RNAi analysis reveals an unexpected role for topoisomerase II in chromosome arm congression to a metaphase plate

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Summary

DNA topoisomerase II (Topo II) is a major component of mitotic chromosomes and an important drug target in cancer chemotherapy, however, its role in chromosome structure and dynamics remains controversial. We have used RNAi to deplete Topo II in *Drosophila* S2 cells in order to carry out a detailed functional analysis of the role of the protein during mitosis. We find that Topo II is not required for the assembly of a functional kinetochore or the targeting of chromosomal passenger proteins, nonetheless, it is essential for anaphase sister chromatid separation. In response to a long-running controversy, we show that Topo II does have some role in mitotic chromatin condensation. Chromosomes formed in its absence have a 2.5-fold decrease in the level of chromatin compaction, and are morphologically abnormal. However, it is clear that the overall programme of mitotic chromosome condensation can proceed without Topo II. Surprisingly, in metaphase cells depleted of Topo II, one or more chromosome arms frequently stretch out from the metaphase plate to the vicinity of the spindle pole. This is not kinetochore-based movement, as the centromere of the affected chromosome is located on the plate. This observation raises the possibility that further unexpected functions for Topo II may remain to be discovered.

Key words: Mitosis, Topoisomerase, Chromosome segregation, Chromosomal passengers, Condensin, Heterochromatin

Introduction

Type II DNA topoisomerases (Topo II) are essential enzymes that modulate the topology of DNA. Topo II function is necessary for virtually all processes involving double stranded DNA, including replication, transcription, recombination and decatenation of sister chromatids prior to anaphase of mitosis (Wang, 1996).

Yeasts and *Drosophila* have a single isoform of Topo II. No *Drosophila* Topo II mutant has been described to date. Yeast Topo II mutants die in mitosis when anaphase fails as the result of a large mass of unresolved lagging chromatin (DiNardo et al., 1984; Uemura and Yanagida, 1984; Holm et al., 1985). In *Saccharomyces pombe*, this phenotype was called 'cut' [cell untimely torn (Uemura and Yanagida, 1986)].

Vertebrates have two type II topoisomerases [α and β (Drake et al., 1989)], either of which can complement yeast Topo II function (Adachi et al., 1992; Jensen et al., 1996), but which have different locations in mitosis. Topo IIα associates with chromosomes from metaphase to telophase, whereas Topo IIβ remains mainly cytosolic until anaphase onset, when low levels of the protein associate with the chromatids (Christensen et al., 2002). There is an ongoing controversy over whether Topo II is concentrated more in axial regions (Earnshaw and Heck, 1985; Gasser et al., 1986; Tavormina et al., 2002; Maeshima and Laemmli, 2003), or spread diffusely throughout the chromatid arms (Hirano and Mitchison, 1993; Swedlow et al., 1993; Christensen et al., 2002).

Topo IIα is enriched at centromeres in prometaphase and metaphase, and a number of studies suggested that the protein may have a role in regulating kinetochore structure (Rattner et al., 1996; Christensen et al., 2002) and/or centromeric cohesion (Bachant et al., 2002). This view has been strengthened by studies of two different human centromeres. A conserved Topo II cleavage site was found near the Y chromosome centromere, and this was absent from two inactive derivatives of this centromere (Floridia et al., 2000). Subsequent functional dissection of the X centromere identified a minimal region of <50 kb that was found in all active centromere derivatives. This region contained a strongly preferred cleavage site for Topo II (Spence et al., 2002).

The function of Topo II has long been controversial, particularly concerning its role, if any, in regulating mitotic chromosome structure. Topo II is required for the final stages of chromosome condensation in fission yeast (Uemura et al., 1987), *Xenopus* cell-free extracts (Adachi et al., 1991) and in *Drosophila* syncitial embryos (Buchenau et al., 1993). However, it is not required for the nocodazole-induced condensation of rDNA in *Saccharomyces cerevisiae* (Lavoie et al., 2002) and drug studies have suggested that Topo II activity is not involved in generating mitotic chromosomes with two morphologically distinct chromatids (Andreassen et al., 1997).

We have used RNAi to successfully deplete Topo II from *Drosophila* cells. This has enabled us to show that in the absence of Topo II, mitotic chromosomes can condense,

although the detailed structures produced are not entirely normal. Thus, Topo II is not essential for mitotic chromatin condensation, but it may have a role in the establishment of chromosome architecture. Topo II is not essential for centromere/kinetochore assembly or function, but it is required for sister chromatid separation at anaphase. Quite surprisingly, Topo II is required for the formation of a compact metaphase plate. This latter result raises the possibility that further unexpected functions for Topo II may remain to be discovered.

Materials and Methods

dsRNA interference

dsRNAi experiments were performed as described previously (Adams et al., 2001). Two fragments from the 5′ end of DmTopo II fused to the T7 RNA polymerase promoter were used as PCR primers. A random human intronic sequence was used as control dsRNA as described previously (Adams et al., 2001). The EST clone LD24716 was used as a PCR reaction template. The PCR fragments obtained (about 700 bp) were used as templates for RNA synthesis using the Megascript kit (Ambion). dsRNAi experiments were performed in six-well plates. At each time point, experimental and controls cells were collected for scoring, immunoblotting and immunofluorescence.

Immunofluorescence staining and immunoblotting

For immunoblotting, cells were collected by centrifugation, resuspended in SDS sample buffer, boiled for 5 minutes and sonicated. For immunostaining, cells were transferred onto poly-lysine-treated slides and left to attach for 20 minutes. Slides were centrifuged for 15 minutes at 4000 rpm before fixation. Cells were fixed in 4% paraformaldehyde in cytoskeleton buffer (CB: 1.1 mM Na_2HPO_4, 0.4 mM KH_2PO_4, 137 mM NaCl, 5 mM KCl, 2 mM $MgCl$, 2 mM EGTA, 5 mM Pipes, 5.5 mM glucose, pH 6.1) for 10 minutes at 37°C. They were permeabilised in 0.1% Triton X-100 in CB for 5 minutes and then rinsed in PBS. Cells were blocked for 30 minutes at room temperature in PBS+10% FBS. Antibody incubations were performed in PBS+1% FBS for 1 hour at 37°C, followed by four 10-minute washes in PBS at room temperature. DNA was stained with 0.1 µg/ml DAPI for 5 minutes at room temperature and rinsed with PBS. Slides were mounted in Vectashield mounting medium (Vectra) and sealed using nail varnish.

Antibodies

The primary antibodies used were as follows. Anti-α-tubulin (mouse mAb B512, used at 1:2000; Sigma-Aldrich); anti-phosphorylated histone H3 (rabbit polyclonal IgG, used at 1:500, Upstate Biotechnology); anti-DmINCENP rabbit polyclonal R801, used at 1:500 (Adams et al., 2001); anti-DmAuroraB R963, used at 1:500 (Adams et al., 2001); anti-Cid chicken polyclonal serum used at 1:200 (Blower and Karpen, 2001); anti-Topo II (rabbit polyclonal antibodies used at 1:500, a gift from Neil Osheroff, Vanderbilt University School of Medicine, Nashville, Tennessee and Donna Arndt-Jovin Dept. of Molecular Biology MPI for Biophysical Chemistry Goettingen, Germany); anti-Barren [rabbit polyclonal used at 1:1000 (Bhat et al., 1996)].

