Depletion of topoisomerase IIα leads to shortening of the metaphase interkinetochore distance and abnormal persistence of PICH-coated anaphase threads

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Summary

Topoisomerase II (topo II) is a major component of mitotic chromosomes, and its unique decatenating activity has been implicated in many aspects of chromosome dynamics, of which chromosome segregation is the most seriously affected by loss of topo II activity in living cells. There is considerable evidence that topo II plays a role at the centromere including: the centromere-specific accumulation of topo II protein; cytogenetic/molecular mapping of the catalytic activity of topo II to active centromeres; the influence of sumoylated topo II on sister centromere cohesion; and its involvement in the activation of a Mad2-dependent spindle checkpoint. By using a human cell line with a conditional-lethal mutation in the gene encoding DNA topoisomerase IIα, we find that depletion of topo IIα, while leading to a disorganised metaphase plate, does not have any overt effect on general assembly of kinetochores. Fluorescence in situ hybridisation suggested that centromeres segregate normally, most segregation errors being chromatin bridges involving longer chromosome arms. Strikingly, a linear human X centromere-based minichromosome also displayed a significantly increased rate of missegregation. This sensitivity to depletion of topo IIα might be linked to structural alterations within the centromere domain, as indicated by a significant shortening of the distance across metaphase sister centromeres and the abnormal persistence of PICH-coated connections between segregating chromatids.

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Key words: Topoisomerase, PICH, Chromosome, Centromere, Condensation, Segregation

Introduction

DNA topoisomerases have essential roles through altering the structure of double-stranded DNA. They are classified into two categories, topo I and II, based on the mode of the enzymatic reaction. Only topoisomerase II (topo II) can both remove positive or negative supercoils and catenate or decatenate DNA duplexes. Owing to its unique, ATP-dependent, DNA-strand-passing activity, topo II plays a role in virtually all processes involving double-stranded DNA and functions at multiple steps in the assembly of mitotic chromosomes from interphase nuclei (Wang, 2002; Porter and Farr, 2004).

Whereas yeasts and *Drosophila* have a single gene encoding topo II, there are two distinct isoforms in higher eukaryotes, designated topo IIα (170 kDa) and topo IIβ (180 kDa) that are encoded by different genes. The two isoforms have identical catalytic activities in vitro and both can complement yeast topo II function. However, the two isoforms show differences in cell cycle expression and have different distributions in vivo. Topo IIα is associated with mitotic chromosomes and is preferentially expressed in proliferating cells, with levels varying throughout the cell cycle, increasing in S phase, peaking in G_2-M and diminishing in G_1 (Heck et al., 1988). The IIβ isoform is expressed in both proliferating and differentiated cells and its amount and stability show no significant fluctuations through the cell cycle. The localisation of the IIβ isoform, however, is controversial; while the bulk is diffusely nucleoplasmic in interphase and mitosis (Chaly et al., 1996; Meyer et al., 1997), a minor fraction of IIβ appears to be associated with mitotic chromatin (Christensen et al., 2002; Null et al., 2002). Nevertheless, while topo IIα is essential for mitosis and cell division, topo IIβ is dispensable in cultured cells (Woessner et al., 1991; Chaly et al., 1996; Grue et al., 1998; Yang et al., 2000; Akimitsu et al., 2003).

A distinctive feature of topo II (especially IIα) is that it accumulates at mitotic centromeres in prometaphase and remains there until early anaphase (Taagepera et al., 1993;

Gorbsky, 1994; Rattner et al., 1996; Sumner, 1996; Christensen et al., 2002; Null et al., 2002). A number of studies have suggested that topo II might have a role in influencing organisation of the centromere (Rattner et al., 1996; Toyoda and Yanagida, 2006), centromeric cohesion (Bachant et al., 2002; Takahashi et al., 2006) and a Mad2-dependent spindle checkpoint (Mikhailov et al., 2004; Skoufias et al., 2004; Toyoda and Yanagida, 2006). This view has been strengthened by the identification, through cytogenetic and molecular studies, of topo II cleavage activity at active centromeres in vertebrates and in parasitic protozoa (Floridia et al., 2000; Andersen et al., 2002; Spence et al., 2002; Agostinho et al., 2004; Spence et al., 2005; Kelly et al., 2006; Obado et al., 2007). However, no overt effect of depletion of topo II on general kinetochore assembly, or on anaphase centromere separation, has been reported (Chang et al., 2003; Sakaguchi and Kikuchi, 2004; Toyoda and Yanagida, 2006). Here, we investigate the role of topo II in mitotic chromosome dynamics using a human cell line (designated HTETOP) that is conditionally null mutant for topo IIα (Carpenter and Porter, 2004). Our focus has been on the influence of topo II within the centromere domain and on anaphase chromosome segregation. The latter is of particular interest given the recent description of ultrafine DNA connections extending between the centromeres of otherwise well-separated chromatids in normal anaphases, which stain for the Plk-1-interacting checkpoint (PICH) and Bloom's syndrome (BLM) helicases (Baumann et al., 2007; Chan et al., 2007).

Whereas no gross effects on mitotic chromosome structure or general kinetochore assembly were detected, the segregation behaviour of a 'sensitised' minichromosome in the topo-IIα-depleted background suggested a centromere effect. Further analysis revealed that depletion of topo IIα, as well as promoting bridging through a failure to decatenate chromosome arms efficiently, also leads to an altered centromere topology. This is reflected in a shortening of the distance across sister centromeres under tension, together with the abnormal persistence of PICH-coated connections between segregating chromosomes in topo-IIα-depleted human cells.

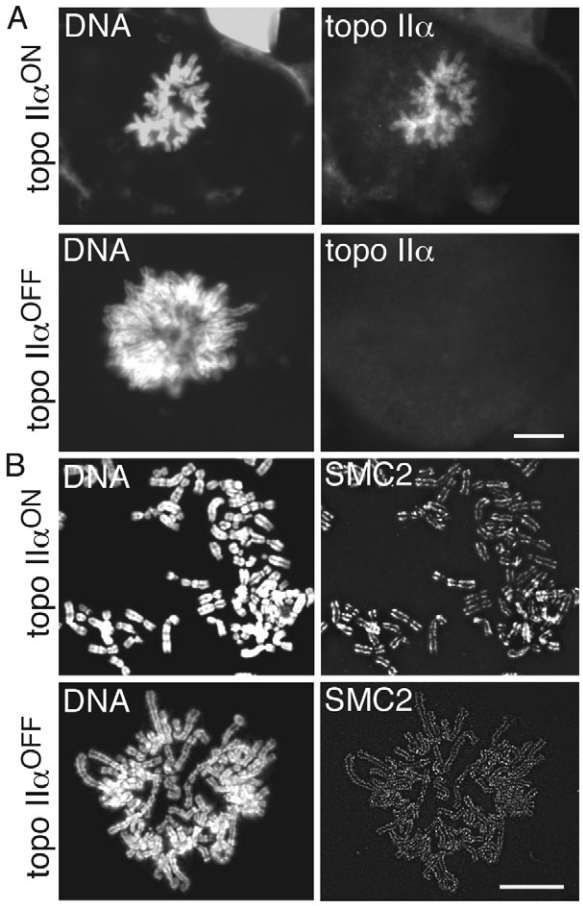

Fig. 1. (A) Immunofluorescence for topo IIα on HTETOP metaphase cells grown in the absence (topo IIα^{ON}) and presence (topo IIα^{OFF}) of dox for 48 hours. (B) Immunofluorescence for the SMC2 subunit of condensin on HTETOP metaphase spreads isolated from cells grown in the presence and absence of dox for 72 hours. The normal axial distribution of SMC2 is detected following depletion of topo IIα. Bar, 10 μm.

Results

Chromosomes depleted of topo IIα retain a structural memory

Topo II and the condensin complex are major non-histone components of the mitotic chromosome scaffold. Recently, it was shown that depletion of condensin results in mitotic chromosomes that are structurally disrupted and in which other scaffold proteins, including topo IIα, are mislocalised (Hudson et al., 2003). We therefore examined the effect of depletion of topo IIα [72 hours exposure to doxycycline (dox)] on the localisation of condensin. Immunofluorescence (IF) using antibodies against the condensin I and II subunit SMC2 revealed a characteristic axial distribution in mitotic chromosomes, even when depleted of topo IIα (Fig. 1). Some variation in the intensity of SMC2 staining was seen between mitotic cells, but this appeared to be independent of exposure to dox. To investigate whether a condensin phenotype would be exposed if both topo IIα and β were depleted simultaneously, RNAi was used to deplete topo IIβ transiently from HTETOP cells. (Depletion of topo IIβ was confirmed by IF, western blotting and real-time PCR and levels estimated to be ~20% of normal at 72 hours post transfection with siRNA; supplementary material Fig. S1.) The localisation of condensin was unaffected by the depletion of both topo II isoforms (data not shown).

