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Scc1/Rad21/Mcd1 Is Required for Sister Chromatid Cohesion and Kinetochore Function in Vertebrate Cells

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Summary

Proteolytic cleavage of the cohesin subunit Scc1 is a consistent feature of anaphase onset, although temporal differences exist between eukaryotes in cohesin loss from chromosome arms, as distinct from centromeres. We describe the effects of genetic deletion of Scc1 in chicken DT40 cells. Scc1 loss caused premature sister chromatid separation but did not disrupt chromosome condensation. Scc1 mutants showed defective repair of spontaneous and induced DNA damage. Scc1-deficient cells frequently failed to complete metaphase chromosome alignment and showed chromosome segregation defects, suggesting aberrant kinetochore function. Notably, the chromosome passenger INCENP did not localize normally to centromeres, while the constitutive kinetochore proteins CENP-C and CENP-H behaved normally. These results suggest a role for Scc1 in mitotic regulation, along with cohesion.

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Introduction

In order to ensure that both daughter cells possess the same genetic material after mitotic division, anaphase in eukaryotes does not initiate until paired sister chromatids are attached to opposite spindle poles via their kinetochores. For this to be coordinated properly, sister chromatids must remain linked after replication (Bickel and Orr-Weaver, 1996; Koshland and Guacci, 2000; Nasmyth et al., 2000; Orr-Weaver, 1999). In budding yeast, this sister chromatid cohesion requires a multiprotein complex called cohesin. Cohesin contains at least four subunits: Scc1p/Mcd1p (Rad21p in fission yeast), Scc3p, Smc1p, and Smc3p (Guacci et al., 1997; Michaelis et al., 1997; Toth et al., 1999). In yeast, cohesin remains tightly associated with chromosomes until the metaphase to anaphase transition, whereupon Scc1p is cleaved in a reaction dependent on a cysteine protease called separase (also known as Esp1p; Cut1p in fission yeast; Nasmyth et al., 2000; Uhlmann et al., 1999). Current models for anaphase have this event destroying the connection between sister chromatids and thereby permitting the microtubules to segregate the chromatids to opposite poles (Uhlmann et al., 2000).

Sister chromatid cohesion is also thought to be important for the spindle checkpoint, which monitors either tension between sister centromeres or their occupancy by spindles (Nicklas, 1997; Waters et al., 1998) and is activated by any sister kinetochores that have not established bipolar attachments to the spindle apparatus. Since the activation of separase is dependent on the activity of the anaphase-promoting complex/cyclosome (APC/C; Ciosk et al., 1998; Cohen-Fix et al., 1996; Funabiki et al., 1996; Glotzer, 1999; Uhlmann et al., 1999), the control of the APC/C by the spindle checkpoint integrates metaphase chromosome alignment with the onset of anaphase.

This integrated model appears applicable to eukaryotes in general. Cohesin complexes containing orthologs of Smc1p, Smc3p, Scc1p/Mcd1p, and Scc3p also exist in fission yeast (Tomonaga et al., 2000), *Xenopus laevis* (Losada et al., 1998), and human cells (Sumara et al., 2000). Temperature-sensitive budding yeast cohesin mutants exhibit lethality and highly abnormal mitoses when shifted to the restrictive temperature and show very high rates of chromosome loss when growing at temperatures permissive for growth (Guacci et al., 1997; Michaelis et al., 1997; Tatebayashi et al., 1998; Toth et al., 1999). Proper sister chromatid cohesion requires the cohesin complex in *Xenopus* (Losada et al., 1998) and

fission yeast (Tomonaga et al., 2000). However, despite these similarities, some mechanistic differences may exist. The majority of vertebrate cohesin dissociates from chromosomes during prophase, well before the sisters separate (Darwiche et al., 1999; Hoque and Ishikawa, 2001; Losada et al., 1998; Sumara et al., 2000). Although most cohesin complexes located on vertebrate chromosome arms are lost during prophase in a process that does not appear to involve Scc1 cleavage, residual amounts of the cohesin Scc1 remain associated with centromeres until the onset of anaphase (Hoque and Ishikawa, 2001; Waizenegger et al., 2000; Warren et al., 2000) when a similarly small amount of Scc1 is cleaved by separase (Waizenegger et al., 2000). The cohesin enriched at centromeres or the centromere-proximal region (Blat and Kleckner, 1999; Megee et al., 1999; Tanaka et al., 1999) appears to have an essential role in generating a dynamic tension between microtubules and sister chromatids in yeast (Goshima and Yanagida, 2000; He et al., 2000; Tanaka et al., 2000). In animal cells, this tension is required for the establishment of stable kinetochore-microtubule attachments (Ault and Nicklas, 1989; Koshland et al., 1988; Nicklas and Ward, 1994; Skibbens et al., 1995) and appears to be involved in downregulating the spindle checkpoint.

