DNA decatenation activity was also compromised. We showed, using an in vitro assay, that DNA decatenation activity of the endogenous Topo II is significantly reduced when DmSMC4 is depleted. Although these results are compatible with a direct interaction between DmSMC4 and Topo II we were unable to detect co-immunoprecipitation (data not shown) indicating that the interaction might be indirect. Nevertheless, our results suggest that proper activity of the enzyme requires condensin. A more direct interaction was reported previously since it was shown that Barren interacts in a yeast two-hybrid assay with Topo II and promotes its decatenating activity (Bhat et al., 1996). However, it has been shown that BRN1, the yeast homologue, is not required for Topo II activity in vivo (Lavoie et al., 2000). Furthermore, it is unlikely that depletion of DmSMC4 completely abolishes Topo II activity since mutation or inhibition of its activity has been shown to cause arrest at the metaphase-anaphase transition, a phenotype not produced by DmSMC4 depletion (Clarke et al., 1993; Uemura et al., 1986; Uemura et al., 1987). Accordingly, we believe that the chromatin bridges observed after depletion of DmSMC4 are due to inappropriate activity of Topo II resulting in the maintenance of catenated DNA between sister chromatids.

Previous reports have suggested a possible mechanistic interaction between cohesins and condensins (Castano et al., 1998; Lavoie et al., 2002). However, we show here that depletion of DmSMC4 does not alter the localisation or removal of cohesins from mitotic chromatin in *Drosophila S2* cells. Similarly, in *S. cerevisiae* it has been shown that although sister chromatid separation does not occur normally in *Ycs4* mutants, MCD1/SCC1 is released from chromosomes at the metaphase-anaphase transition (Bhalla et al., 2002). Conversely, depletion of cohesins in higher eukaryotes does not appear to affect chromosome condensation (Losada et al., 1998; Sonoda et al., 2001; Vass et al., 2003) and in *Xenopus* extracts the release of cohesin during prophase is not required for chromatin compaction mediated by condensin (Losada et al., 2002). Taken together, these results indicate that the removal of cohesins during mitosis is independent of condensin activity.

Depletion of cohesins causes premature sister chromatid separation and a significant prometaphase arrest (Sonoda et al., 2001; Vass et al., 2003). This prometaphase arrest could be due to the activity of the spindle checkpoint, which prevents exit from mitosis if proper chromosome orientation and organisation of a metaphase plate is not achieved. Strikingly, we observed that simultaneous depletion of DmSMC4 and DRAD21 does not lead to premature sister chromatid separation or arrest during prometaphase but cells progress into anaphase and telophase showing extensive chromatin bridges. We propose that sister chromatids do not separate prematurely in the absence of cohesins because, as shown above, depletion of DmSMC4 prevents sister chromatid resolution by compromising Topo II activity. These cells then proceed into prometaphase, kinetochores can now bind spindle microtubules and chromosomes congress to the metaphase plate, allowing cells to satisfy the spindle checkpoint and initiate mitotic exit. Thus, in the absence of DmSMC4, abnormal decatenation of sister chromatids appears to provide an alternate mechanism to hold sisters together during early stages of mitosis.

From our results we propose that condensin is essential to organise a clearly defined axial structure of sister chromatids where Topo II can localise. In the absence of this specific localisation, Topo II can still bind chromatin but its decatenation activity is not specifically directed and sister chromatids cannot resolve properly.

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