We then examined the effect of depletion of topo IIα on chromosome integrity. The assay used relies on the ability of chromosomes to recover their morphology following complete unfolding of the chromatin (Cole, 1967; Hudson et al., 2003). Chromosomes, in cells grown on coverslips and arrested overnight with colcemid, are first unfolded by exposure to a solution of low ionic strength lacking divalent cations and containing EDTA. They are then refolded by the addition of a buffer of low ionic strength containing 5 mM Mg^{2+}. Under this assay, the term 'structural integrity' is defined as being 'the ability of chromosomes to remain morphologically indistinguishable at the level of the light microscope during repeated cycles of swelling and shrinking' (Vagnarelli et al., 2004). HTETOP cells in which topo IIα had been depleted (72-120 hours exposure to dox) were subjected to successive cycles with chromatin-unfolding and chromatin-compacting buffers. No effect on the ability of mitotic chromosomes to expand and

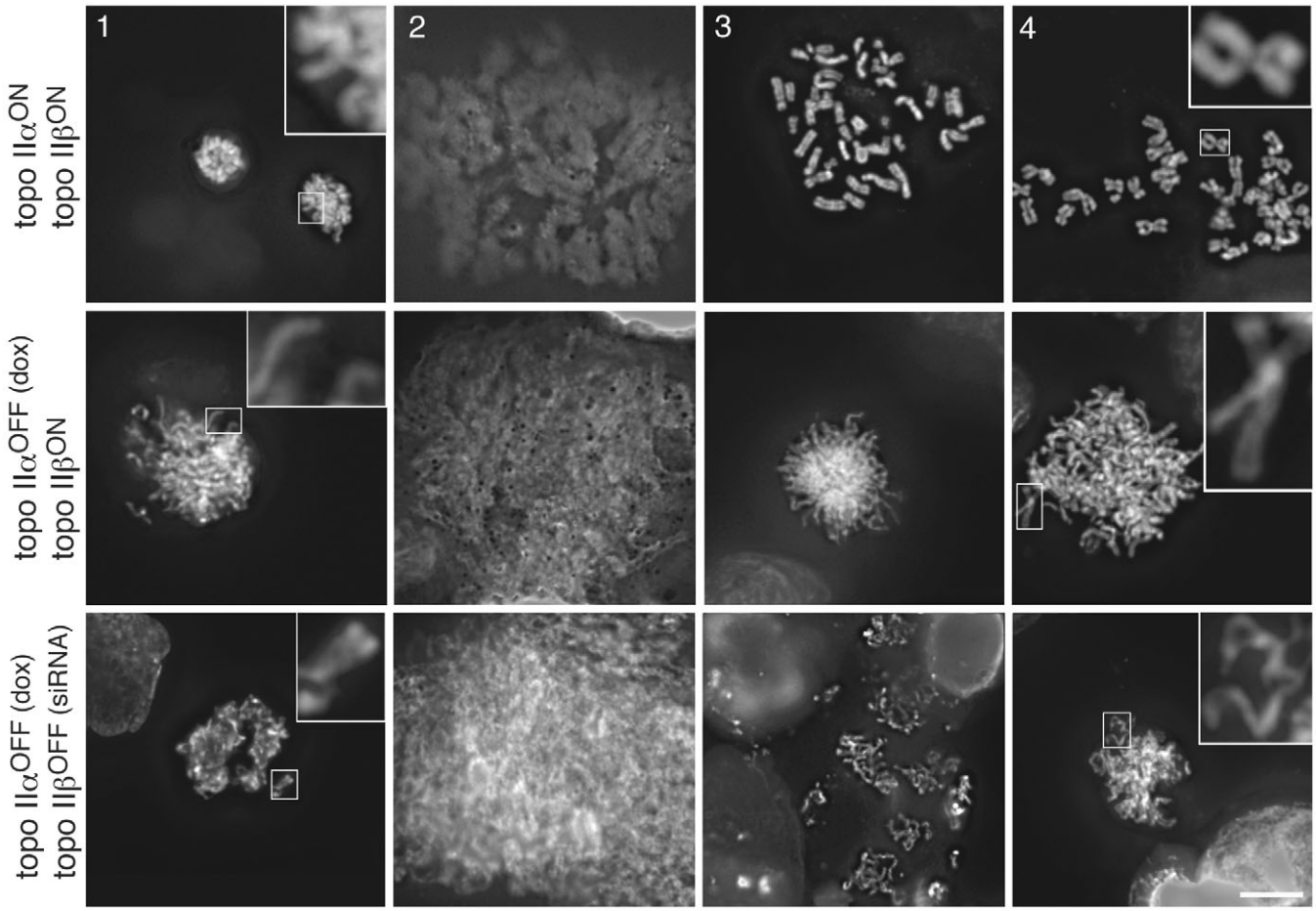

Fig. 2. Topo II is not required for the structural integrity of mitotic chromosomes. Shown are representative images following the expansion and refolding assay. Stages (1) to (3) represent the first cycle of expansion and refolding, and (4) is the refolded status after cycle 2. Stage (1): colcemid-arrested metaphase before treatment; stage (2): first-round expansion in the low-salt plus EDTA TEEN buffer; stage (3): first-round contraction in the low-salt plus Mg^{2+} RSB buffer; stage (4): appearance after a second round of expansion and contraction. Topo-II-depleted chromosomes [both α-only depleted (120 hours exposure to dox) and α- plus β-depleted (120 hours exposure to dox plus 72 hours exposure to siRNA)] recover their normal starting morphology, which is a slightly thinner and more ribbon-like appearance than that displayed by the control chromosomes (Carpenter and Porter, 2004). All images are shown at the same magnification, with some regions enlarged for clarity. Bar, 10 μm.

to subsequently recover their gross structural integrity was detected following depletion of topo IIα (Fig. 2). Consistent with previous observations, the extent of contraction achieved in this assay by chromosomes depleted of topo IIα was slightly less than for normal chromatin, with chromosomes generally being thinner and with sister chromatids that were less well resolved (Fig. 2). However, ⩾98% of chromosomes were seen to be contracted following two rounds of incubation in the expansion and contraction buffers, irrespective of whether the cells had been exposed to dox. This suggests that depletion of topo IIα does not affect the ability of chromosomes to recover a compact structure. Depletion of topo IIβ, either in the presence or absence of topo IIα, had no detectable effect on the ability of HTETOP chromosomes to recover their native morphology under these assay conditions (Fig. 2 and data not shown).

Many topo-IIα-depleted cells have a disorganised metaphase plate

In many metaphase cells following depletion of topo IIα, one or more chromosome arms extended away from the compact mass of chromatin, often stretching towards the pole (Fig. 3B-E). This behaviour, which previously has been described in *Drosophila* cells transiently depleted of topo II (Chang et al., 2003), was particularly clear in dox-treated cells arrested in metaphase under tension using the proteasome inhibitor MG132. After 48-72 hours exposure to dox, 20-30% of MG132-arrested metaphases had at least one arm protruding polewards, compared with a background frequency of 0-4%. Similar observations were made following the depletion of both topo IIα and IIβ (72 hours exposure to dox plus siRNA) (Fig. 3D,E). Staining for the inner kinetochore protein CENP-C suggested that, in most cases, these protrusions were not associated with the pulling activity of the centromere.

Missegregation following depletion of topo IIα arises from effects both on chromosome arms and at centromeres

After 48-72 hours of topo IIα repression, most HTETOP anaphase cells are aberrant, with frequent lagging and bridging of chromatin (Carpenter and Porter, 2004). Nevertheless,

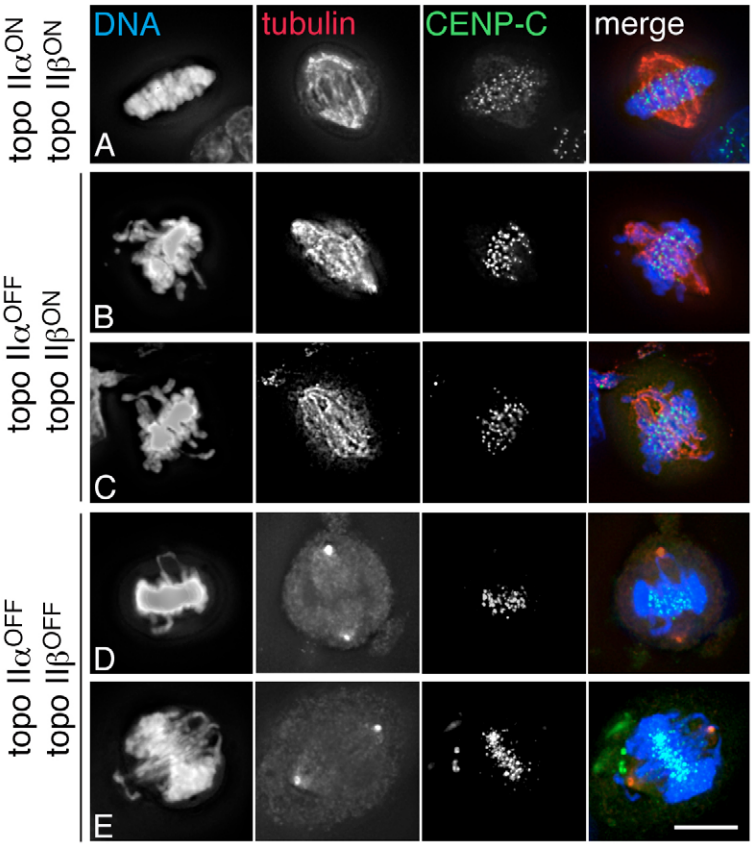

Fig. 3. Many metaphases in topo-II-depleted cells have one or more elongated chromosome arms stretched towards the spindle pole. (A) A control (expressing topo IIα and IIβ) HTETOP metaphase cell; (B,C) HTETOP topo-IIα-depleted metaphase cells (48 hours exposure to dox); (D,E) topo IIα-depleted (72 hours exposure to dox) and topo IIβ-depleted (72 hours exposure to siRNA) cells arrested in metaphase with MG132 (DNA/DAPI, blue; tubulin, α or γ as indicated, red; CENP-C, green). Bar, 10 μm.

despite the lethal nature of topo IIα depletion, these cells do manage to separate the bulk of their chromosomes. This is in contrast to the situation when both topo II isoforms are depleted simultaneously, where the bulk of the chromatin remains unsegregated (Sakaguchi and Kikuchi, 2004; Toyoda and Yanagida, 2006) (data not shown). This suggests that topo IIβ contributes substantially to sister chromatid decatenation, as has been reported previously (Sakaguchi and Kikuchi, 2004).

