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Analysis of Scc1-deficient cells defines a key metaphase role of vertebrate cohesin in linking sister kinetochores

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Cleavage of the cohesin subunit Scc1p/Mcd1p/Rad21 permits sister chromatid separation and is considered to trigger anaphase onset. It has also been suggested that the cohesin complex is essential for chromosome condensation and for assembling fully functional kinetochores. Here, we used vertebrate cells conditionally deficient in *Scc1* to probe cohesin function in mitosis. Cells lacking cohesin arrest in prometaphase, with many chromosomes failing to align at a metaphase plate and high levels of the spindle assembly checkpoint protein, BubR1, at all kinetochores. We show that the structural integrity of chromosomes is normal in the absence of Scc1. Furthermore, specific inhibition of topoisomerase II, which is required for decatenation of replicated chromosomes, can bypass the cohesin requirement for metaphase chromosome alignment and spindle checkpoint silencing. Since the kinetochore effects of Scc1 deficiency can be compensated for by topoisomerase II inhibition, we conclude that Scc1 is not absolutely required for kinetochore assembly or function, and that its principal role in allowing the onset of anaphase is the establishment of sufficient inter-sister tension to allow biorientation.

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INTRODUCTION

To ensure the faithful transmission of chromosomes in mitosis, replicated chromatids remain paired until both sister kinetochores attach to opposite poles of the mitotic spindle, a state known as biorientation. This pairing requires cohesin, a multiprotein complex that ensures proper sister chromatid pairing until anaphase onset (Nasmyth, 2002).

Genetic and biochemical studies in yeast, *Xenopus* and human systems have shown that the cohesin complex contains two SMC (structural maintenance of chromosomes) proteins, Smc1 and Smc3, and two non-SMC proteins, Scc1 and Scc3 (Hirano, 2002; Nasmyth, 2002). Cleavage of the cohesin subunit Scc1p/Mcd1p/Rad21 (hereafter Scc1) by separase triggers sister chromatid separation (Uhlmann *et al*, 2000; Hauf *et al*, 2001). Cohesin complexes localize to the centromeres in yeasts (Blat & Kleckner, 1999; Tanaka *et al*, 1999) and vertebrates (Gregson *et al*, 2002). However, the association of the Scc1 subunit with chromosome arms persists until anaphase onset in *Saccharomyces cerevisiae* (Guacci *et al*, 1997; Michaelis *et al*, 1997; Uhlmann *et al*, 1999), but is lost during prophase in metazoan cells (Waizenegger *et al*, 2000; Warren *et al*, 2000).

Several observations have suggested that cohesin might be required for kinetochore assembly or function during mitosis. Fission yeast cohesin mutants exhibit prolonged Bub1 binding to centromeres and appear to be defective in kinetochore–microtubule interactions (Toyoda *et al*, 2002). Chicken DT40 cells, conditionally null for *Scc1*, arrest in mitotic prometaphase following *Scc1* repression, with most chromosomes properly aligned on the metaphase plate but with a significant number scattered throughout the spindle (Sonoda *et al*, 2001). Depletion of the *Drosophila* Scc1 orthologue Drad21 by RNA interference also results in mitotic defects (Vass *et al*, 2003). In the absence of Scc1, the chromosomal passenger INCENP was mislocalized in both chicken and *Drosophila* cells (Sonoda *et al*, 2001; Vass *et al*, 2003). Finally, the expression of an N-terminally truncated form of human Scc1 leads to mitotic abnormalities (Hoque & Ishikawa, 2002).

An additional role for cohesin might be in establishing chromosome higher-order structure. Chromosome condensation defects have been described in Scc1-deficient yeast (Guacci *et al*, 1997; Lavoie *et al*, 2002). We have recently shown that mitotic chromosome formation involves two separable steps: condensation of the chromatin and formation of a stable higher-order structure (Hudson *et al*, 2003). Condensin is required only for the

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latter. Although Scc1-deficient DT40 cells underwent chromatin condensation, and appeared to bind condensin normally (Sonoda *et al*, 2001), we could not exclude the possibility that, as in yeast, cohesin might be required for some aspect of the chromosome higher-order structure.

Here we show that the structural integrity of mitotic chromosomes remains intact in the absence of Scc1. We also demonstrate that specific inhibition of topoisomerase II (topo II) can bypass the cohesin requirement in metaphase chromosome alignment and silencing of the spindle assembly checkpoint. These findings show that the cohesin complex is not absolutely required for kinetochore function in mitotic chromosomes.