Here we report the conditional inactivation of *SCC1* in chicken DT40 cells to evaluate the in vivo function of Scc1 in higher eukaryotes. This report shows that vertebrate Scc1 is an essential protein and is required for sister chromatid cohesion in interphase and mitosis, but not for chromosome condensation. Loss of sister chromatid cohesion also impedes the homologous recombinational repair of DNA damage. Chromosome misalignment at metaphase results in a mitotic arrest or delay in Scc1-deficient cells, with aberrant disjunction at anaphase potentially implicating cohesion defects in aneuploidy. Surprisingly, we also found that Scc1 deficiency disrupts the centromere localization of the chromosome passenger, INCENP. These findings suggest additional roles for Scc1 at centromeres, in the control of kinetochore function per se.

Results

Generation of Conditionally Scc1-Deficient Chicken DT40 Cells

Chicken Scc1 shows a high degree of identity to its human (92%) and budding yeast (27%) orthologs, suggesting that Scc1 function is conserved across eukaryotes. To investigate the role of Scc1 in higher eukaryotes, we generated conditionally Scc1-deficient clones in which cells with the *SCC1* gene disrupted were kept alive by expression of a cDNA under control of a tetracycline (tet)-repressible promoter. An *SCC1* deletion construct was generated such that a 2 kb genomic fragment encoding amino acids 81–91 would be replaced with selection marker cassettes (Figure 1A). The chicken *SCC1* gene is located on chromosome 2, which is trisomic in DT40 cells. We sequentially transfected Scc1 disruption constructs containing either the blasticidin or histidinol resistance cassettes into DT40 cells (Figure 1A) and isolated $SCC1^{+/-/-}$ clones (Figure 1B). The structure of the disrupted Scc1 locus was confirmed by Southern blotting (Figure 1B). An $SCC1^{+/-/-}$ clone was transfected with a chicken *SCC1* transgene under the control of a tet-repressible promoter. $SCC1^{+/-/-}$*tetSCC1* clones showing tet-dependent suppression of transgene expression were transfected with the puromycin *SCC1* disruption construct to disrupt the remaining *SCC1* allele. We obtained four $SCC1^{-/-/-}$Scc1^{+} clones, which expressed slightly more Scc1 than wild-type cells (Figure 1D). All of these clones were indistinguishable from wild-type cells in terms of their proliferative characteristics (Figure 1E) and exhibited essentially the same phenotype following tet-mediated Scc1 depletion. The phenotype of a single clone is described hereafter. Western blot analysis confirmed that the Scc1 protein level in $SCC1^{-/-/-}$*tetSCC1* cells was reduced by at least two orders of magnitude 24 hr after the addition of tet (Figures 1C and 1D).