To address the nature of the segregation errors arising from depletion of topo IIα and the origin of the DNA in the chromatin bridges, fluorescence in situ hybridization (FISH) was undertaken using the following probes: a pan-centromere DNA probe; centromere-specific DNA probes; paints for human chromosomes 6, 19, 20 and the X chromosome; a PNA telomeric (TTAGGG)n repeat; and a rDNA probe. This work was undertaken on HTETOP and a hybrid derivative carrying an extra human X centromere-based minichromosome.

Analysis of bridged anaphases using a pan-centromeric DNA suggested that centric regions segregate normally following the depletion of topo IIα (Fig. 4A). We then examined the segregation behaviour of the ribosomal RNA gene arrays. In *Saccharomyces cerevisiae*, TOP2-dependent decatenation of the rDNA appears to occur later than for the rest of the genome (Sullivan et al., 2004) and, in top2 mutants, hyper-recombination within the rDNA cluster has been described (Christman et al., 1988). However, in HTETOP cells, there was no evidence for the rDNA loci being disproportionately involved in chromatin bridges (Fig. 4B). For example, although 96% of anaphases in topo-IIα-depleted (72 hours exposure to dox) cells exhibited chromatin bridging (48/50 anaphase cells), rDNA was detected in the chromatin bridge in only 6% of cases (3/50). In all remaining cells, the nucleolar organiser regions segregated correctly. Telomeric DNA was frequently observed within the chromatin bridges arising from depletion of topo IIα, consistent with a failure to decatenate the distal regions of some chromosome arms. The occasional detection of chromatin bridges that lack (TTAGGG)n hybridisation signals could be due either to a hybridisation failure or could reflect bridging events arising from chromosomal rearrangements, such as breakage-fusion-bridge cycles, rather than from a failure of decatenation (Fig. 4C).

Chromosome-specific paints, used in conjunction with centromere-specific α-satellite probes, revealed that, following depletion of topo IIα, segregation errors increased for all chromosomes studied, but the errors were especially marked for the larger chromosomes (Fig. 4D-F). For example, for chromosome 6 (overall size ~176 Mb, with arms of ~65 and ~111 Mb) and the X (~160 Mb, with arms of ~60 and ~100 Mb), 7.7% and 5.9% of chromatids were missegregated in cells depleted of topo IIα, whereas, for chromosomes 19 and 20 (overall size ~70 Mb, with arms of ~30 and ~40 Mb), depletion of topo IIα induced missegregation rates of only 1.3% and 2.3% of chromatids, respectively. For chromosome 6 and the X, chromatin bridging made up the bulk of the missegregation events (95% and 89%, respectively), whereas, for chromosomes 19 and 20, bridging events contributed only 44% and 39% of the total errors (the rest being nondisjunction, chromosome lagging or apparent 1:0 segregation events).

To test further the notion that the bridging caused by depletion of topo IIα is a function of the length of chromosome arms, we examined the segregation of an 'armless' 2.7 Mb human minichromosome following its transfer into HTETOP cells. This linear minichromosome is a truncated version of the human X chromosome: the arms have been deleted and its non-telomeric DNA is composed totally of centric and pericentromeric sequences (Spence et al., 2006). Two independently derived hybrids were analysed and gave similar results. Consistent with a correlation between arm length and chromosome bridging, the minichromosome displayed no bridging, before or after depletion of topo IIα. Strikingly, however, other forms of missegregation, to which the minichromosome is particularly prone, were exacerbated by topo IIα depletion. Thus, when topo IIα was depleted (48 hours exposure to dox), the missegregation rate of the minichromosome increased from 2.2% to 6.5%, compared with <0.2% to 5.6% for the X (Fig. 4G). While, for the X chromosome, most errors involved

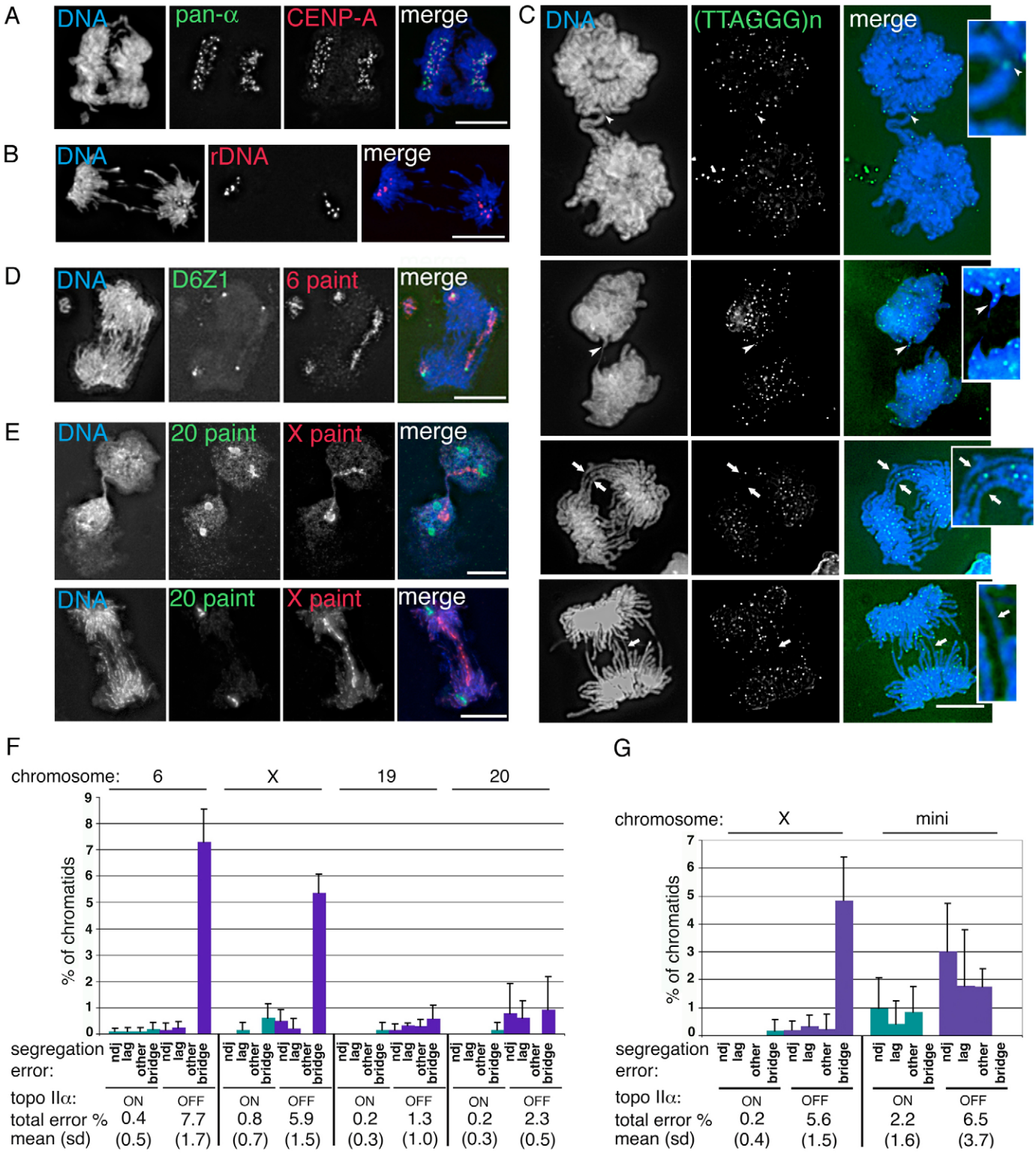

Fig. 4. See next page for legend.

chromatin bridging (86%), for the minichromosome, the errors detected were a mixture of nondisjunction, lagging and apparent 1:0 segregation events. To exclude the possibility that exposure to dox might by itself affect chromosome segregation, the behaviour of the minichromosome was assessed in the parental HT1080 background following 72 hours exposure to dox – there was no detectable effect on the rate of minichromosome missegregation (<1% both in the presence and absence of dox). These data suggest therefore that, in addition to a crucial role in preventing the