RESULTS

In this study, we have used a conditionally null chicken DT40 cell line, genotype *Scc1*$^{-/-/-}$ *tet-Scc1* (Sonoda *et al*, 2001). In standard culture media, the cells are healthy and will be referred to as Scc1^{ON}. The addition of doxycycline shuts off expression of the *Scc1* transgene, and under these conditions cells will be referred to as Scc1^{OFF}.

Scc1 depletion does not affect condensin function

We recently developed an assay that probes the stable higher-order structure of mitotic chromosomes. This assay relies on the ability of chromosomes to recover their morphology following complete unfolding of the chromatin to the 10 nm fibre ('beads-on-a-string') (Hudson *et al*, 2003). In this assay, chromosomes are first unfolded by exposure to a low ionic strength solution lacking divalent cations and containing EDTA (Fig 1A). They are then refolded by the addition of a low ionic strength buffer containing 5 mM Mg^{2+} (Cole, 1967; Earnshaw & Laemmli, 1983). We define the term 'structural integrity' as the ability of chromosomes to remain morphologically indistinguishable at the level of light microscopy during repeated cycles of swelling and shrinking. We previously showed that condensin is essential for this structural integrity (Hudson *et al*, 2003).

In order to investigate whether the loss of cohesin might have an impact on this structural integrity of mitotic chromosomes, we performed the assay on chromosomes of Scc1^{ON} and Scc1^{OFF} cells. As shown in Fig 1B, even though the loss of Scc1 caused sister chromatids to separate, it did not perturb the ability of chromosomes to recover their condensed morphology following unfolding of the chromatin. These data are quantified in Fig 1C. In contrast, 95 and 100% of condensin-deficient chromosomes are abnormal at cycles 1 and 2, respectively, in this assay (Hudson *et al*, 2003). Thus, the loss of cohesin does not disrupt significantly the structural integrity of mitotic chromosomes.

Topo II inhibition rescues chromosome biorientation

Scc1^{OFF} cells in mitosis accumulate in prometaphase, with most macrochromosomes aligned at a metaphase plate but with a number of the microchromosomes scattered throughout the spindle (Fig 2A) (Sonoda *et al*, 2001). This phenotype is consistent with defective kinetochore function, but could equally well be explained by defects in the ability of sister kinetochores to orient properly to opposite spindle poles. To test whether cohesin is indeed required for kinetochore function, we have artificially imposed cohesion in Scc1^{OFF} DT40 cells by inhibiting topo II. Topo II activity is thought to be required to separate catenated