All fluorescently conjugated secondary antibodies (Jackson ImmunoResearch Laboratories) were used according to the manufacturer's instructions.

Fluorescent in situ hybridisation (FISH)

1×10^6 RNAi-treated cells were transferred to poly-lysine-coated slides and left for 10 minutes at room temperature. Cells were fixed in 4% paraformaldehyde for 5 minutes at room temperature then in

methanol-acetic acid (1:3) at –20°C for 20 minutes, air dried and then aged for 2 days.

Various heterochromatic probes were used: X-chromosome 359 satellite chromosome 2 AACAC satellite and chromosome 3 dodecasatellite (Abad et al., 1992). Euchromatic BAC clones were obtained from the MRC UK HGMP resource centre, Cambridge, UK (BACH47E07, 10A-10A –X chromosome–; BACHN09, 30C5-30D –chromosome 2L–; BACH47E02, 50A1-50A5 –chromosome 2R–; BACH47K04, 71A-71A –chromosome 3L–; BACN01B01, 90C7-90C10 –chromosome 3R–.

BACs were reduced to ~200 bp in length by sonication. Probes were prepared from the sonicated DNA using DIG-high Prime DNA Labelling and Detection Starter Kit II (Roche). After removing excess nucleotides (ProbeQuant G-50 Micro Columns, Amersham), the quality and quantity of each probe was determined according to the protocol of the kit. For hybridisation, 300 µg of each digoxigenin-dUTP-labelled probe was ethanol-precipitated with 20 µg human cot-1 DNA (sonicated to 200 bp length; Roche) and resuspended in 40 µl hybridisation mix per slide (50% deionised formamide, 2× SSC, 5% dextran sulphate). Probes were denatured at 85°C for 10 minutes. The aged cells on the slides were rehydrated in 2× SSC and digested with 100 µg/ml RNAse A for 1 hour at 37°C, then dehydrated for 5 minutes each in 70% and 100% ethanol. After air drying, the cells were denatured for 5 minutes at 80°C in 50% deionised formamide, 2× SSC, washed for 5 minutes on ice in 2× SSC, and dehydrated for 5 minutes each at 0°C in 70%, 95% and 100% ethanol. After air drying, the denatured probes were applied to the slides, covered with coverslips and slides were incubated in a humid box at 37°C overnight. After hybridisation, the slides were washed for 2× 5 minutes at 42°C in 50% deionised formamide, 2× SSC and 5 minutes in 50% deionised formamide, 0.1× SSC, then 5 minutes each in 2× SSC, PBS at room temperature. The cells were blocked for 1 hour in 10% FBS in PBS, then rinsed with PBS and incubated in sheep anti-digoxigenin-rhodamine (used at 1:200, Roche) for 1 hour at 37°C. The slides were washed 3× 10 minutes in PBS and stained for 5 minutes with 0.1 µg/ml DAPI and washed for 10 minutes in PBS. Slides were mounted in Vectashield mounting medium (Vectra) and sealed using nail polish.

Microscopy

Imaging was performed using a Zeiss Axioplan 2 or an Olympus IX-70 microscope controlled by Delta Vision SoftWorx (Applied Precision, Issequa, WA, USA). Image stacks were deconvolved, quick-projected and saved as tiff images to be processed using Adobe Photoshop.

Measurement of chromatin density

In order to quantify DNA staining density the ten central sections of an image stack were deconvolved and projected using an averaging algorithm. The total integrated intensity of a 20×20 pixel box was measured at the appropriate wavelengths using the Data Inspector tool. For each metaphase cell analysed, three measurements were taken on the chromosomes within the cell and three of the background outside the cell. Values were corrected by subtracting the background of the appropriate wavelength.