Scc1 Is Required for Completion of Mitosis in Vertebrate Cells

The proliferative properties of wild-type and $SCC1^{-/-/-}$ cells were monitored by growth curves and cell cycle analysis. The growth curve of the $SCC1^{-/-/-}$Scc1^{+} cells was indistinguishable from that of wild-type cells, which divided every ~8 hr. We next examined cell proliferation and viability following the addition of tet (doxycycline) to the medium. $SCC1^{-/-/-}$Scc1^{-} cells stopped proliferating 24 hr after tet addition (Figure 1E). Cell cycle analysis showed that cells started to accumulate in G2/M phase 21 hr after tet addition, with extensive cell death occurring at 24–30 hr (data not shown). Mitotic indices began to increase at 18 hr and reached 26% 27 hr after tet addition (Figure 1F). These data suggest that $SCC1^{-/-/-}$Scc1^{-} cells accumulate in M phase rather than in G2 phase and that they are arrested or delayed in mitosis. To examine more closely the effects of Scc1 depletion on cell cycle progression, $SCC1^{-/-/-}$ cells were synchronized at the G1/S boundary by sequential nocodazole-mimosine block (see Experimental Procedures), so that 95% of the cell population was at the G1/S boundary. When these cells had been treated with tet for 27 hr, their Scc1 expression was undetectable by Western blot (Figures 1D and 2A, time 0). The plant amino acid mimosine blocks the cell cycle reversibly in late G1 phase by upregulation of the CDK inhibitor $p27^{kip1}$ and acts as an effective inhibitor of DNA replication (Wang et al., 2000). Upon release from mimosine arrest, cells reentered the cell cycle in a synchronous manner. S phase progression in Scc1^{-} cells was indistinguishable from that of Scc1^{+} cells (Figure 2A). We also analyzed the cell cycle progression following synchronization at the G1 phase by centrifugal elutriation and obtained the same result (data not shown). These data extend the previous findings that DNA replication in vitro takes place nearly normally in *Xenopus* nuclei assembled in cohesin-depleted extract (Losada et al., 1998) and in the Rad21-K1ts mutant of fission yeast at the restrictive temperature (Tatebayashi et al., 1998). These results indicate that Scc1 is not primarily required for DNA replication. However, in contrast to Scc1^{+} cells, which began to exit mitosis 5–6 hr after release from the mimosine block as manifested by the appearance of cells in the G1 phase, most Scc1^{-} cells remained in G2/M phase even at 7–8 hr after release from the G1 block, suggesting that Scc1 is essential for the timely completion

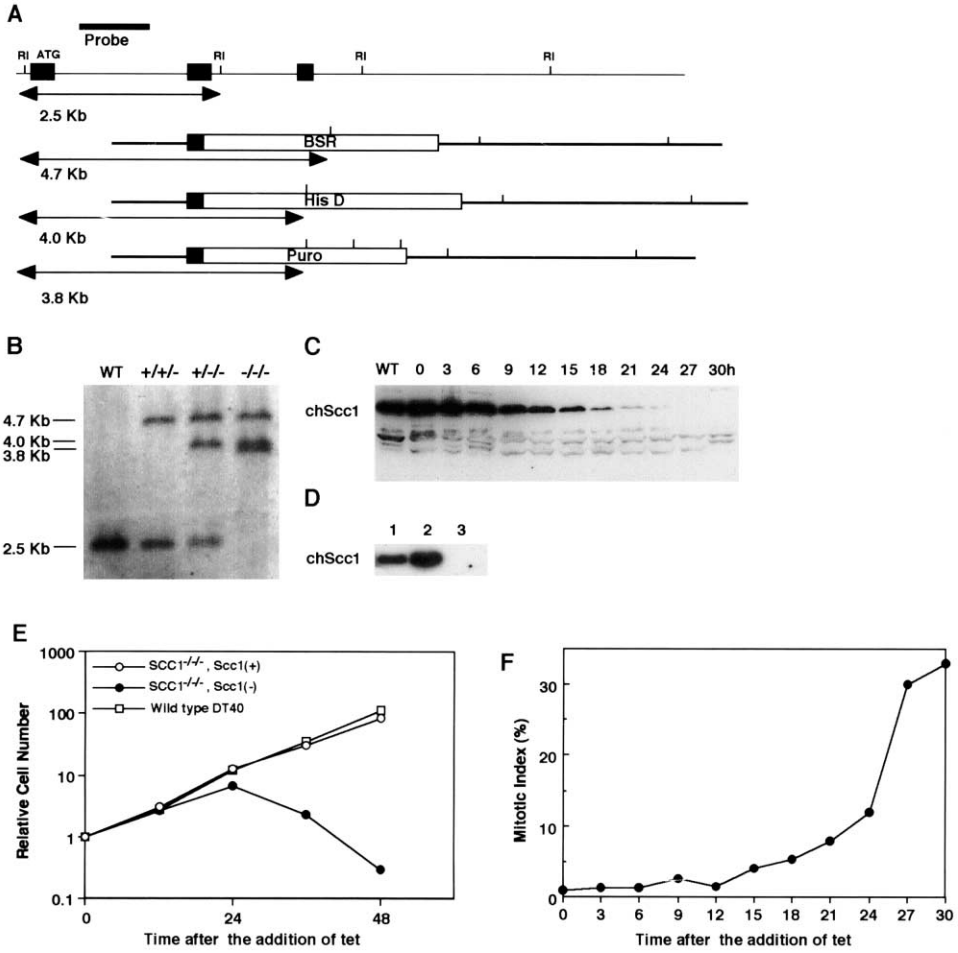

Figure 1. Generation of *SCC1*$^{-/-/-}$ DT40 Clones

(A) Schematic representation of the chicken *SCC1* locus, the three gene disruption constructs, and the configuration of the targeted loci. Black boxes indicate the positions of exons that were disrupted. RI indicates EcoRI restriction sites.