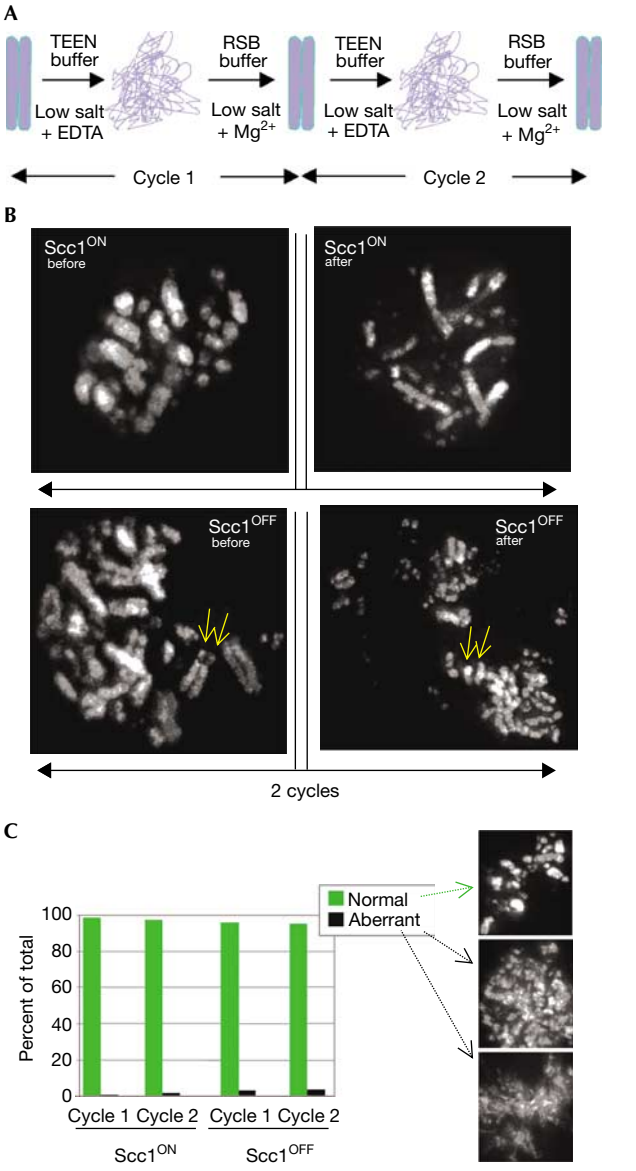

Fig 1 | Scc1-deficient chromosomes have a stable higher-order structure. **(A)** Assay for mitotic chromosome structural integrity (Hudson *et al*, 2003). Note that this is a morphological assay and might not detect the possible loss or alteration of associated proteins during the refolding cycles. **(B)** Even though the sister chromatids (arrows) show an abnormal degree of premature separation, cohesin (Scc1)-depleted chromosomes have normal structural integrity. **(C)** Quantitation of the results presented in panel **(B)**. Examples of normally refolded and aberrantly refolded chromosomes are shown at the right. Scc1-depleted chromosomes recover normally.

sister chromatids during mitosis in yeast (DiNardo *et al*, 1984; Uemura & Yanagida, 1986; Holm *et al*, 1989) and mammalian cells (Gorbsky, 1994; Ishida *et al*, 1994; Sumner, 1995).

To test whether topo II inhibition might improve metaphase chromosome alignment, we treated Scc1^{OFF} DT40 cells with ICRF-159 or ICRF-187, which are highly specific

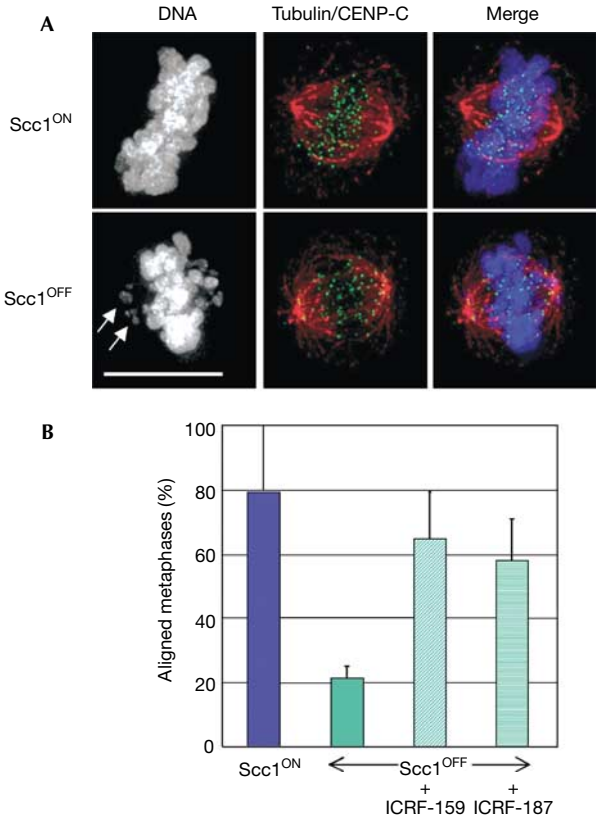

Fig 2 | Successful alignment of Scc1-deficient chromosomes at a metaphase plate following treatment with topo II inhibitors. (**A**) A metaphase cell was defined as a cell with its chromosomes all aligned on the plate, as shown at the top, whereas cells with chromosomes scattered about the plate (arrows, lower panels) were defined as prometaphase. Cells on polylysine-treated slides were fixed and incubated with antibodies to α-tubulin (red) and CENP-C (green), along with DAPI (grey in left panels). Scale bar, 10 μm. (**B**) Histogram showing the relative percentage of metaphase $Scc1^{OFF}$ cells following the treatment of synchronized cells with the topo II inhibitors ICRF-159 or ICRF-187 for 1 h before analysis. Cells in which the spindle axis was not parallel to the slide were excluded from this analysis. Data shown are the mean ± standard deviation of at least three experiments in which at least 25 cells with appropriately aligned spindles were scored blind. Levels of alignment under all conditions differed significantly from those observed in $Scc1^{OFF}$ conditions (χ^2-test; $P<0.01$). A control experiment on 25 $Scc1^{ON}$ metaphases showed 85 and 84% aligned at metaphase after treatment with ICRF-159 and ICRF-187, respectively.