Results

Effect of Topo II RNAi on cell growth and mitosis

Immunofluorescence analysis of Topo II in S2 cells shows that the enzyme is localised diffusely on the chromosomes, but also accumulates in specific chromosomal regions, some of which apparently coincide with centromeres (Fig. 1C,E). Following the addition of specific dsRNA to *Drosophila* S2 cells, Topo II
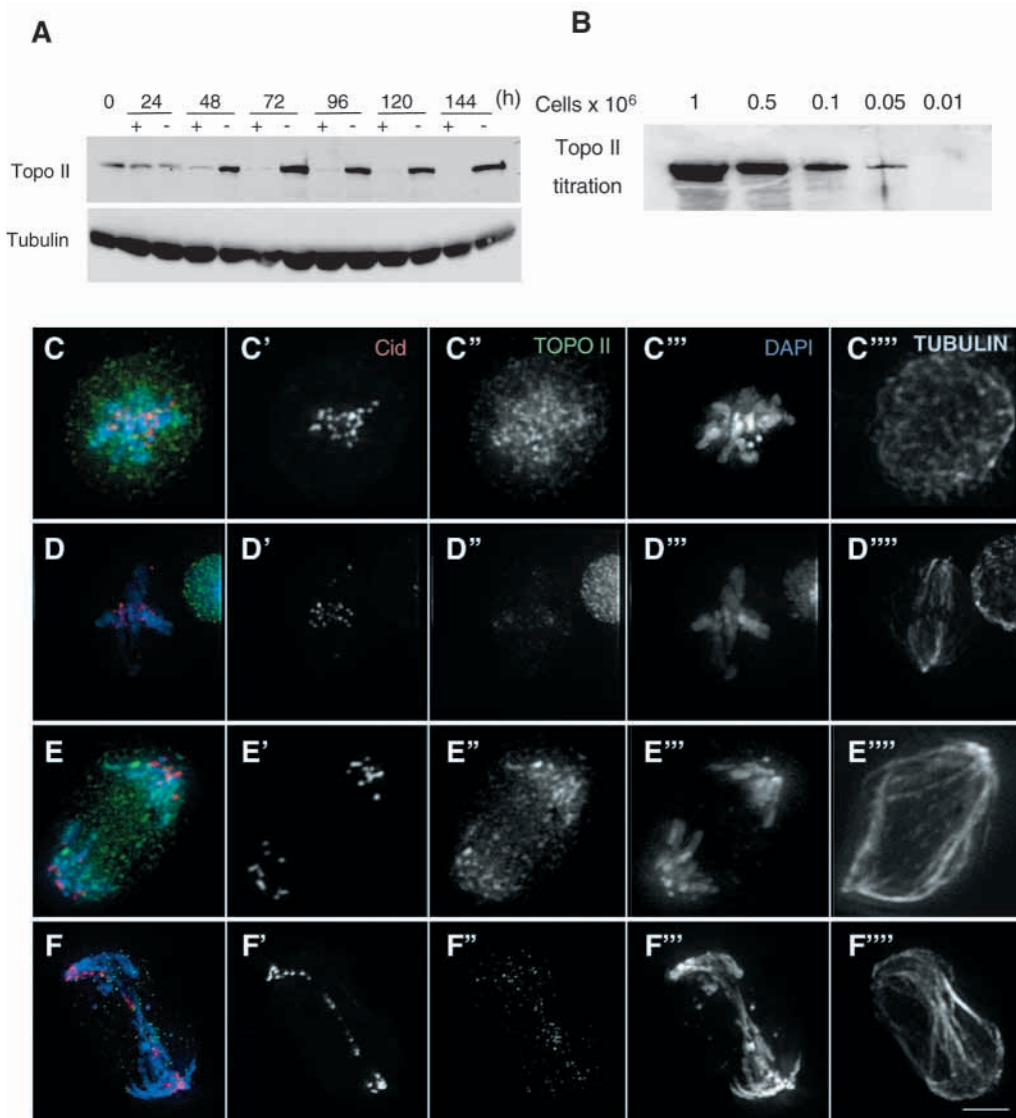
Fig. 1. Efficient depletion of Topo II in *Drosophila* S2 cells using RNAi. (A) Immunoblots: (upper) Topo II levels begin to fall by 48 hours after the addition of dsRNA and become undetectable by 72 hours (1×10^6 cells loaded per lane); (lower) loading control (anti-tubulin). –, control RNAi; +, Topo II RNAi treated. (B) The anti-Topo II antibody used can detect Topo II in 5×10^4 cells, but not 1×10^4 cells. (C-F) Immunofluorescence analysis showing Topo II depletion at the 72nd hour after treatment; (C,E) controls, (D,F) treated cells, (C,D) prometaphase, (E,F) anaphase. In all merged images, DAPI is blue, Cid is red and Topo II is green. Scale bar: 5 µm.
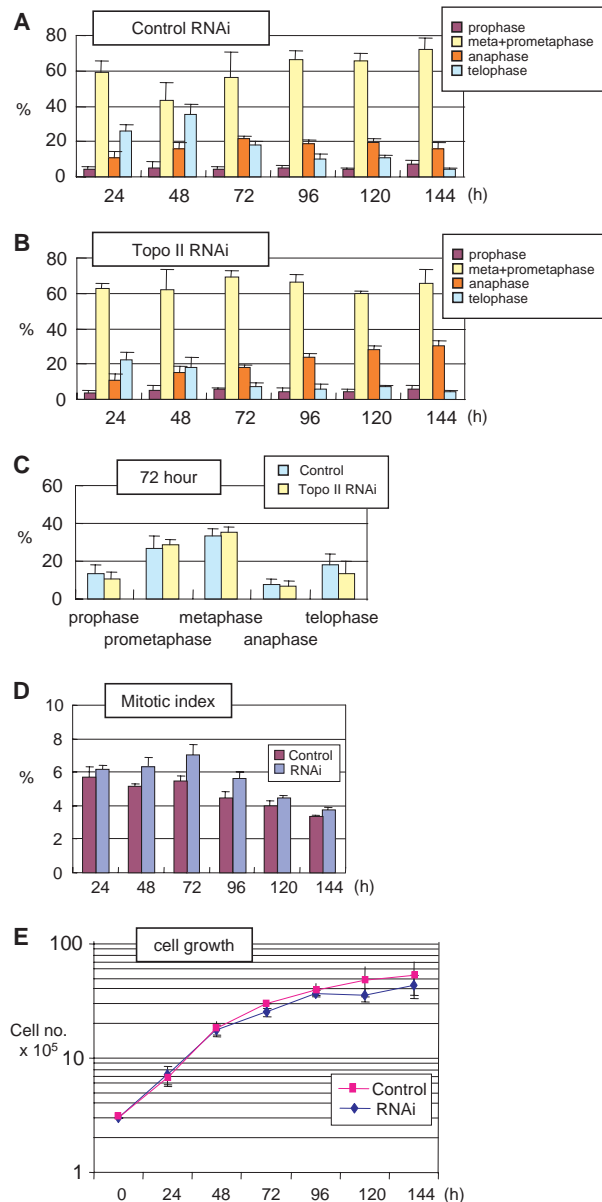
few differences were observed between experimentals and controls (Fig. 2A,B). If we scored only cells where the spindle axis was perpendicular to the optical axis (where metaphases and prometaphases could be distinguished), again no reproducible difference was seen (Fig. 2C). In fact, the only significant difference seen in these experiments was a slight increase in the percentage of anaphase cells in the 96-, 120- and 144-hour time points (Fig. 2B). The frequency of cells in cytokinesis was also elevated relative to controls, and >85% of these cells had chromatin bridges across the midbody (data not shown).

These observations reveal that *Drosophila* S2 cells do not have a checkpoint that arrests the cell cycle in response to loss of Topo II function, and that progress through mitosis is not delayed significantly by lack of Topo II activity.

Topo II function is required for normal mitotic chromosome morphology but not histone H3 phosphorylation

One of the most persistent questions about Topo II function is whether the protein has a role in mitotic chromosome structure. This has been difficult to answer, because the drugs and antibodies used to inhibit Topo II function in previous published studies might not necessarily block the function of Topo II as a structural element in chromosomes. Furthermore, chromosome architecture is extremely dependent on specimen preparation conditions and is difficult to assess quantitatively. We have avoided these problems by, firstly, generating cells where the protein is effectively absent, and secondly, using a

levels were severely reduced by 48 hours and the protein became undetectable by both immunoblotting and immunofluorescence after 72 hours (Fig. 1A,D,F). A titration experiment revealed that the antibody used in this study could detect Topo II from 5×10^4 cells, but not from 1×10^4 cells (Fig. 1B). Overall, we estimate that levels of the protein in the culture fell by at least a factor of 20 in response to the dsRNA treatment. The protein did not re-accumulate in the cells even by 144 hours after addition of dsRNA (Fig. 1A).

There was no significant difference in mitotic index between cultures treated with control or Topo II-specific dsRNA (Fig. 2D). Both populations displayed a slight decline in mitotic index over the course of the experiment, presumably because the medium was depleted of essential ingredients.

Detailed analysis of the distribution of mitotic phases in cells following Topo II RNAi revealed surprisingly few differences between the Topo II-depleted cells and cells treated with a control dsRNA. If prometaphases and metaphases were grouped together (this was because spindles are oriented randomly in these small non-adherent cells, and it is frequently difficult to distinguish between metaphase and prometaphase),