Fig. 4. The effect of depletion of topo IIα on human chromosome segregation. (A-E) HTETOP topo-IIα-depleted (48 hours exposure to dox) segregating cells. (A) immuno-FISH showing colocalisation of segregating signals for the DNA pan-alphoid probe (green) and anti-CENP-A (red); (B) typical FISH image showing segregation of the rDNA loci (red); (C) four post-metaphase cells showing the segregation behaviour of telomeric regions, detected using a PNA anti-telomere probe (green). White arrowheads indicate telomeric DNA in chromatin bridges. White arrows point to chromatin bridges that lack detectable (TTAGGG)n signals. These regions have been enlarged for clarity. (D) in this anaphase, the centromeres (green) of both copies of chromosome 6 have segregated, while the chromosome 6 paint (red) reveals that, for one of these chromosomes, the sister chromatids are bridged. This cell also appears to contain an acentric fragment of chromosome 6. (E) Two examples of segregating cells in which chromosome 20 (green) has separated correctly (although the second cell appears to have only one copy of this chromosome), while the sister chromatids of the single X chromosome (red) form a bridge. DNA is counterstained with DAPI (blue). Bar, 10 μm. (F) Chromosome paints and centromere-specific DNA probes were used to analyse the segregation of four of the endogenous HTETOP chromosomes: 6, the X, 19 and 20. For each chromosome, the numbers of sister chromatids analysed from either untreated (topo IIα^{ON}) or 48 hours exposure to dox (topo IIα^{OFF}) cells ranged from 141 to 447 per experiment. Shown are the means from three independent experiments (with s.d.). The total numbers of chromatids assessed for each chromosome under topo IIα ON/OFF conditions were: Chr. 6: 1230 and 1183; Chr. X: 657 and 592; Chr. 19: 1146 and 1058; Chr. 20: 1298 and 1006. Segregation errors were classified as non-disjunction (ndj), lagging, bridging or others (uncharacterised, apparent 1:0, segregation events, which might reflect absence of replication, chromosome loss or hybridisation failure). The combined segregation errors for each chromosome are summarised below the graph (mean percentage and s.d.). (G) The segregation behaviour of a 2.7 Mb human X centromere-based minichromosome was analysed following its transfer (by cell fusion) into HTETOP cells. Segregation data for the endogenous X chromosome in this hybrid background are also presented (mean and s.d.). For both the mini and X chromosomes, the total number of sister chromatids examined from either untreated cells or cells exposed to dox for 48 hours was ~1300 (based on data collected from three experiments for each of two independently derived hybrid cell lines). The total levels of chromatid missegregation following depletion of topo IIα are significantly higher, both for the whole X and for the minichromosome (Paired Student's *t*-test, $P \leqslant 0.005$).

accumulation of catenations in chromosome arms, topo IIα is required for normal centromere function.

Prominent PICH connections in topo-IIα-depleted anaphase cells

To investigate general kinetochore assembly, HTETOP cells in which topo IIα had been depleted (72-96 hours exposure to dox) were examined by IF using antibodies against CENP-A, -C, -E, -F, Aurora B and acetyl-histone H3. The depletion of topo IIα does not have any obvious effect on the localisation of these proteins, or on the under-acetylated nature of the centromere region (Fig. 5). Quantification of the centromere-specific histone H3 variant CENP-A at randomly selected centromeres, by indirect IF, revealed no significant change in cells depleted of topo IIα (n=459) relative to cells expressing topo IIα (n=906): topo IIα^{OFF} median=3608 fluorescence units (FU) (average absolute deviation 912) compared with topo IIα^{ON} median=3560 FU (average absolute deviation 799). The localisation of CENP-A was also examined in HTETOP cells depleted of IIβ and in cells depleted of both isoforms simultaneously. CENP-A was detected at centromeres in all cases (data not shown) Thus, there appears to be no obvious effect of depletion of topo II on the localisation of CENP-A or of the other kinetochore components examined.

We then examined the effect of depletion of topo IIα on the localisation of PICH (Baumann et al., 2007). PICH was found at the centromere in metaphase, and PICH-positive threads were detected in anaphase irrespective of the presence or absence of topo IIα (Fig. 6). Strikingly, the number of PICH-positive threads detected post-metaphase increased substantially in cells depleted of topo IIα (48 hours exposure to dox) (Fig. 6B,C). Thus, following depletion of topo IIα, the mean number of threads per anaphase cell increased fivefold (from 0.9 to 4.8 PICH threads/cell), with the proportion of PICH thread-negative anaphases decreasing from 74% to 10%. In some cases, these PICH-positive threads could be distinguished from the DAPI-stained chromatin bridges (examples indicated in Fig. 6B).

Both isoforms contribute to centromeric topo II cleavage activity

Topo IIα protein has been shown to be concentrated at the centromere of mitotic vertebrate chromosomes and to have major cleavage sites within centromeric DNA. To examine the effect of depleting topo IIα on centromeric DNA cleavage, this activity was assayed within the centromere of the 2.7 Mb human X centromere-based minichromosome. This allowed us to examine a molecularly defined haploid centromere, resolvable using pulsed-field gel electrophoresis (PFGE). Two independently derived HTETOP-minichromosome hybrids were analysed and gave similar results.

There was a noticeable difference in the ethidium bromide staining pattern of high molecular weight (HMW) DNA that depended on whether the cells had been treated with dox. In the absence of etoposide (irrespective of dox exposure), the bulk of the DNA was restricted to the slots and region of the gel above the compression zone, consistent with it being intact HMW DNA. Following etoposide exposure, the DNA extracted from cells expressing topo IIα showed a substantial accumulation of fragments in the 200 kb to 2 Mb size range. In cells from which most of the topo II had been depleted, the bulk of the DNA from etoposide-exposed cells was >2 Mb. This is consistent with all, or most, of the double-strand breaks trapped by etoposide being created by the action of topo II (Fig. 7A).

Southern blotting allowed us to focus on etoposide-specific cleavage fragments originating from within the centromeric α-satellite (DXZ1) DNA array on the minichromosome. Previous work has shown that exposure of growing cells to etoposide (0, 100 or 500 μM, 60 minutes at 37°C) results in partial cleavage of the minichromosome, generating 1.85 and 0.85 Mb DXZ1-hybridising fragments, together with a smear of more randomly cleaved products (this smear often masks the weaker 0.85 Mb hybridisation band) (Spence et al., 2002; Spence et al., 2005). In the HTETOP background following 72 hours exposure to dox, the amount of DXZ1 cleavage product was reduced substantially (and was barely detectable using 100 μM etoposide), although cleavage could still be detected using 500

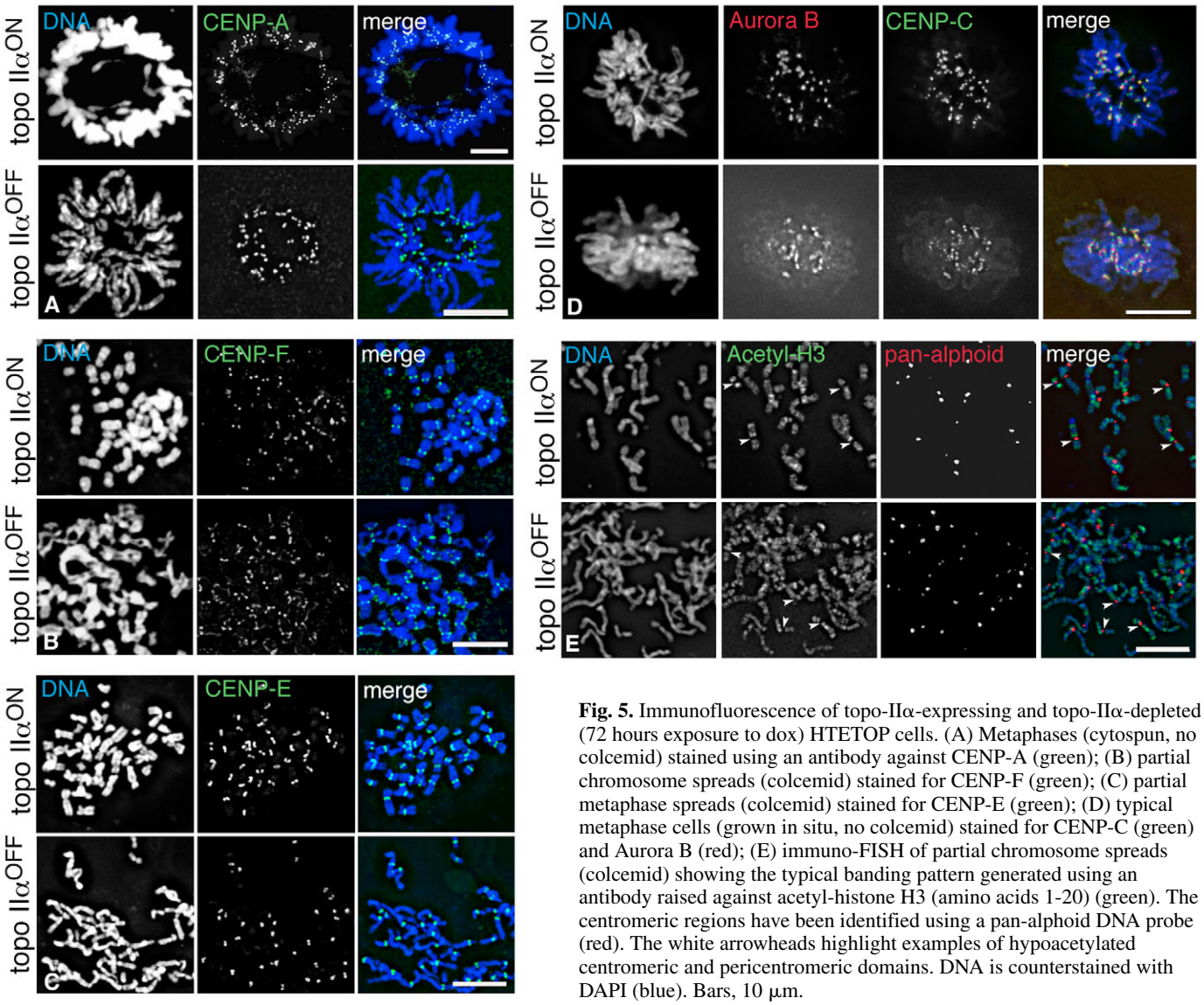

Fig. 5. Immunofluorescence of topo-IIα-expressing and topo-IIα-depleted (72 hours exposure to dox) HTETOP cells. (A) Metaphases (cytospun, no colcemid) stained using an antibody against CENP-A (green); (B) partial chromosome spreads (colcemid) stained for CENP-F (green); (C) partial metaphase spreads (colcemid) stained for CENP-E (green); (D) typical metaphase cells (grown in situ, no colcemid) stained for CENP-C (green) and Aurora B (red); (E) immuno-FISH of partial chromosome spreads (colcemid) showing the typical banding pattern generated using an antibody raised against acetyl-histone H3 (amino acids 1-20) (green). The centromeric regions have been identified using a pan-alphoid DNA probe (red). The white arrowheads highlight examples of hypoacetylated centromeric and pericentromeric domains. DNA is counterstained with DAPI (blue). Bars, 10 μm.