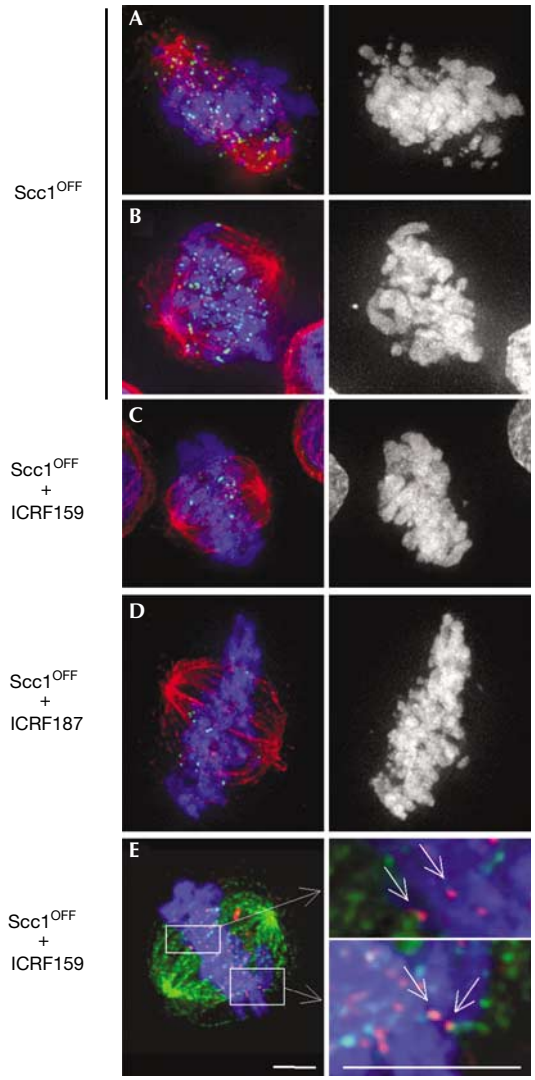

Fig 3 | Decrease in kinetochore-associated BubR1 in Scc1-deficient cells following topo II inhibitor treatment. (**A**, **B**) All $Scc1^{OFF}$ cells in early mitosis have levels of BubR1 accumulation at kinetochores characteristic of prometaphase for both aligned and nonaligned chromosomes. (**C**, **D**) Treatment of $Scc1^{OFF}$ cells with the topoisomerase inhibitors ICRF-159 (**C**) and ICRF-187 (**D**) promotes the alignment of chromosomes at a metaphase plate and results in a downregulation of BubR1 levels at the kinetochores. Synchronized, Scc1-deficient cells were treated as for Fig 2. Staining is shown with antibodies to α-tubulin (red) and BubR1 (green), along with DAPI (grey in the right panels). (**E**) Treatment of $Scc1^{OFF}$ cells with ICRF-159 promotes the bipolar attachment of sister kinetochores to opposite spindle poles. Synchronized, Scc1-deficient cells were treated as for Fig 2. Staining is shown with antibodies to α-tubulin (green) and CENP-C (red), along with DAPI (blue). Scale bars, 5 μm.

bisdioxopiperazine drugs that catalytically inhibit topo II without causing a significant G2–M arrest (Andoh & Ishida, 1998). It was necessary to perform these analyses on synchronized DT40 cells treated only for short periods, as long-term exposure to either drug interfered with mitotic chromosome condensation (Gorbsky, 1994) and caused dramatic levels of apoptosis (data not shown). Both drugs significantly improved metaphase chromosome alignment in $Scc1^{OFF}$ cells (Fig 2B), and in high-resolution images sister kinetochores could be seen to be bioriented (Fig 3E arrows in insets). We conclude that cohesin is not essential for microtubule binding by kinetochores or for chromosome biorientation on the mitotic spindle. Our data are consistent with results recently obtained in budding yeast, where inhibition of topo II was able to suppress partially defects in sister kinetochore biorientation following depletion of Scc1 (T. Tanaka, personal communication).

Topo II inhibition restores tension at kinetochores

The accumulation of Scc1^{OFF} cells in prometaphase is probably due to activation of the spindle assembly checkpoint, as kinetochores of all chromosomes in these cells exhibit high levels of BubR1 (Fig 3A,B). This accumulation of BubR1 at kinetochores could reflect either defects in kinetochore structure or an inability to shut off the spindle assembly checkpoint when sister kinetochores are linked by chromatin lacking cohesin. To examine whether the improved metaphase alignment induced by inhibition of topo II was sufficient to silence this checkpoint signal, we stained ICRF-159- or ICRF-187-treated Scc1^{OFF} cells for BubR1. Both treatments greatly reduced the levels of kinetochore-associated BubR1 (Fig 3C,D).