not correlate with any obvious abnormalities in histone H3 serine[10] phosphorylation, even in the most abnormal looking chromosomes (Fig. 3B,C).
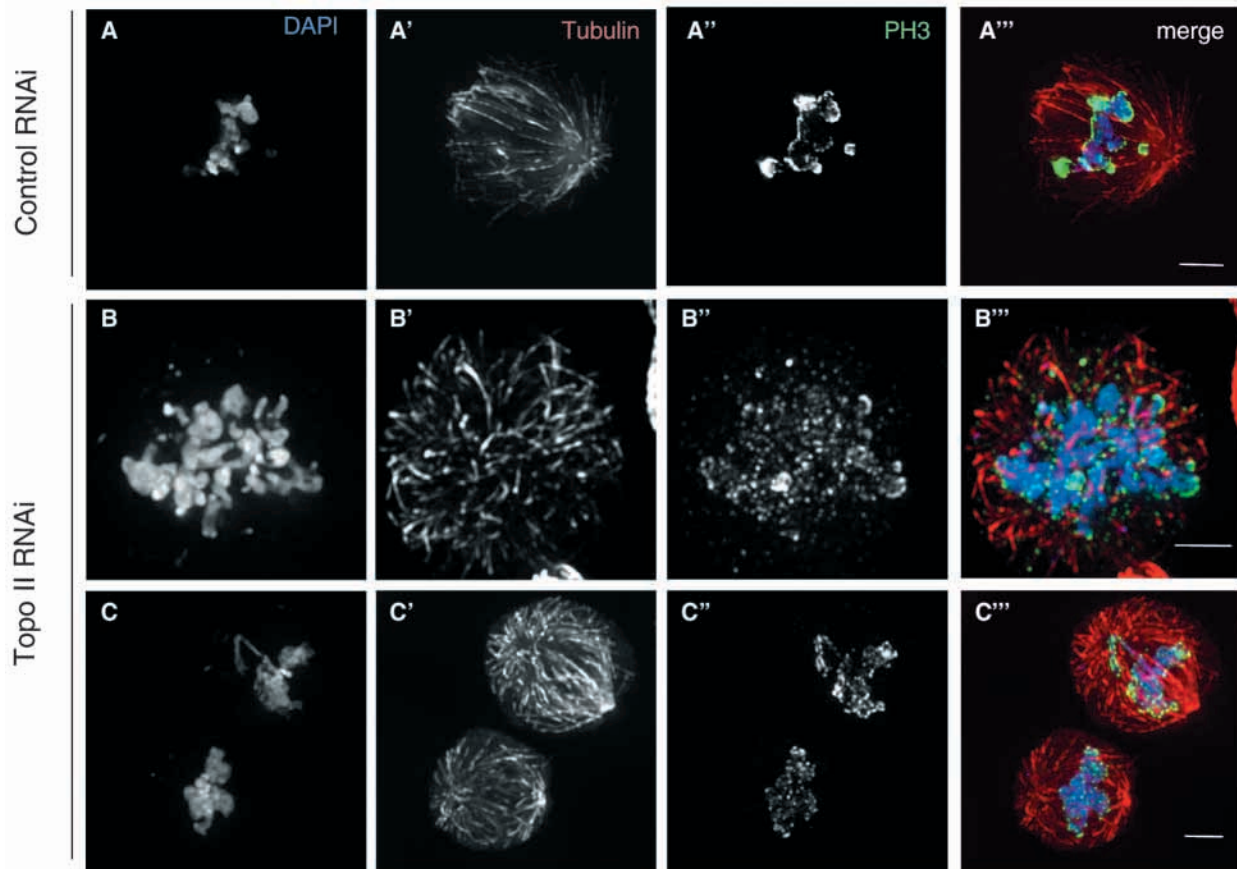
It is now widely accepted that mitotic chromosome condensation is regulated by the condensin complex (Hirano and Hirano, 2002), one component of which, the non-SMC subunit Barren, has been proposed to interact functionally and physically with Topo II (Bhat et al., 1996). It was therefore possible that the alterations of chromatin packaging and morphology seen in the absence of Topo II might be due to effects on condensin targeting or function. However, we found that the behaviour of Barren is apparently unaffected by the depletion of Topo II: the protein becomes selectively concentrated at or near centromeres during metaphase before its levels decline significantly during anaphase (Fig. 4).

These experiments reveal that although the distribution of condensin appears normal and some degree of mitotic chromosome condensation can occur in the absence of Topo II, the enzyme does contribute both to the extent of chromatin condensation and to the apparent quality of the structures produced.

Topoisomerase II is required for formation of a compact metaphase plate

In many metaphase cells observed following Topo II depletion, one or more chromosome arms extended outwards from the compact mass of chromosomes, often stretching towards the spindle pole (Fig. 5B,C; examples of this characteristic configuration are also shown in Fig 3C, Fig. 4C,D and Fig. 6A-D). This phenotype was first observed at 48 hours, when Topo II levels had begun to fall significantly, and it was maximal at 96 hours, when roughly 25% of metaphases had one or more protruding chromosome arms (Fig. 5D).

In many cases, the protruding chromosome had a V- or J-shaped appearance, resembling a chromatid engaged in anaphase A poleward movement. However, precocious movement of the centromere towards the pole cannot be the explanation for this phenomenon. In the great majority of cases, when the centromere of the protruding chromosome was identified by immunostaining for CENP-A/Cid (Fig. 5B") it was positioned normally at the metaphase plate. Immunolocalisation of the condensin subunit Barren also

quantitative assay to examine whether the degree of chromatin condensation within mitotic chromosomes is normal in the absence of Topo II.

We performed a quantitative analysis of the amount of DAPI-stained DNA in a defined volume on image stacks (see Materials and Methods for details). A previous analysis had shown that this method was sensitive enough to detect the increases in chromosome condensation that normally occur from prophase through metaphase, and the subsequent decondensation that begins in anaphase (Adams et al., 2001). The present analysis showed that in the absence of Topo II, the chromatin was roughly 2.5-fold less condensed than normal (DAPI density, 2.15 ± 0.68 versus 5.09 ± 0.87 (arbitrary units); $n=15$). In addition to this difference in condensation, many of the chromosomes in Topo II-depleted cells in prometaphase were morphologically abnormal, often having a mass of chromatin without defined sister chromatids (Fig. 3B,C). The change in chromatin compaction following loss of Topo II did

Fig. 3. Topo II depletion causes abnormalities in chromosome structure, but does not affect histone H3 phosphorylation on serine[10]. All images are from cultures at 72 hours after addition of Topo II dsRNA. (A-A''') Control RNAi-treated metaphase cell. (B-B''') Prometaphase cell with abnormal chromosome morphology viewed parallel to spindle axis. (C-C''') Two metaphase cells, one of which has a highly elongated chromosome arm extending to one spindle pole. Both are viewed nearly perpendicular to the spindle axis. (A-C) DAPI staining for DNA; (A'-C') anti-tubulin shows the mitotic spindle; (A'',B'') histone H3 phosphorylated on serine[10] is stained with a specific antibody; (A'''-C''') merged images (DAPI is blue, tubulin is red, histone H3 is green. Scale bar: 5 μm.

allowed us to visualise the normal metaphase alignment of the centromeres (Fig. 4C''). In this case, the entire elongated chromosome arm had detectable levels of the condensin subunit but the centromere-enriched staining for Barren was found on the metaphase plate. We could also observe INCENP staining all along the protruding chromosome arm (data not shown).

These observations reveal that the movement of the chromosome arm towards the spindle pole cannot be due to centromere activity, but it was possible that some other specialised structural feature of the chromosome arms, for example, the rRNA locus, might be responsible for this positioning. We therefore performed FISH experiments using BAC clones to identify the chromosome arm(s) involved in this unusual morphology. This analysis revealed that the protruding chromosome arm could be the X, or either arm of chromosome 3 (Fig. 6A,B). These three chromosome arms share no obvious feature that could explain this association with the spindle pole. Chromosome 2 was never observed to stretch towards the pole (*n*=22, Fig. 6C).

Despite the lack of a centromere close to the spindle pole, the protruding chromosome arm appeared to be under tension. This was most clearly seen when we used heterochromatic

probes in our FISH experiments. Occasionally we could observe centromeric heterochromatin in the protruding arm, and when we did, it was often abnormally elongated (compare signals from the protruding chromosome and its homologue, Fig. 6D). In the absence of evidence for a kinetochore actively pulling the arm, this is most consistent with the arm being somehow trapped at the pole, and then the combined forces of kinetochore congression to the metaphase plate, chromatin condensation and the 'polar wind' (presumably primarily chromokinesins) actively trying to push/pull the arm away from the pole.