μM etoposide (but the 1.85 Mb signal was reduced relative to that of the intact 2.7 Mb minichromosome). In order to determine the origin of the residual cleavage, we investigated the effect of depleting IIβ, which has been estimated by others to be present at ~13% of the levels of topo IIα protein (Sakaguchi and Kikuchi, 2004). Depletion of topo IIβ alone had no detectable effect in this assay, but, following depletion of both isoforms simultaneously very little, if any, DXZ1 cleavage was detectable, even at the higher etoposide concentration (Fig. 7B). This is consistent with etoposide trapping double-strand breaks generated by both isoforms of topo II, and suggests that both isoforms can contribute to cleavage within centromeric DNA.

A shortening in the distance across topo IIα-depleted bi-oriented sister centromeres is consistent with impeded decatenation

To determine whether depletion of topo IIα has any effect on the structure of sister kinetochores under tension, HTETOP cells were arrested using MG132, fixed and subjected to FISH using a chromosome-11-specific centromere probe (D11Z1). The α-satellite DNA of bi-oriented sister chromosomes appeared either as separate dots or stretched continuously across the centromere domain. The distance across sister centromere pairs, to the outer edges of the D11Z1 signal, was measured after 72 hours exposure to dox, in parallel with untreated controls (Fig. 8A,B).

The distances across paired centromeres under tension in HTETOP cells decreased significantly (~10-25%) in topo-IIα-depleted cells during metaphase (Fig. 8B). In topo IIα^{ON} HTETOP cells grown in the absence of dox and arrested using MG132, the median distance between sister centromeres was 1.76 μm (average absolute deviation 0.39, n=163), whereas, in topo-IIα^{OFF} HTETOP cells, the distance was significantly smaller (1.46 μm, average absolute deviation 0.35, n=188, P<0.0001), based on the three independent experiments presented in Fig. 8B. No significant difference in the distance across sister centromeres was detected in the absence of tension (data collected from colcemid-arrested topo IIα^{ON} and topo α^{OFF} HETETOP cells), or following transient

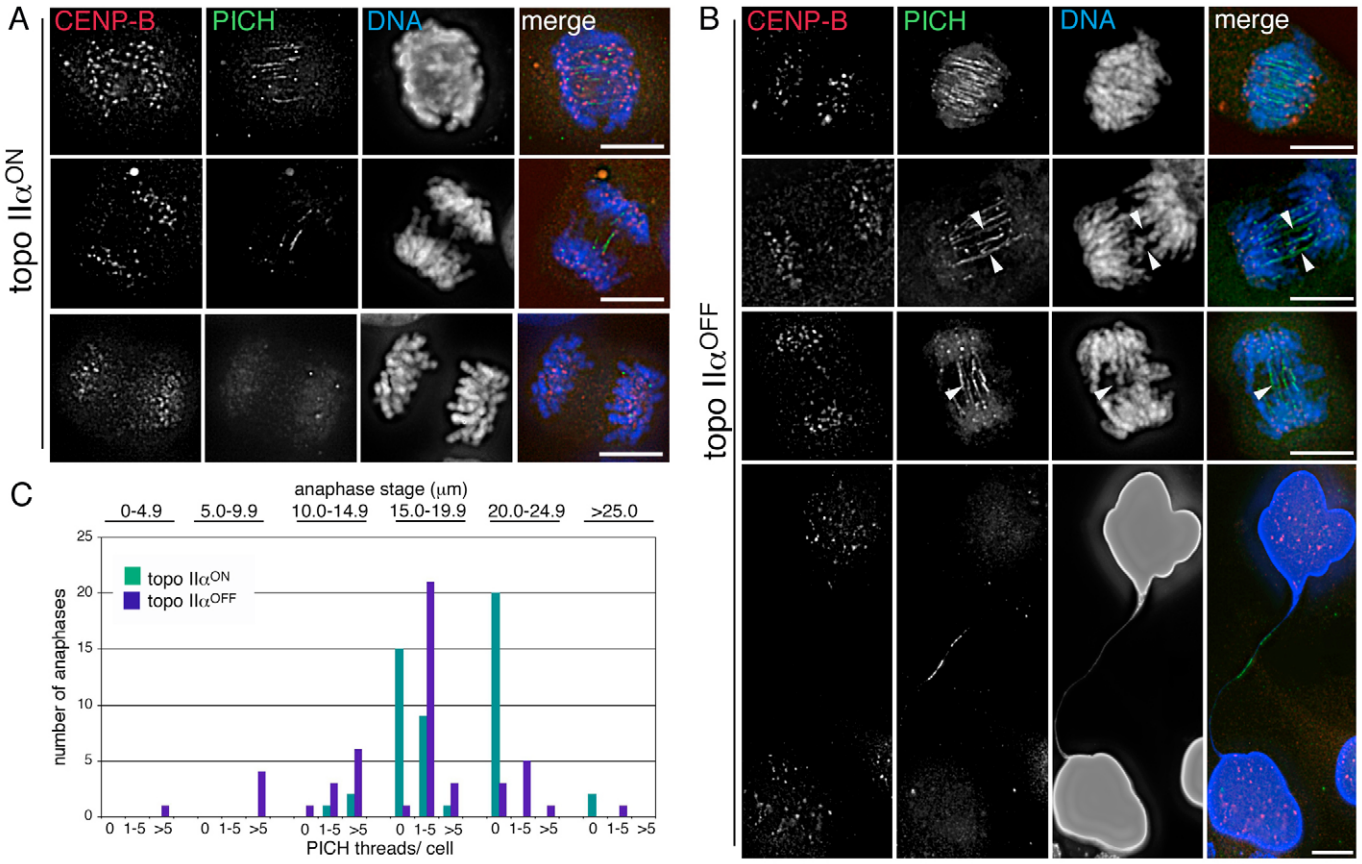

Fig. 6. The effect of depletion of topo IIα on anaphase PICH-coated threads. HTETOP cells expressing topo IIα (A), or depleted of topo IIα (48 hours exposure to dox) (B) were fixed and permeabilised before being co-stained for PICH (green) and CENP-B (red). Shown are cells in anaphase or undergoing aberrant cytokinesis. Arrowheads indicate examples of PICH threads that are not associated with DAPI-stained chromatin bridges. Bar, 10 μm. (C) Quantification of the number of PICH threads for anaphase cells expressing topo IIα (topo IIα^{ON}) and for cells treated for 48 hours with dox (topo IIα^{OFF}). 50 anaphase cells were analysed for each, and the stage was determined by the distance between segregating sister kinetochores.

depletion of topo IIβ (72 hours exposure to siRNA plus MG132).

To exclude the possibility that this decrease was due to an effect of dox exposure, rather than being due to depletion of topo IIα, distances were measured in the same way in the parental HT1080 cell line grown with and without dox addition and arrested using MG132. There was no significant difference in the inter-kinetochore distance, indicating that the decrease is specific to depletion of topo IIα (Fig. 8B). Further support for this is provided by the observation that, in HTETOP cells rescued from dox sensitivity by constitutive expression of the topo IIα protein [as a fusion with the C-terminus of eGFP in cell line HTETOP-GFP-topoIIα Clone J (Carpenter and Porter, 2004)], the distance across sister centromeres is restored (i.e. there is no significant difference in sister centromere distances in clone J cells grown in the presence or absence of dox, arrested using MG132) (Fig. 8B).

As influences that perturb the organisation of the centromere sometimes have an impact on the metaphase spindle, spindle length was examined in HTETOP cells stained with antibody against γ-tubulin. Data collected from three independent experiments revealed a small, but significant, increase in the pole-to-pole spindle distance in cells depleted of topo IIα: in topo IIα^{ON} cells (0 hours exposure to dox), the mean spindle length was 8.29 μm (s.d. 2.25, n=154), whereas, in topo IIα^{OFF} cells (72 hours exposure to dox), the mean spindle length was 9.91 μm (s.d. 3.38, n=150; P=0.0001) (Fig. 8C). A similar significant increase in spindle length was detected where distances between focused spindle poles were estimated based on immunostaining for α-tubulin (data not shown). Although, following 72 hours of depletion of topo IIα, many nuclei had an abnormal appearance (Carpenter and Porter, 2004), estimates of nuclear size and polyploidy in the two cell populations (based on the DAPI-stained area in interphase cells) revealed no significant change (topo IIα^{ON}: mean area 271 μm^2, s.d. 89.1, n=329, compared with topo IIα^{OFF} mean 275 μm^2, s.d. 111, n=343).