DISCUSSION

Scc1 is dispensable for mitotic chromosome structure

Our findings confirm and extend previous observations (Losada *et al*, 1998; Sonoda *et al*, 2001) by showing that Scc1 is not required for mitotic chromatin condensation or for the structural integrity (as defined by the assay shown in Fig 1) of vertebrate mitotic chromosomes. These results are in contrast to the close interdependence between cohesin and chromosome condensation seen in budding yeast (Guacci *et al*, 1997; Lavoie *et al*, 2002). The key condensin component ScII/SMC2 localizes to the chromosome scaffold in Scc1^{OFF} cells (Sonoda *et al*, 2001). Since condensin loss in vertebrate mitotic chromosomes significantly impairs the structural integrity of mitotic chromosomes (Hudson *et al*, 2003), these observations suggest that condensin in Scc1-depleted cells is functional. Therefore, our findings provide support for a model in which the condensation of vertebrate mitotic chromosomes is largely independent of cohesin.

Cohesin not absolutely required for kinetochore function

The present results also resolve an ongoing controversy about the role of cohesin in kinetochore assembly and function. Previous studies had suggested that cohesin might be required for the assembly of fully functional kinetochores (Tanaka *et al*, 2000; Sonoda *et al*, 2001; Hoque & Ishikawa, 2002; Toyoda *et al*, 2002). Here, we have shown that if topo II is inhibited using the highly specific inhibitors ICRF-159 or ICRF-187, chromosomes deficient in cohesin can (i) bind microtubules from opposite spindle poles, (ii) bind elevated levels of BubR1 when not properly aligned at the spindle midzone, (iii) congress to a metaphase plate and (iv) downregulate BubR1 levels at the kinetochore (and presumably therefore silence the metaphase spindle assembly checkpoint) once properly bioriented. Thus, by these four criteria, fully functional kinetochores can be assembled in the effective absence of Scc1.

Speculation: role of topo II in sister chromatid cohesion?

Our results raise the possibility that topo II could have a role in regulating levels of tension between sister kinetochores during normal prometaphase and metaphase. The enzyme is concentrated in the centromeric heterochromatin at the appropriate time (Rattner *et al*, 1996; Christensen *et al*, 2002; Tavormina *et al*, 2002) and can be phosphorylated by Aurora B kinase (Morrison *et al*, 2002). The role of topo II at centromeres remains open; however, the present results showing that topo II activity can apparently influence the activation of the spindle assembly checkpoint raise the possibility that regulation of topo II activity might modulate the development of spindle tension across sister kinetochores. Indeed, sumoylation of topo II in budding yeast appears to have a key role in regulating centromeric cohesion (Bachant *et al*, 2002). Furthermore, abnormal regulation of topo II activity may perturb anaphase onset. Regardless of the role of topo II, our data show that the role of cohesin in achieving chromosome biorientation is indeed to establish cohesion (Nasmyth, 2002) and not to perform some secondary function at the kinetochore.

METHODS

Cell culture and microscopy. DT40 cells were cultured and synchronized as described (Sonoda *et al*, 2001). ICRF drugs were from Adria SP (Albuquerque, NM) and were dissolved in 0.2 M HCl. Working concentrations were 10 μg/ml. Nocodazole was used at 0.25 μg/ml and colcemid at 0.1 μg/ml. For immunostaining, whole cells were fixed with 4% formaldehyde and permeabilized with 0.15% Triton X-100 (both in cytoskeleton buffer: 137 mM NaCl, 5 mM KCl, 1.1 mM Na_2HPO_4, 0.4 mM KH_2PO_4, 2 mM $MgCl_2$, 2 mM EGTA, 5 mM PIPES, 5.5 mM glucose). DAPI was used at 1 μg/ml. Analysis of chromosome structure by treatment of cells with chromosome compacting/unfolding buffers was performed as described (Hudson *et al*, 2003). The micrographs shown consist of single-plane projections of deconvolved, three-dimensional data sets taken with an Olympus IX-70 microscope driven by the SoftWorx system (Applied Precision, Issaquah, WA).

Antibodies. Polyclonal anti-CENP-C antiserum WCE30B was raised in rabbit against an N-terminal peptide CMAERLDHLK-KYYRAR, synthesized at the Centre Hospitalier de l'Université Laval (Québec, Canada). After purification over protein A-agarose, serum was used at 1:500 dilution. Anti-BubR1 antibody (Nishihashi *et al*, 2002) was raised against amino acids 650–765 of the chicken protein N-terminally fused to glutathione S-transferase and was used at 1:500 dilution. Monoclonal anti-α-tubulin B512 from Sigma was used for immunofluorescence at 1:2,000 dilution. Fluorescently labelled secondary antibodies from Jackson Immunoresearch Laboratories (West Grove, PA) were used at 1:200 dilution.

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