Topo II is not required for centromere/kinetochore assembly or function, but is required for sister chromatid segregation

A number of studies have shown that Topo IIα in mammalian cells is concentrated at centromeres during prometaphase and metaphase (Floridia et al., 2000; Spence et al., 2002; Christensen et al., 2002; Tavormina et al., 2002), and one study showed that kinetochore structure was apparently abnormal in cells treated with Topo II inhibitors (Rattner et al., 1996). However, all aspects of centromere behaviour examined were
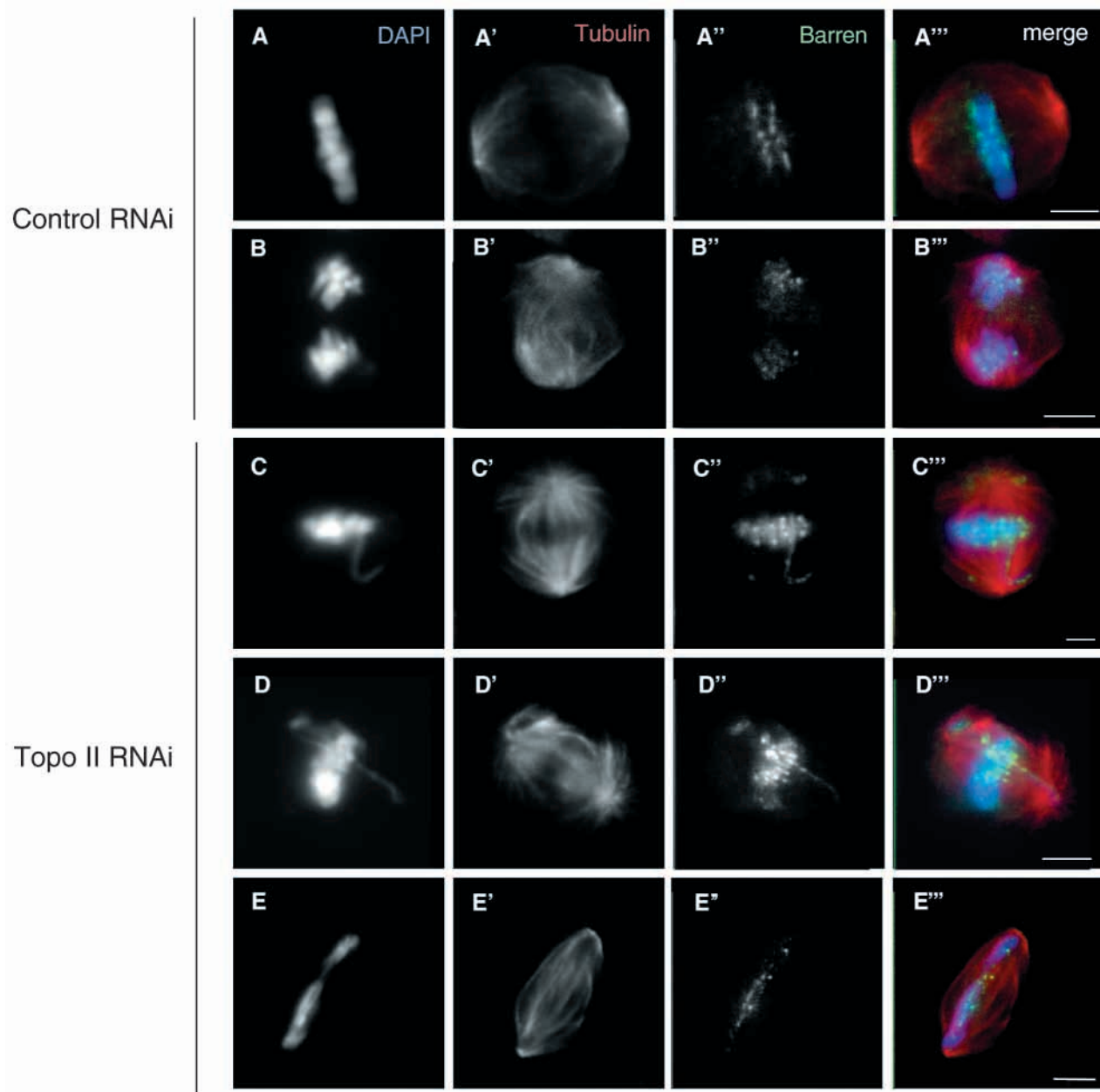
Fig. 4. The condensin subunit Barren associates normally with mitotic chromosomes in Topo II-depleted cells. (A,B) Control RNAi-treated normal metaphase and anaphase cells. (C,D) Topo II RNAi-treated metaphase cells with a highly elongated chromosome arm extending to one spindle pole. (E) Topo II RNAi-treated anaphase cell with the bulk of the chromatin stretched out between the separating kinetochores. (A-E) DAPI staining for DNA. (A'-E') anti-tubulin shows the mitotic spindle; (A''-E'') staining for Barren shows the position of the condensin complex; (A'''-E''') merged images (DAPI is blue, tubulin is red, Barren is green). Scale bar: 5 µm.

normal in Topo II-depleted *Drosophila* S2 cells. These included the ability to assemble a kinetochore (detected with Cid/CENP-A – Fig. 1D, Fig. 5B,C), targeting of chromosomal passenger proteins (Fig. 7), binding of spindle microtubules and migration towards the spindle poles (Fig. 8B''; see also Fig. 1F).

Since the chromosomal passenger proteins INCENP, Survivin and Aurora-B are essential for numerous chromosomal functions during mitosis, we examined their targeting in some detail in cells depleted of Topo II. No obvious defects were observed (Fig. 7). Furthermore, in the majority of cells, INCENP/Aurora B transferred normally to the central spindle at the transition to anaphase even though sister chromatids did not disjoin normally. However, in some anaphases INCENP localisation was aberrant, with the protein distributed both along the segregating chromatids and parallel to the spindle microtubules (Fig. 7E). The reason for this is unknown, but it could be a result of defective release of sister chromatid cohesion in these cells.