(B) Southern blot analysis of wild-type (WT), heterozygous mutant (+/+/−) and (+/−/−), and homozygous mutant (−/−/−) clones. EcoRI enzyme-digested genomic DNA was hybridized with the probe DNA shown in (A).

(C) Western blot analysis of the time course of Scc1 repression following the addition of the tetracycline analog doxycycline at time 0. Extracts from 10^6 cells prepared from wild-type (WT) or Scc1$^-$ cells at the times indicated following tet addition were immunoblotted with a polyclonal antibody against Scc1.

(D) Western blot analysis of the Scc1 protein in cells synchronized at the G1/S boundary. Immunoblot for Scc1 of total protein prepared from 10^6 asynchronous wild-type cells (lane 1), G1/S phase synchronized *SCC1*$^{-/-/-}$Scc1$^+$ cells (lane 2), or G1/S phase synchronized *SCC1*$^{-/-/-}$Scc1$^-$ cells (lane 3).

(E) Growth curves of cultures of the indicated genotypes in the absence or presence of doxycycline (tet). Representative curves from three separate experiments are shown.

(F) Mitotic index in *SCC1*$^{-/-/-}$Scc1$^-$ cells at various times following tet addition. Two hundred cells were scored following analysis by DAPI staining.

of mitosis (Figure 2A). Eventually, a few Scc1$^-$ cells seemed to exit mitosis, as shown by the appearance of a small, broader G1 peak at 7–8 hr.

Scc1 Is Required for Chromosome Alignment at Metaphase and Segregation at Anaphase

To examine mitotic progression in the absence of Scc1, cells were either fixed and stained with DAPI and anti-tubulin antibody, or centromeres were labeled by tagging the endogenous CENP-H protein with green fluorescent protein (CENP-H/GFP) by gene targeting (Fukagawa et al., 2001). CENP-H is a constitutive component of the centromere that colocalizes with CENP-A and CENP-C throughout the cell cycle. It is notable that both CENP-H (Figure 3) and CENP-C (Figure 6) appear normal in the absence of Scc1, indicating that kinetochore assembly is not severely compromised. In addition, the spindle checkpoint protein Mad2 localized to the unaligned metaphase chromosomes in Scc1-deficient cells, indicating that this aspect of kinetochore function is normal and accounting for the block in prometaphase (see Supplemental Figure S1 at http://www.developmentalcell.com/cgi/content/full/1/6/759/DC1).

DT40 cells have 11 autosomal macrochromosomes, the ZW sex chromosomes, and 67 microchromosomes. This corresponds to the normal chicken karyotype except for a trisomy of chromosome 2 and one additional microchromosome. In Scc1$^+$ cells, mitosis proceeded