Discussion

Topo IIα is not essential for the maintenance of gross structural integrity

Several reports have now shown that, in living cells, extensive chromosome condensation occurs in the absence of topo II, although in general the process seems to take longer, with the final extent of compaction being less (Chang et al., 2003; Carpenter and Porter, 2004; Sakaguchi and Kikuchi, 2004;

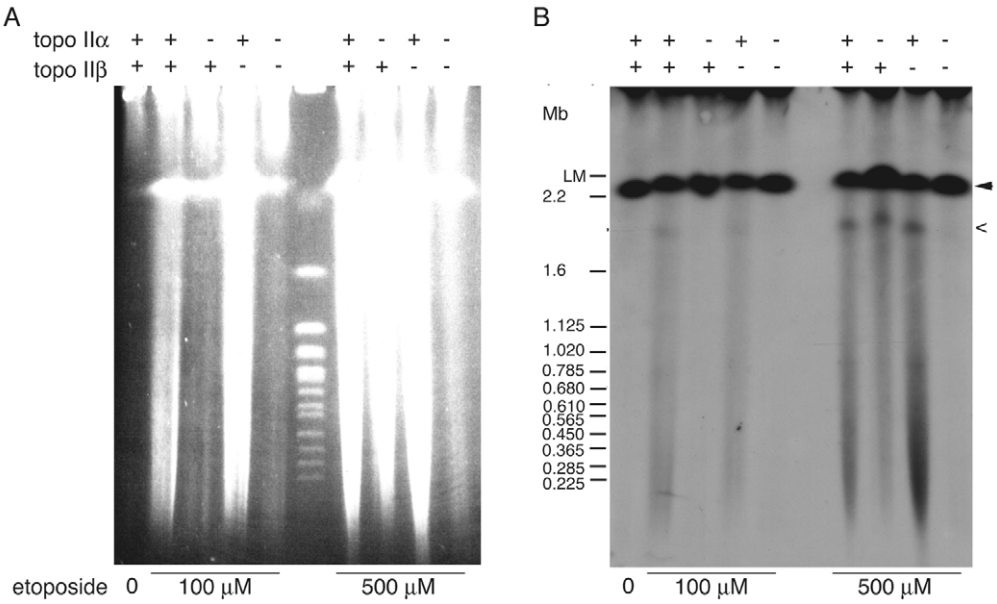

Fig. 7. The effect of depletion of topo II on centromeric topo II cleavage activity. The HTETOP-minichromosome hybrid cells expressing topo II or depleted (72 hours exposure to dox) for topo IIα and/or topo IIβ (72 hours exposure to siRNA) were exposed in culture to etoposide [0 (DMSO only), 100 or 500 μM] for 60 minutes at 37° C and embedded in agarose. (A) Undigested HMW DNA was resolved by PFGE and stained using ethidium bromide. (B) After transfer, the Southern blot was probed using the X α-satellite DNA DXZ1 to detect the 2.7 Mb X centromere-based minichromosome (position indicated by black arrowhead). An etoposide-specific DXZ1-hybridising fragment of ~1.85 Mb (position indicated by '<') could be detected, in addition to a more general smear of hybridisation (this was more noticeable in DNA from cells expressing topo IIα). At the lower etoposide concentration (100 μM), a signal in the 1.85 Mb range could only be detected in cells expressing topo IIα (either alone, or together with the β isoform); at the higher etoposide concentration (500 μM), the 1.85 Mb signal was barely detectable after depletion of both isoforms, but could still be detected following depletion of topo IIα alone (although the signal was reduced relative to that of the intact minichromosome in the same sample). This suggests that both isoforms of topo II contribute to this cleavage site within the centromeric DNA. LM, limit mobility.

Toyoda and Yanagida, 2006), Nevertheless, the chromosomes look remarkably normal. Moreover, unlike depletion of condensin, which leads to altered localisation of topo II (Hudson et al., 2003; Vagnarelli et al., 2006), depletion of topo II (in either human or *Drosophila* cells) does not affect the axial distribution of condensin (this report) (Chang et al., 2003). Therefore, in order to probe topo-IIα-depleted chromosomes for possible effects on other aspects of structure, they were exposed to buffer conditions that, when applied to normal chromosomes, result in the alternate disruption and restoration of the chromosome architecture. Following depletion of condensin from vertebrate cells, chromosomes are unable to recover their condensed morphology after unfolding under these conditions (Hudson et al., 2003). However, upon removal of topo IIα (alone, or together with removal of topo IIβ), mitotic chromosomes, although generally longer and thinner than normal, nevertheless retained the ability to recondense after extensive unfolding. Although we do rule out the possibility that these compacted structures might be, to some extent, abnormal, our observations suggest that, in human cells, topo II does not have an essential scaffolding role. This is consistent with earlier observations that topo II can be extracted from condensed chromosomes assembled in vitro without any observed change in structure (Hirano and Mitchison, 1993) and that it interacts very dynamically with mitotic chromatin (Christensen et al., 2002; Tavormina et al., 2002).

Topo IIα is required for a compact metaphase plate

When we examined the organisation of the metaphase plate in the absence of topo IIα, we observed that, in many cells, one or more chromosome arms extended away from the compact mass of chromatin, often stretching towards the pole, apparently independent of centromere activity. A similarly disorganised metaphase plate has been reported in topo-II-depleted *Drosophila* S2 cells (Chang et al., 2003; Savvidou et al., 2005). This suggests that the chromosome arms are trapped in the vicinity of the pole and are unable to retract, or be pushed back, towards the metaphase plate. Whether this chromosome arm congression defect reveals a conserved role for the centrosomal topo II protein described by others (Barthelmes et al., 2000) remains to be explored.

Topo IIα ensures that long chromosome arms are resolved in anaphase

The major topo-IIα-depletion phenotype is a defect in chromosome segregation, with virtually all cells displaying one or more anaphase chromatin bridges. We have shown that chromosomes with longer arms are involved in bridging more frequently (five- to tenfold increase) than smaller chromosomes. We found no evidence of either centromeric DNA or the rDNA loci being disproportionately involved in these bridges. One explanation for the positive correlation between bridging and chromosome arm length would be that, as sister chromatids are drawn apart by the polewards spindle forces, residual catenations are either ruptured, or slide along the arms, allowing separation to proceed from the centromere outwards. The longer the arm, the more likely it is that residual intertwinings, accumulating in