Despite the apparently normal centromere/kinetochore function virtually all anaphase and telophase cells depleted of Topo II (72 hours after addition of dsRNA) exhibited
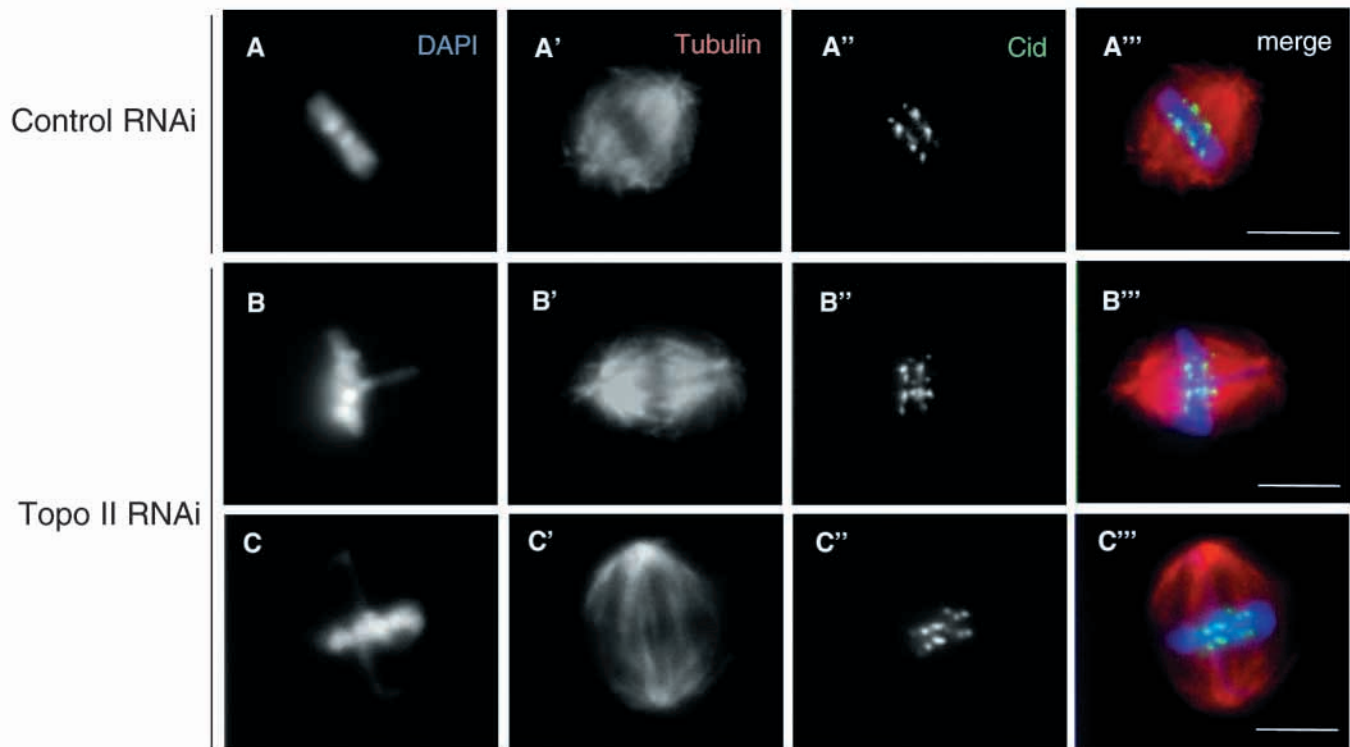
Fig. 5. Many metaphases in Topo II-depleted cells have an unusual phenotype in which one or more chromosome arm(s) becomes highly elongated and stretches towards the spindle pole. (A) Control RNAi-treated normal metaphase cell. (B) Topo II RNAi-treated metaphase cell with a highly elongated chromosome arm extending to one spindle pole. (C) Topo II RNAi-treated metaphase cell with J-shaped chromosome arms extending to spindle poles. (D) Statistical analysis of the protruding arm phenotype. (A-C) DAPI staining for DNA; (A'-C') anti-tubulin shows the mitotic spindle; (A''-C'') staining for Cid/CENP-A shows the position of kinetochores; (A'''-C''') merged images (DAPI is blue, tubulin is red, Cid is green). Scale bar: 5 μm.

abnormalities in chromosome segregation (Fig. 8B,C). Anaphase and telophase cells had normal mitotic spindles with centromeres approaching the poles, but sister centromeres were linked by massive chromatin bridges (Figs 1F, 4E, 7F, 8B). This led to failures in cytokinesis, and the number of binucleate cells in cultures also increased fivefold (data not shown). This is in agreement with previous genetic analyses of Topo II function in yeasts, and with inhibitor studies in metazoan cells (DiNardo et al., 1984; Holm et al., 1985; Uemura and Yanagida, 1986; Gorbsky, 1994).

In control cells, kinetochores migrated towards the spindle poles in a compact line perpendicular to the spindle axis (Fig. 8A''). In contrast, kinetochores of Topo II-depleted cells in anaphase appeared to be distributed in a linear fashion along the spindle axis (Fig. 8B''; see also Fig. 1F''). This was reminiscent of the behaviour of kinetochores in *top2* mutants of *S. pombe* (Funabiki et al., 1993), and may reflect a 'tethering' effect, where kinetochores migrate towards the poles until they are halted by unresolved catenations in the trailing chromatin.

Thus Topo II is required for efficient sister chromatid segregation at anaphase, but this is probably primarily because of a requirement for decatenation of chromatid arms. Topo II appears not to be required for either centromere or kinetochore assembly or function.

Discussion

The role of DNA topoisomerase II (Topo II) in mitotic chromosome structure and dynamics has been controversial for well over a decade, at least partly because functional studies to date have involved the use of antibodies and drugs that may not have produced a straightforward null phenotype. We have used RNAi to deplete *Drosophila* cultured cells of their single Topo II isoform. This analysis has revealed that the function of Topo II differs in several substantial ways from that predicted by previous studies. For example, Topo II is not required for inactivation of a checkpoint that monitors chromatin catenation in G2, or for the assembly or function of kinetochores, however, it is required for alignment of the chromosomes at a compact metaphase plate. With regard for one of the most contentious issues, we found that Topo II is required for mitotic chromatin to reach a normal level of compaction, but not for the global process of mitotic chromosome condensation.
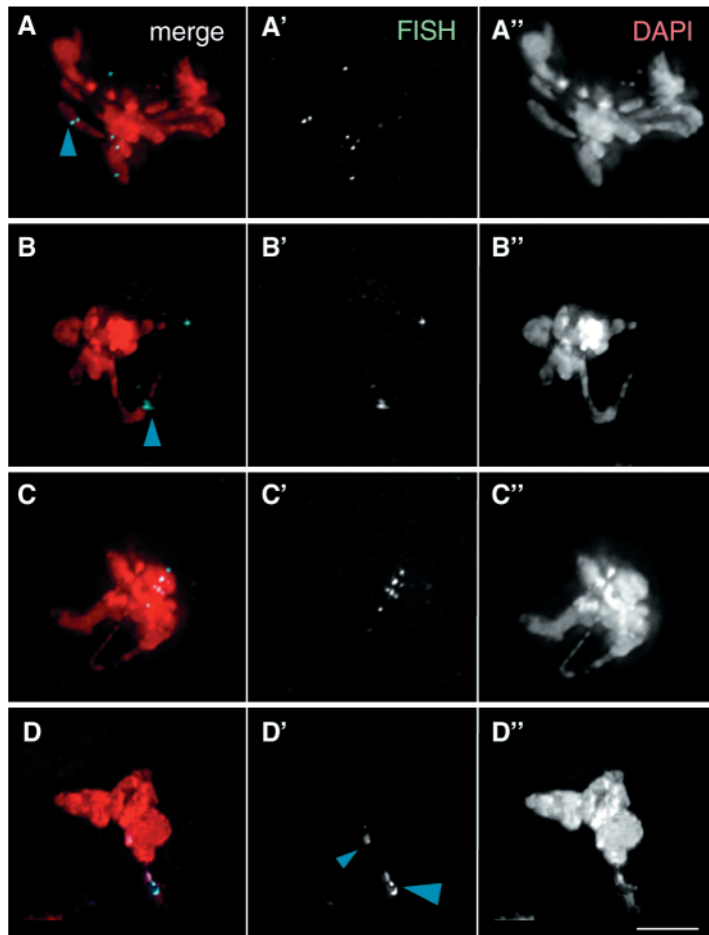
Fig. 6. Identification of the chromosomal component in the protruding arm phenotype. Analysis by FISH at 72 hours after the addition of Topo II dsRNA using the following probes. (A) Euchromatic chromosome 3 probe (BACH47K04); (B) Euchromatic chromosome X probe (BACH47E07). In A and B, arrowheads indicate FISH signal in protruding arm. (C) Euchromatic chromosome 2 probe (BACHN09); (D) Heterochromatic chromosome X probe (359 satellite); large arrowhead points to extended FISH signal in protruding arm; small arrowhead points to condensed FISH signal in metaphase plate. In all merged images, the probe is green and the DNA is red. Scale bar: 5 μm.