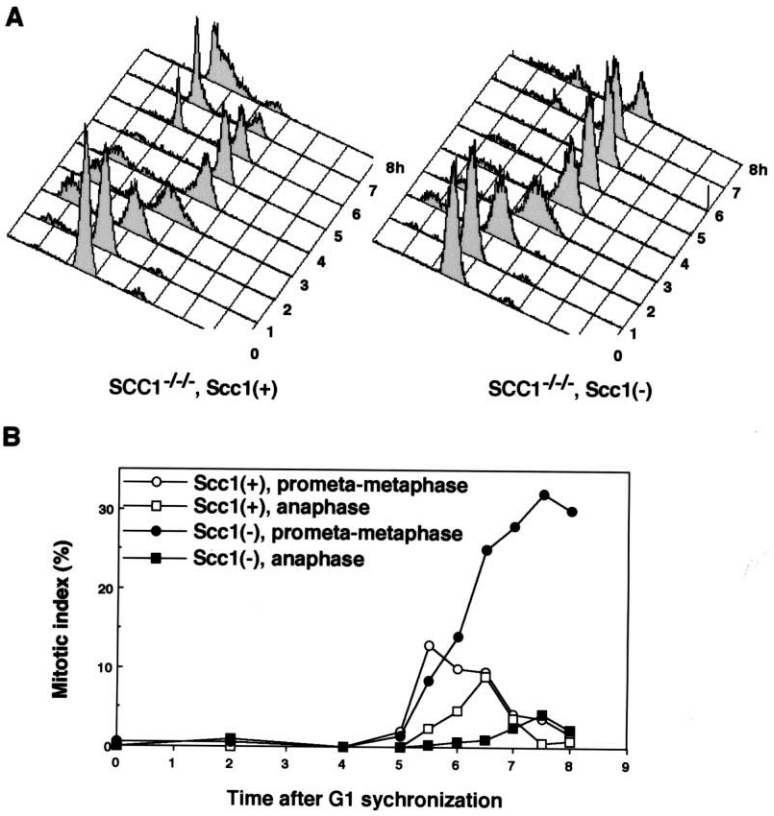

Figure 2. Cell Cycle Analysis of Scc1-Deficient DT40 Cells

(A) Flow cytometric analysis of synchronized *SCC1*$^{-/-/-}$ cell cultures with (Scc1^{-}) or without (Scc1^{+}) addition of doxycycline. Cells were harvested and stained with propidium iodide to detect total DNA (horizontal axis, linear scale) at the time points indicated after their release from nocodazole-mimosine G1/S phase arrest.

(B) Time course analysis of mitotic progression in Scc1^{+} and Scc1^{-}*SCC1*$^{-/-/-}$ cells following release from synchronization at the G1/S boundary.

normally (Figures 3A–3E and 3K–3O); in metaphase, the centromere regions were aligned at a metaphase plate, while in anaphase, the sister chromatids segregated in two distinct groups to the spindle poles. In contrast, Scc1 deficiency imposes a mitotic block or delay at prometaphase or metaphase (Figure 2B), with a small proportion of cells undergoing anaphase. Although Scc1 is not essential for bipolar spindle formation (Figures 3G–3I), substantial fractions of the chromosomes failed to achieve a bipolar attachment and were scattered either between the spindle poles or occasionally outside the spindle (Figures 3G, 3H, 3Q, and 3R). These unaligned chromosomes, which were seen in more than 90% of prometaphase cells ($n \geq 100$), appeared to establish a monopolar connection to microtubules (Figures 3G and 3H). In anaphase, which was seen in ~5% of Scc1-deficient cells at 7.5 hr after release from the mimosine block, we did not observe a sharp separation between chromosomal masses, indicative of successful segregation of the sister chromatids (Figures 3D and 3N). Instead, we observed many lagging chromosomes at the midzone or the midbody, suggestive of defects in either kinetochore attachment or function (Figures 3I, 3S, and 3T).

Scc1 Is Required for Sister Chromatid Cohesion in Interphase Nuclei and during Mitosis

To test whether Scc1 is involved in sister chromatid cohesion in interphase, we examined the distance between sister chromatids in interphase nuclei. The relative positions of alleles of the chicken *OVALBUMIN* locus, which is located on the long arm of chromosome 2, were examined by in situ hybridization of fixed nuclei. Interphase Scc1^{+} cells showed two closely located signals on each of the three chromosomes (Figures 4A and 4B). In contrast, the sister chromatids in Scc1^{-} cells were significantly further apart (Figures 4C and 4D), indicating that Scc1 is required for sister chromatid pairing in interphase nuclei.

To examine sister chromatid cohesion in mitotic cells, we performed karyotype analysis on Scc1^{+} and Scc1^{-} cells that had been synchronized at the G1/S boundary and entered prometaphase 6 hr after synchronization. While chromosomes from Scc1^{+} cells retained close cohesion between their sister chromatids (Figures 4E and 4F), the chromosomes from Scc1^{-} cells consisted of sister chromatids that were separated at least partially (Figures 4G and 4H). Interestingly, the manner of the partial separation showed considerable variability between chromosomes. Some sister chromatids lost arm cohesion while maintaining cohesion at the centromere. Others appeared to be connected only at telomere regions. Sister chromatid cohesion was further investigated employing atomic force microscopy. We examined the chromosomes of synchronized cells beginning to enter prometaphase and noted a marked separation of sister chromatids in chromosomes from Scc1^{-} cells relative to controls (Figures 4I–4L). It should be noted that the loss of cohesion was distributed randomly along the whole chromosome, including at centromere regions. This observation is in marked contrast to the chromosomes of wild-type cells, where only the arms of chromosomes can be separated artificially, that is, by prolonged colcemid treatment. Thus, Scc1 appears to play an important role in sister cohesion both along