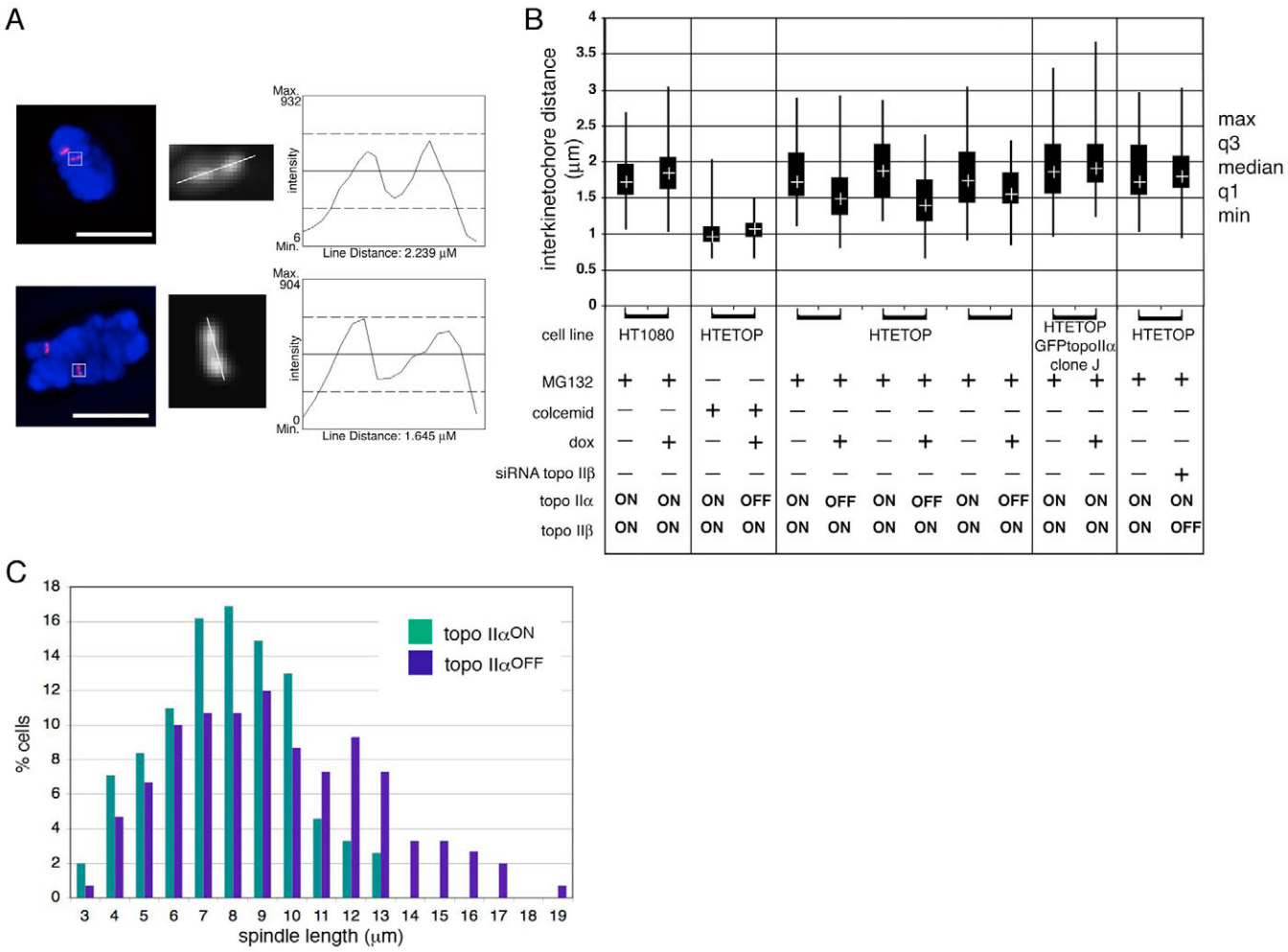

Fig. 8. The effect of depletion of topo IIα on the metaphase inter-kinetochore distance. (A) Distances between sister centromeres of human chromosome 11 detected by FISH using D11Z1 DNA as a probe were assessed using Leica Deblur software. Bar, 10 μm. (B) Measurements made of treated cells in parallel with untreated controls (treated/untreated pairs indicated by brackets) are presented as a boxplot showing the third and first quartiles, with the median indicated by a cross in each box. The maximum and minimum values are indicated by the ends of the vertical lines. Each plot is based on ⩾40 measurements. In HTETOP cells, topo IIα was depleted by 72 hours exposure to dox. Cells were arrested using MG132 (10 μM for 2 hours 30 minutes), allowing distances under tension to be measured. For the dox-treated, topoIIα-depleted HTETOP cells, data from three independent experiments are presented. In each case, the decrease in the distance across the centromere domain under tension following depletion of topo IIα is significant (P=0.001, 0.0001, 0.01, respectively). To deplete topo IIβ, HTETOP cells were transiently transfected with siRNA (72 hours exposure). Colcemid-treated HTETOP cells served as a control where no spindle or tension existed. The lack of any effect from dox treatment itself was confirmed by analysis of MG132-arrested parental HT1080 cells. Clone J is a derivative of HTETOP that constitutively expresses topo IIα as a fusion with the C-terminus of eGFP (Carpenter and Porter, 2004).
(C) Quantification of spindle lengths in HTETOP cells arrested using MG132 (10 μM for 2 hours 30 minutes) following 0 (topo IIα^{ON}) or 72 hours (topo IIα^{OFF}) exposure to dox. Measurements of the pole-to-pole distance, based on γ-tubulin and DAPI staining, were collected from three independent experiments (topo IIα^{ON}, n=154; topo IIα^{OFF}, n=151).

the distal regions, might fail to be resolved during anaphase, resulting in a bridge. It is also possible that reduced chromosome condensation in topo-IIα-depleted cells contributes to the bridging phenotype. Similar observations concerning long arms preventing intertwinings from passively resolving off the end during segregation have been made in topo II mutants of *S. cerevisiae* (Spell and Holm, 1994).

A linear minichromosome reveals a topo-IIα-depletion effect at the centromere

The concentration of topo IIα at the mitotic centromere raises the question as to whether this isoform has a specific role in some aspect of centromere biology. In our system, topo IIα is gradually and asynchronously depleted from cells, and, as a result, it is not possible to study the impact of depletion of topo IIα on the centromere independently from its effects on arm decatenation. However, we were able to study the segregation behaviour of a linear minichromosome derived from the centromeric and pericentromeric regions of the human X chromosome. As FISH had revealed a positive correlation between bridging and arm length and suggested normal centromere separation, we were surprised at the relatively high level of missegregation displayed by the minichromosome. Given that this structure would have no, or few, arm catenations

to resolve, this suggests the existence of a topo-IIα-depletion defect at the centromere, to which our minichromosome is sensitised compared with normal chromosomes. This observation, together with earlier reports of drug inhibition of topo II affecting the structure of the kinetochore (Rattner et al., 1996) and the more recent detection of major sites of topo II cleavage activity within human centromeric DNA (Floridia et al., 2000; Spence et al., 2002; Spence et al., 2005), led us to examine centromere behaviour in the absence of topo IIα in more detail.

Topo IIα depletion is associated with a shortening in the distance across bi-oriented sister centromeres

We detected a significant shortening (~10-25%) in the distance across sister centromeres following the depletion of topo IIα. Transient depletion of topo IIβ alone had no detectable effect. Depleting both isoforms simultaneously resulted in a highly disordered metaphase plate, making the identification of sister centromere pairs difficult. However, where bi-oriented sister centromere pairs could be identified, the distance between them was found to decrease to a similar extent to that observed following depletion of topo IIα (data not shown). This is consistent with the 20-30% shortening reported previously following either the depletion, or the ICRF193-induced inhibition, of both topo II isoforms simultaneously (Toyoda and Yanagida, 2006).

This decreased distance across bi-oriented centromeres argues against a role for topo II in centromere cohesion. Instead, it would be consistent with depletion of topo II impeding decatenation and resulting in the persistence of highly intertwined duplexes with reduced flexibility (Skibbens et al., 1993; Shelby et al., 1996). Intriguingly, a small but significant increase in the length of the spindle was also detected following depletion of topo IIα. While many nuclei had an abnormal appearance, the increased spindle length did not appear to be accounted for by any significant increase in nuclear size. Thus, depletion of topo IIα results in decreased stretching of bi-oriented chromosomes and elongation of the mitotic spindle. In this regard, it is intriguing that recent affinity purification experiments have revealed a potential interaction between *Drosophila* topo II and the outer kinetochore protein Ndc80 (Przewloka et al., 2007). This raises the possibility that topo IIα exerts an effect on kinetochore-MT interactions through the Ndc80 complex. Further work is required to elucidate the function of topo IIα within the centromere domain and to understand the mechanism underlying the decreased distance across bi-oriented sister centromeres.

Topo IIα has a role in the generation and/or resolution of anaphase PICH connections

Consistent with observations on the depletion of the single topo II isoform in S2 cells (Chang et al., 2003), we found that depletion of topo IIα (alone, or together with topo IIβ) had no detectable effect on generalised kinetochore assembly in human cells. There was also no obvious effect, at the level of the condensed metaphase chromosome, on the hypoacetylated status of the centromere/pericentromeric domain. We then examined the distribution of the recently identified inner centromere protein PICH (Baumann et al., 2007). PICH could be detected at the centromere domain of metaphase chromosomes and was seen extending, in thread-like structures, between segregating chromosomes in anaphase cells. Strikingly, when the levels of topo IIα were depleted, the number of PICH threads increased substantially in segregating cells. This is consistent with the observation that, if topo II is inhibited using ICRF193 or ICRF159, PICH threads become very prominent and supports the idea that PICH, a putative chromatin-remodelling enzyme, associates with catenated DNA, which is stretched under tension, until it is resolved during anaphase (Baumann et al., 2007; Chan et al., 2007). At least some of the PICH threads detected in topo-IIα-depleted anaphases were clearly distinct from chromatin bridges, suggesting that this protein is not simply associated with residual arm catenations. Others have shown that the PICH-coated ultrafine anaphase connections contain DNA and colocalise with the BLM helicase and its cellular partners topo IIIα and hRMI1, suggesting that topo IIIα might have a key role in their resolution (Chan et al., 2007). Moreover, these extensions are often capped by immunofluorescence signals for the outer kinetochore protein Hec1, suggesting that they involve DNA originating from the centromere (Baumann et al., 2007; Chan et al., 2007). Intriguingly, evidence has been presented, from vertebrates and parasitic protozoa, for a concentration of topo II cleavage activity within centromeric DNA (Floridia et al., 2000; Spence et al., 2002; Spence et al., 2005; Kelly et al., 2006; Obado et al., 2007). We have shown here that, in human cells, both topo II isoforms contribute to this cleavage activity. Establishing what relationship, if any, exists between this topo-II-susceptible centric subdomain and the PICH/BLM-coated ultrafine anaphase connections awaits future investigation.

Concluding remarks

It is well known that perturbation of topo IIα activity has serious consequences for chromosome segregation. This study has shown that the DNA caught up in anaphase chromatin bridges arises predominantly from a failure in chromosome arm decatenation, with longer chromosome arms being particularly susceptible. In addition to this, however, we detected a surprisingly high rate of missegregation by a <3 Mb 'arm-less' minichromosome. Closer examination of the impact that depletion of topo IIα has on chromosome segregation revealed a number of more subtle effects, including a defect in chromosome arm congression, a shortening of the distance across bi-oriented sister centromeres, an increase in the length of the mitotic spindle and an increase in the incidence of PICH-coated connections in anaphase cells. Currently, we do not understand which of these phenotypes impacts on the mitotic stability of the minichromosome, how they are related or the underlying mechanism(s). It is possible that the shortening of the interkinetochore distance and lengthening of the spindle reflect the effects of depletion of topo IIα on the stability of kinetochore-microtubule attachments. Previous work has indicated that the minichromosome has a compromised kinetochore (based on reduced levels of CENP-A and Aurora B) (Spence et al., 2006), which might make it especially susceptible to destabilising effects.