Topo II is not required to inactivate a G2 checkpoint or to assemble functional kinetochores in *Drosophila* cells

In mammals, entry into mitosis is thought to be regulated by a Topo II-dependent checkpoint that monitors the level of catenation of sister chromatids (Downes et al., 1994; Clarke and Gimenez-Abian, 2000). This checkpoint involves signalling through the ATR kinase following inactivation of Topo II by ICRF-193 (Deming et al., 2001).

The results of the present study reveal that *Drosophila* S2 cells do not appear to have a checkpoint mechanism that couples mitotic entry or progression with Topo II activity. Cells traversed mitosis with relatively normal kinetics, even though mitotic events were grossly abnormal, particularly in the closing stages. We have noted previously that S2 cells have weak checkpoints controlling behaviour in mitosis (see Adams et al., 2001), and the possibility remains that other cell types might have this checkpoint.

Previous studies suggested that Topo II could have an essential role in kinetochore structure (Rattner et al., 1996; Bachant et al., 2002). Our results identify no key function for Topo II in kinetochore assembly or function. Following Topo II depletion we observed normal chromosomal targeting of the centromere-specific histone H3 subtype Cid/CENP-A. Furthermore, kinetochores interacted with microtubules and migrated towards the spindle poles in anaphase. The different patterns of kinetochore distribution observed in control and Topo II-depleted anaphases probably reflect the high levels of residual catenation of sister chromatids.

Topo II is required for complete chromatin condensation and a normal chromosomal morphology

Chromosome morphology did not look normal following Topo II depletion. Local levels of chromatin condensation were roughly 2.5-fold less in the Topo II-depleted cells, and many metaphases had a mass of condensed chromatin that lacked well-defined sister chromatids. This phenotype could be either a direct consequence of the loss of Topo II activity, or due to interference with targeting of factors required for proper mitotic chromosome morphology. These include the condensin complex (Hirano and Hirano, 2002) and the chromosomal passenger proteins INCENP and Aurora-B.

Depletion of the chromosomal passenger proteins has previously been shown to result in production of dumpy, irregularly shaped chromosomes (Adams et al., 2001), possibly because Aurora-B is required both for chromosome targeting of Barren, a non-SMC subunit of the condensin complex (Giet and Glover, 2001), and for phosphorylation of histone H3 on serine[10] (Gurley et al., 1978; Bradbury, 1992; Adams et al., 2001; Murnion et al., 2001). However, INCENP and Aurora-B target normally to mitotic chromosomes in Topo II-depleted cells.

Our results show that Topo II is also not required for the normal targeting of Barren to chromosomes, however we cannot exclude a secondary role for Topo II in condensin function. For example, Topo II-mediated decatenation activity might be required for the resolution of the two sister chromatids during condensin-mediated chromosome condensation (Holm, 1994; Steffensen et al., 2001).