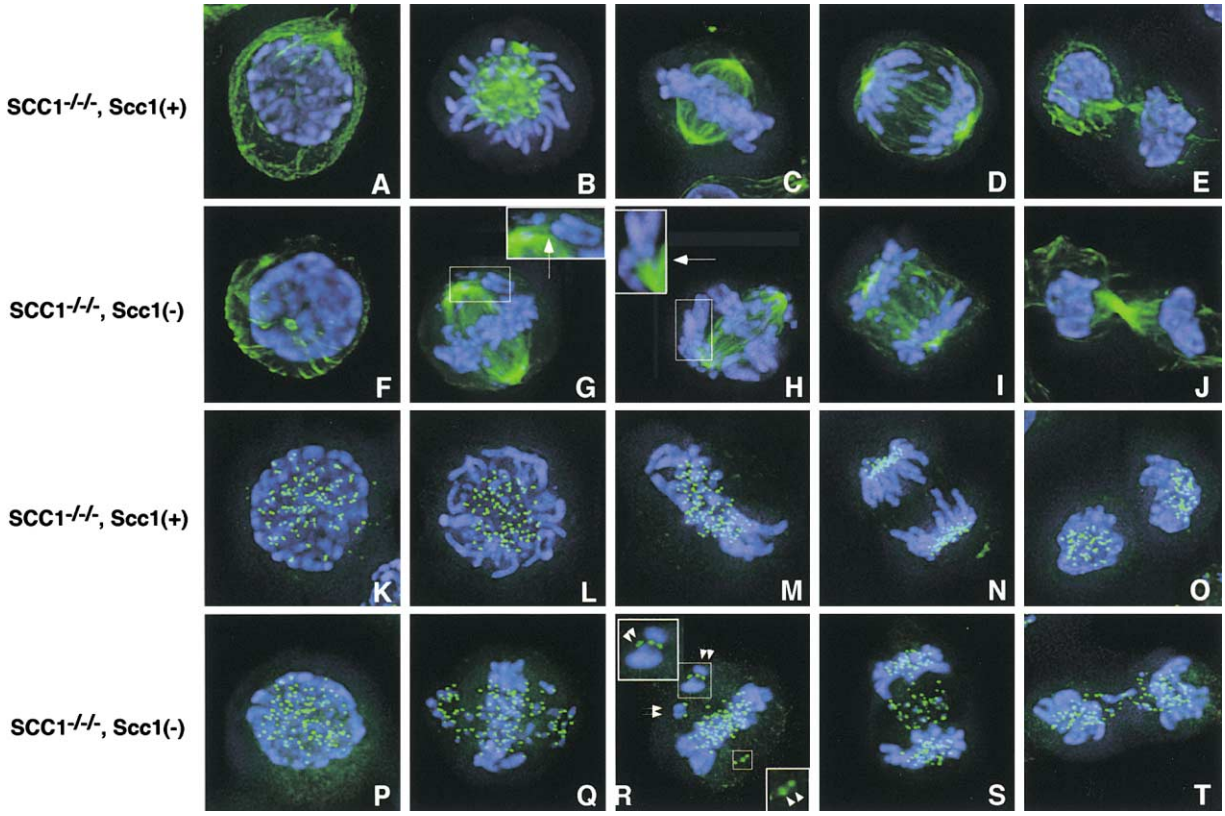

Figure 3. Phenotypic Characterization of Mitotic Scc1-Deficient Cells

(A–J) Synchronized *SCC1*$^{-/-/-}$ cells cultured in the presence or absence of doxycycline, as indicated, were stained for tubulin (green) and DNA (blue) 6–7 hr after release from the G1/S phase block. Boxes show close-ups of chromosomal regions in which the monopolar attachment can be seen more clearly.

(K–T) Synchronized *SCC1*$^{-/-/-}$ cells expressing CENP-H/GFP cultured in the presence or absence of doxycycline, as indicated, were stained for DNA (blue) 6–7 hr after release from the G1/S phase block. CENP-H/GFP fluorescence is shown in green. Arrows indicate paired sister kinetochores.