The concentration of topo IIα at mitotic centromeres should serve to ensure that there is efficient decatenation of residual

intertwinings at the onset of anaphase. This would appear to be crucial for maintaining genome integrity as the powerful forces exerted by the kinetochore microtubules on this part of the chromosome during polewards movement could lead to rupture of the duplex, especially if the capacity for removing centromeric catenations through displacement along the arms is restricted by the presence of the kinetochore. However, identification of the PICH protein revealed that, in mitosis, it is normal for connections to persist between segregating sisters well into anaphase. Why this occurs is perplexing. The suggestion that PICH regulates access of the decatenating activity and/or protects catenated centromeric DNA from nonspecific rupture (Baumann et al., 2007) presents challenges for future experiments to test the link between the various topoisomerases (topo II and topo III), helicases (PICH and BLM) and cohesin in chromosome alignment and separation.

Materials and Methods

Cell culture, fusion and transfection

HTETOP cells were cultured as previously described (Carpenter and Porter, 2004). The 2.7 Mb minichromosome was transferred into the HTETOP background by whole-cell fusion between it and a DT40 hybrid donor cell line (1aA1) (Spence et al., 2006) HTETOP-minichromosome hybrids were selected using 2.5 μg/ml blasticidin S (ICN). The transfer of the minichromosome into two independently derived hybrids was confirmed by FISH using a DXZ1 DNA probe and by PFGE and Southern blotting of uncut HMW DNA.

Etoposide (Sigma) was dissolved in 100% DMSO at 10 mM and stored in the dark at –20°C. The inhibitor was added to exponentially growing cells to the specified final concentration and incubated at 37°C for the time indicated. An equivalent volume of 100% DMSO was added as the no-drug control. Doxycycline (Dox; Sigma) was dissolved in water and stored in the dark at –20°C for up to 2 weeks. It was used at a final concentration of 1 μg/ml. MG132 (Calbiochem) was dissolved in DMSO at 10 mM and stored at –20°C for up to 4 weeks. HTETOP cells were arrested using a final concentration of 10 μM for 2 hours 30 minutes. Colcemid (KaryoMax, Gibco/Invitrogen) was used to arrest HTETOP cells at a final concentration of 100 ng/ml for 1 hour for immunofluorescence (where used), 2 hours for inter-kinetochore measurements and overnight for mitotic chromosome structural integrity assays.

In siRNA experiments, human topo IIβ was depleted using 5′-GGCCCAGAUUUUAAUUAUAtt-3′. Two other target sequences were also selected and gave similar results (5′-GGCAUCGCAUCUUGUUUAGAtt-3′ and 5′-GGUUUAUACAAGAUCUUUGtt-3′) (Ambion). Transfection was done by nucleofection according to the manufacturer's instructions (Amaxa).

PFGE and Southern blot hybridisation

HMW DNA was resolved in 0.7% chromosomal grade agarose (Bio-Rad 162-0136), 0.25× TBE, over 72 hours at 11°C using the following parameters: a pulse time of 350-50 seconds (changing logarithmically), a rotor angle of 110°-100° (decreasing linearly) and a voltage of 120-50 V (decreasing linearly) (Rotaphor TypeV, Biometra). Southern transfers were performed onto nylon membranes (Hybond N+, GE BioSiences) and DNA probes were radioactively labelled by random priming. Southern blot hybridisations were carried out in a Hybaid oven, and membranes were washed at high stringency (0.2× SSC, 0.1% SDS at 65°C). The DNA probe used in hybridisations was DXZ1, a 2.0 kb *Bam*HI α-satellite DNA fragment from pSV_2X5.

Fluorescence in situ hybridisation

FISH was performed as described previously (Spence et al., 2002). Probe DNA was labelled with DIG-11-dUTP (Roche). Specimens were mounted in Vectashield (Vector Labs) with DAPI counterstain. The fluorochromes used were anti-DIG FITC (Roche) and avidin Texas Red (Vector Labs). Telomeric DNA was detected using a fluorescently conjugated telomere PNA probe (DakoCytomation). Other DNA probes used were as follows: a pan-centromere chromosome paint (Cambio); a mouse rDNA probe from plasmid pA; the human centromere probes D11Z1, D6Z1 and DXZ1; and paints for human chromosomes 6, X, 19 and 20 (Star-FISH™ Cambio). For FISH with rDNA, slides were pretreated as follows: RNase (100 μg/ml) in 2× SSC at 37°C for 1 hour), three washes in 2× SSC, one wash in PBS at 37°C, pepsin treated (100 μg/ml in 10 mM HCl, 10 minutes at 37°C), washed in PBS, fixed in 1% formaldehyde-PBS for 10 minutes, then followed by a final rinse in PBS. Slides were dried in an ethanol series before FISH.

For measurements across bi-oriented sister centromeres, MG132-arrested cells growing on slides in QuadriPerm slide chambers (VivaScience Ltd) were fixed using absolute methanol for 10 minutes, followed by 2% formaldehyde-PBS and a final fix in 3:1 methanol:acetic acid. FISH signals were detected for the chromosome 11 centromere, and Z stacks of multi-channel images captured at 0.1 μm intervals using Leica FW4000 software. The distance across sister centromere pairs, to the outer edges of the D11Z1 signal, was measured using Leica Deblur software.

Indirect immunofluorescence

To localise topo IIα, tubulin, Aurora B/AIM-1, CENP-A, CENP-C, CENP-E, CENP-F and acetyl-histone H3, cells were swollen in 75 mM KCl, spun onto poly-L-lysine-coated slides (BDH) in a Cytospin cytocentrifuge (Shandon) and fixed in methanol. Antibodies were diluted in KCM (120 mM KCl, 20 mM NaCl, 10 mM Tris-HCl pH 8.0, 0.5 mM EDTA, 0.01% Triton X-100) plus 10% foetal bovine serum, and washes were in KCM. To localise PICH, cells growing on slides were fixed and permeabilised in PTEMF (20 mM PIPES pH 6.8, 4% fomaldehyde, 0.2% Triton X-100, 10 mM EGTA, 1 mM $MgCl_2$). Antibodies were diluted in 3% BSA in PBS plus 0.05% Tween 20 (PBST), and washes were in PBST. SMC2 IF was carried out using a version of the solvent spreading method (Earnshaw et al., 1989). Colcemid-arrested cells were swollen with 75 mM KCl for 10 minutes and then fixed in three changes of 3:1 methanol:acetic acid before being dropped onto slides and air dried. Spreads were rehydrated in PBS for 15 minutes before being swollen in TEEN (1 mM triethanolamine-HCl pH 8.5, 0.2 mM NaEDTA, 25 mM NaCl) for 7 minutes. The primary and secondary antibodies were diluted (1:100, 1:200) in TEEN plus 0.5% foetal bovine serum, and washes were in TEEN. Final washing was in KB (10 mM Tris-HCl pH 7.7, 0.15 M NaCl, 0.5% foetal bovine serum).

For measurements of spindle length, dox-treated cells (0 or 72 hours) growing on slides were arrested using MG132, fixed in methanol and stained using mouse antibody against tubulin (gamma or alpha). Pole-to-pole measurements for bipolar cells where the spindle was perpendicular to the light path were made using the line tool in the Leica Deblur software on maximum intensity projections of Z stacks. CENP-A quantification was carried out as described previously (Spence et al., 2006).

Antibodies

Primary antibodies for IF were as follows: rabbit anti-human CENP-A (1:400) (Valdivia et al., 1998); mouse anti-human CENP-A (1:500; Abcam); Aurora B/AIM-1 (1:200; BD); acetyl-histone H3 (1:100; Upstate); CENP-C (554, 1:100) (Saitoh et al., 1992); CENP-B (mACA-1, 1:1000) (Cooke et al., 1990); SMC2 (ScII A, 1:200) (Saitoh et al., 1994); topo IIα (1:100; Topogen); β-tubulin (1:200; Sigma); γ-tubulin (1:500; Sigma); α-tubulin (1:500; Abcam); CENP-F (D10, 1:750) (Liao et al., 1995); CENP-E (mAb177, 1:250) (Yen et al., 1991); PICH (raised in rabbit, 1:200) (Baumann et al., 2007). Secondary antibodies for IF (1:200) were as follows: goat anti-mouse Texas Red and goat anti-rabbit Texas Red (Molecular Probes); rabbit anti-mouse FITC, swine anti-rabbit FITC, or swine anti-rabbit conjugated to biotin (Dako); sheep anti-mouse digoxigenin (Chemicon). Biotin-labelled secondary antibodies were visualised using fluorescein-avidin DN (Vector Labs) or avidin Cy5 (Amersham Bioscience) and digoxigenin with sheep anti-digoxigenin FITC (Roche).

Assay of mitotic chromosome structural integrity

For assessment of the structural integrity of mitotic chromosomes by treatment with chromosome compacting/unfolding buffers, cells were grown on slides in Quadriperm slide chambers and assayed as described previously (Hudson et al., 2003). Chromosomes are first unfolded by exposure to a solution of low ionic strength lacking divalent cations and containing EDTA (0.5× TEEN: 0.5 mM triethanolamine-HCl, pH 8.5, 0.2 mM NaEDTA, 12.5 mM NaCl). They were then refolded by the addition of a buffer of low ionic strength containing Mg^{2+} (RSB buffer: 10 mM Tris-HCl pH 7.4, 10 mM NaCl, 5 mM $MgCl_2$). The TEEN-RSB cycle was then repeated. For microscopy, slides were fixed in 2% formaldehyde and stained with DAPI.

Imaging

Images were captured using a Leica DM6000B microscope driven by FW4000 software, or a Zeiss Axioskop 2 using the Cytovision system (Applied Imaging).

Statistics

Comparison of means was done by unpaired Student's *t* tests, assuming equal variance, unless otherwise indicated. Results were handled in Excel.

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