## Topo II is required for a compact metaphase alignment of the chromosomes

The most surprising and novel phenotype observed in this study was that the chromosomes failed to form a compact metaphase plate in about 25% of metaphase cells. Instead, these cells had one or more chromosome arms protruding a substantial distance from the plate, typically stretched along the long axis of the spindle towards one of the poles. Analysis by FISH revealed that the chromosomal component involved in this phenotype is not entirely random, although any of the arms of chromosomes 3 and X could be found near the poles of metaphase cells. In contrast, neither arm of chromosome 2 was ever observed to protrude from the plate in this way.
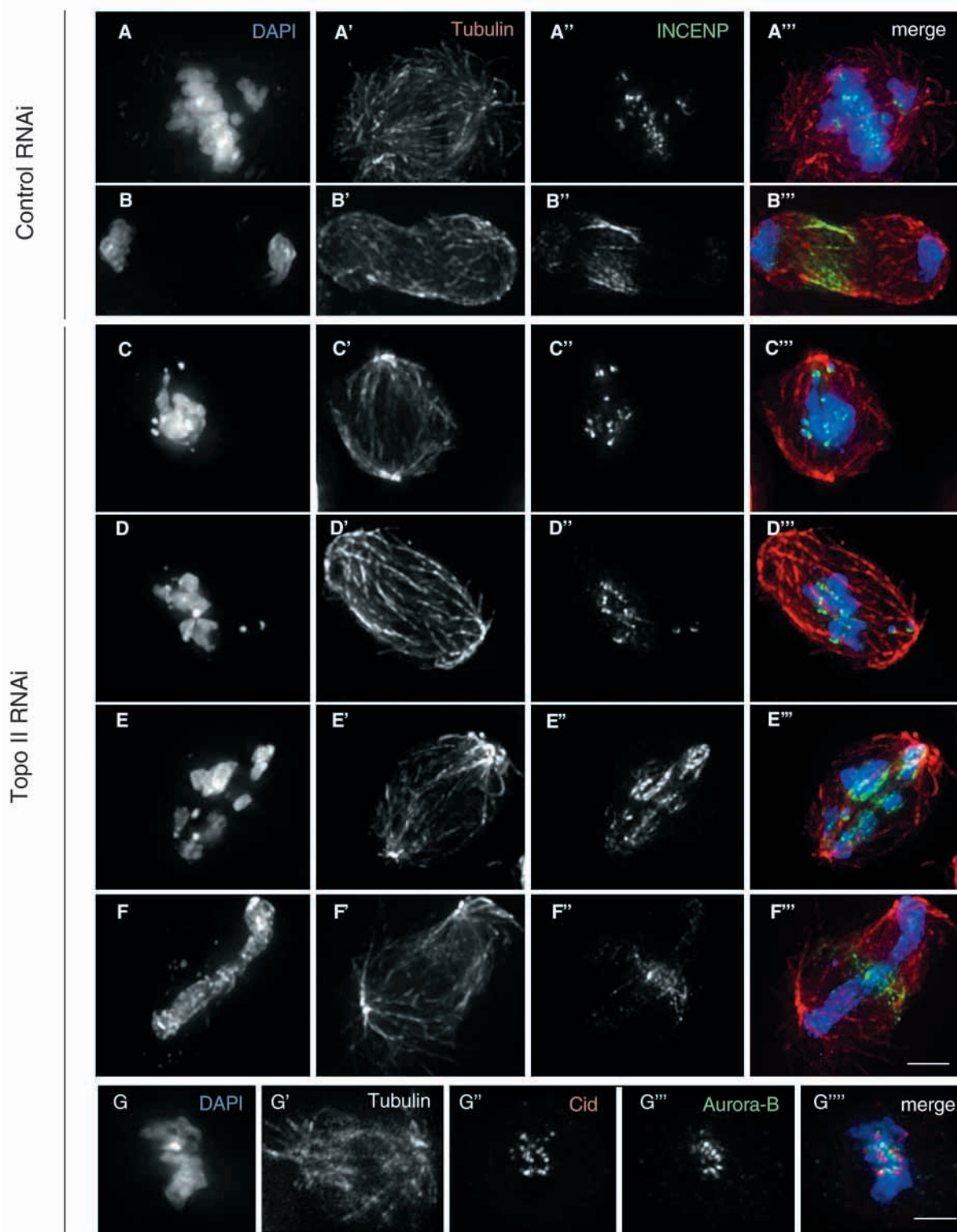
Fig. 7. The chromosomal passenger protein INCENP is localised normally in Topo II-depleted cells with the exception of a few highly abnormal anaphase cells. (A) Control RNAi-treated normal prometaphase cell. (B) Control RNAi-treated normal telophase cell. (C) Topo II RNAi-treated prometaphase cell with abnormal chromosomal morphology. (D) Topo II RNAi-treated late prometaphase cell with abnormal chromosomal morphology. (E) Topo II RNAi-treated abnormal cell with INCENP stretched along the chromosome arms. (F) Topo II RNAi-treated anaphase cell with massive chromatin bridging, but INCENP located normally on the central spindle. (G) Topo II RNAi-treated late prometaphase cell with abnormal chromosomal morphology: Aurora-B localises normally on the centromeres. (A-G) DAPI staining for DNA; (A'-G') anti-tubulin shows the mitotic spindle; (A''-F'') staining for INCENP; (G'') staining for Cid shows the positions of the kinetochores; (G''') staining for the chromosomal passenger Aurora B. In A'''-F''' DAPI is blue, tubulin is red, INCENP is green, and in G'''' DAPI is blue, Cid is red, Aurora-B is green. Scale bar: 5 μm.
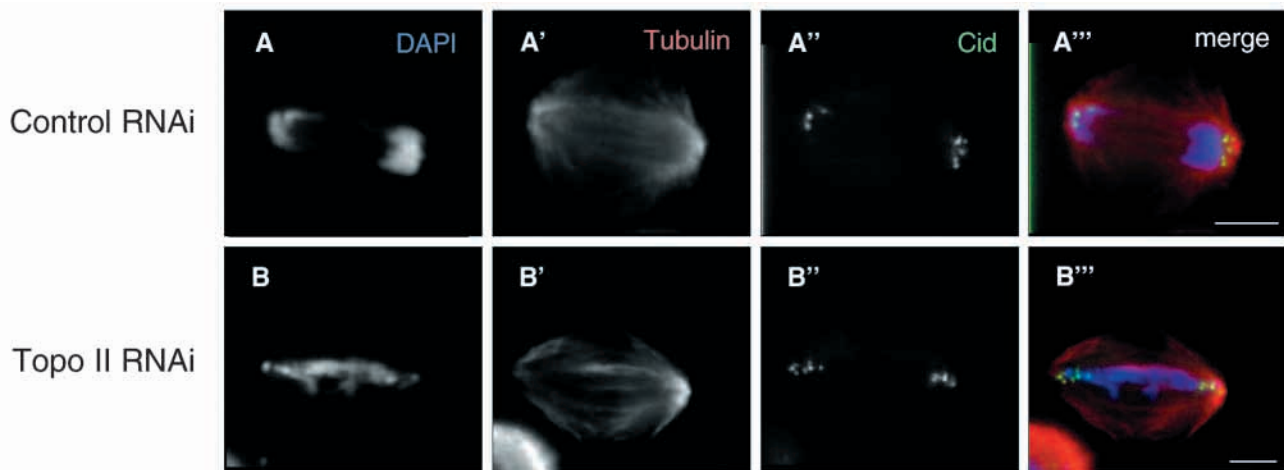
Fig. 8. Anaphase in Topo II-depleted cells is characterised by the presence of massive chromatin bridges, however sister centromeres usually manage to disjoin and move towards the spindle poles. (A) Control RNAi-treated normal anaphase cell. (B) Topo II RNAi-treated anaphase cell with the bulk of the chromatin stretched out between the separating kinetochores. (A-B) DAPI staining for DNA; (A'-B') anti-tubulin shows the mitotic spindle; (A"-B") staining for Cid/CENP-A shows the position of kinetochores; (A'''-B''') merged images (DAPI is blue, tubulin is red, Cid is green). Scale bar: 5 μm. (C) Statistical analysis of the lagging chromosome phenotype.
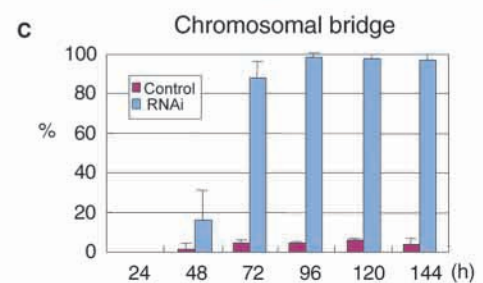
The protruding chromosomes were not precociously separated sister chromatids moving to the pole. In almost every case, the centromeres were clustered normally at the metaphase plate, and no Cid staining was observed on the protruding arm. Furthermore, in favourable instances, the two sister chromatids could be observed. Thus, this phenomenon does not appear to represent abnormal kinetochore-based movement towards the spindle pole.

We have considered three models to explain the origin of the protruding arms in Topo II-depleted cells. First, they could be chromosomal regions that exhibit a persistent failure in condensation. If this was the case, we would expect the arm to be oriented randomly or extruded laterally out of the spindle because of the action of the 'polar wind' (Carpenter, 1991). For example, when chromosome arms are severed with a laser, they are rapidly ejected from the vicinity of the spindle pole (Rieder et al., 1986) as a result of the action of plus-end-directed chromokinesin motors associated with the chromatin as well as collisions with elongating microtubules (Funabiki and Murray, 2000). This is inconsistent with our observation that the stretched arms almost invariably have at least one region in close proximity to a spindle pole.

A second model suggests that the protruding arms move towards the pole because they do in fact have active kinetochores that lack detectable Cid/CENP-A. As the protruding chromosomes derive from three different chromosome arms and have a whole range of morphologies, this hypothesis would require the unlikely possibility of the efficient formation of neocentromeres at a number of different positions on the long arms of chromosomes X and 3.

A third possibility is that the arms are physically trapped close to the pole and cannot retract back to the metaphase plate. This could explain the morphology of the J-shaped chromosomes if the point of entrapment was close to the pole: chromokinesins would try to move the short and long arms of the J away from the pole. This is consistent with the highly elongated appearance of these protruding arms, which would be stretched as they were pulled away from the pole by the associated chromokinesin motors [see the stretched chromosomes in Funabiki and Murray (Funabiki and Murray, 2000)].

It is remarkable that the protruding arms consistently extend towards one pole of the mitotic spindle. Although Topo II is a major component of mitotic chromosomes and is generally considered to be a chromatin protein, one recent study has shown that Topo IIα can be detected as a salt-stable component of centrosomes in a number of human cell lines (Barthelmes et al., 2000). Although that study did not propose a definitive function for Topo II at the centrosome, our results suggest that this centrosomal Topo II could have a role in enabling the arms of metaphase chromosomes to detach from centrosomes and assume their classical condensed structure at the metaphase plate.

Conclusions

A detailed phenotypic analysis of the role of Topo II in mitotic events has confirmed that the enzyme has essential roles in sister chromatid separation at anaphase. The enzyme is not, as was expected, essential for assembly of a functional kinetochore, but it does appear to have a role in the establishment of a normal mitotic chromosome morphology. Most surprisingly, in the absence of Topo II, one or more chromosome arms are frequently trapped in the vicinity of the spindle pole during metaphase. It will be a challenge for future

experiments to test whether Topo II can detach DNA from entanglements with other polymers in addition to DNA.

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