(A, F, K, P), (B, C, G, H, L, M, Q, R), (D, I, N, S), and (E, J, O, T) represent cells in prophase, prometaphase/metaphase, anaphase, and cytokinesis, respectively.

the chromosome arms and at centromeres. This is consistent with the reported association of human SCC1 with centromeres until the metaphase to anaphase transition (Waizenegger et al., 2000).

Scc1 Is Dispensable for Condensin Binding and Chromosome Condensation

In order to assess whether defective cohesion could have an impact on chromosome condensation during mitosis, we performed immunofluorescence localization of the condensin subunit ScII/SMC2 in metaphase spreads of Scc1-deficient cells (Saitoh et al., 1994). As shown in Figure 5, ScII/SMC2 localized all along the chromatids, even in the absence of cohesion. This result, along with the condensed chromosome morphology seen in Scc1^{-} cells (Figures 3 and 4), demonstrates that chromosome condensation is not dependent on sister chromatid cohesion and highlights the independence in targeting of the condensin and cohesin complexes to chromosomes.

Scc1 Is Necessary for the Appropriate Centromere Targeting of the Chromosome Passenger INCENP

The prometaphase/metaphase block in mitosis in the absence of Scc1, along with the aberrant segregation of chromosomes at anaphase, suggests that there is a failure in kinetochore function. Since the constitutive kinetochore components CENP-H and CENP-C appear to behave appropriately, we tested whether the loss of Scc1 affects the chromosomal passenger protein INCENP, which is necessary for normal metaphase chromosome alignment and sister kinetochore disjunction (Adams et al., 2001; Kaitna et al., 2000; Mackay et al., 1998; Oegema et al., 2001). Strikingly, immunofluorescence localization of INCENP in metaphase chromosomes of Scc1-deficient cells revealed that it was not targeted normally to inner centromeres (Figure 6), while immunoblot analysis of mitotic chromosomes showed no difference in INCENP levels following Scc1 depletion (data not shown). Of note, the appropriate centromere targeting of INCENP was not observed even in the sisters that appeared to be tightly associated with each other at the centromere in Scc1-deficient cells. This is important, as it indicates that loss of INCENP targeting from the inner centromere is not dependent on the precocious sister chromatid separation phenotype induced by loss of cohesin function. The fact that Scc1 is essential for the targeting of INCENP to the inner centromere raises the possibility that certain aspects of chromosomal passenger function may lie downstream of cohesin function.

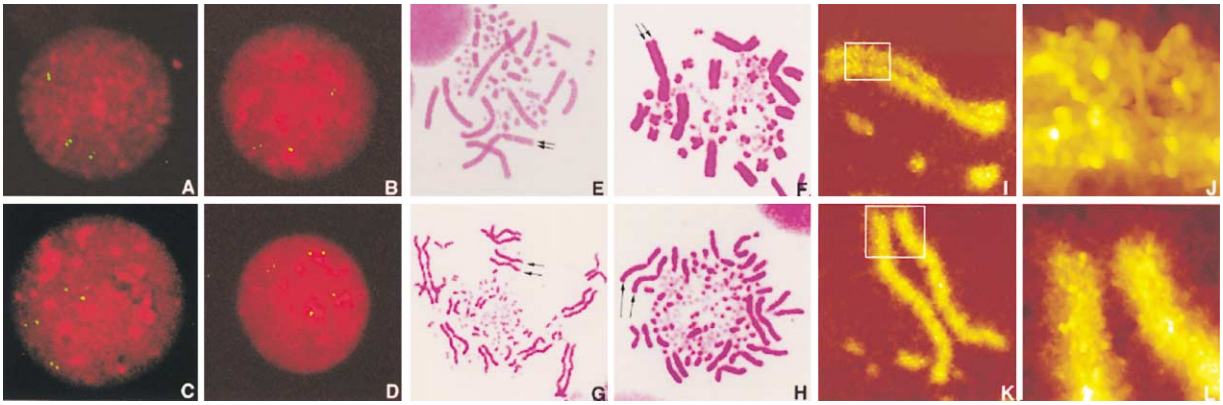

Figure 4. Loss of Sister Chromatid Cohesion in *SCC1*$^{-/-/-}$ Cells

(A–D) Scc1^{+} (A and B) and Scc1^{-} (C and D) cells were subjected to FISH with a probe specific for the trisomic *OVALBUMIN* locus on chromosome 2 (green) 5 hr after release from G1/S phase block. Nuclear DNA was stained with DAPI (red).

(E–H) Metaphase spreads were prepared from Scc1^{+} (E and F) and Scc1^{-} (G and H) cells 6 hr after release from synchronization at the G1/S boundary. Arrows indicate paired sister chromatids. Note the marked separation in Scc1^{-} cells.

(I–L) Synchronized populations of Scc1^{+} (I and J) and Scc1^{-} (K and L) cells were subjected to atomic force microscopy 6 hr after release from synchronization at the G1/S boundary. A close-up of an area along the chromosome is shown in (J) and (L) to emphasize the increased separation between the main body of the sister chromatids in Scc1-deficient cells (L).

Cohesion in Interphase Nuclei Facilitates Homologous Recombinational DNA Repair

Fission yeast Rad21 was originally isolated as one of the radiation-sensitive mutants (Phipps et al., 1985). Genetic studies showed that Scc1/Rad21 is involved in DNA double-strand break (DSB) repair (Birkenbihl and Subramani, 1992; Tatebayashi et al., 1998). Given our data indicating the involvement of Scc1 in interphase sister chromatid cohesion, we hypothesized that Scc1 might promote homologous DNA recombination (HR) between sister chromatids by holding them in close proximity. We previously showed that HR plays an important role in repairing DSBs arising during DNA replication and those induced by ionizing radiation (IR) in the late S to G2 phases (Takata et al., 1998). The level of spontaneously occurring chromosomal breaks was slightly increased in Scc1^{-} cells compared to Scc1^{+} cells (Figure 7A). In addition, the level of IR-induced chromosomal aberrations was significantly increased in Scc1^{-} cells when compared with Scc1^{+} cells (Figure 7A). Thus, the loss of cohesion between sisters reduces the efficiency of DSB repair in interphase nuclei. In order to address HR-mediated repair events more directly, we measured the frequency of sister chromatid exchange (SCE) following exposure of cells to 4-nitroquinoline 1-oxide (4NQO). Our previous data (Sonoda et al., 1999; Takata et al., 2001) showed that this cytologically visible crossing-over event is mediated by HR and likely reflects the repair of DNA damage that arises during replication of a chemically modified template DNA strand, such as a DNA strand associated with 4NQO. We found that the levels of SCEs induced by 4NQO were significantly reduced in the absence of Scc1 (Figure 7B). These observations indicate that sister chromatid cohesion facilitates HR-mediated repair of replication-associated DNA damage. Taken together, these data suggest that Scc1 plays an essential role in HR-mediated repair during S to G2 phases by holding sister chromatids together.

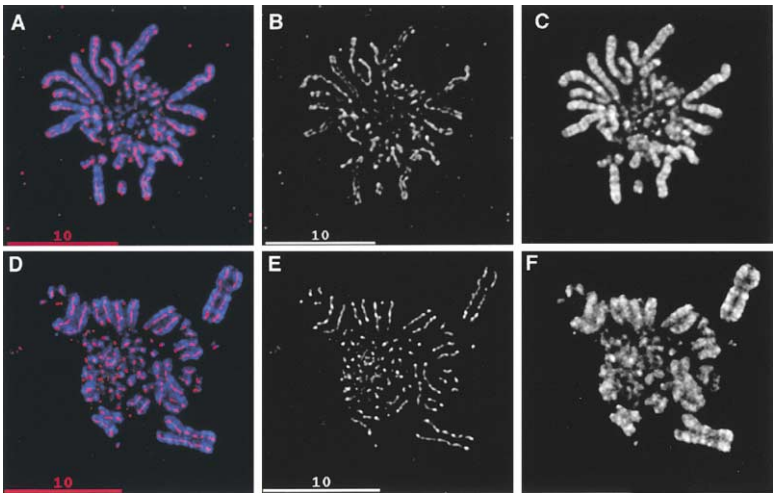

Figure 5. Chromosome Condensation and Condensin Binding in the Absence of Scc1

Chromosome spreads from asynchronous populations of colcemid-blocked *SCC1*$^{-/-/-}$Scc1^{+} cells (A–C) or colcemid-blocked *SCC1*$^{-/-/-}$Scc1^{-} cells that had been treated with tet for 24 hr (D–F) were stained with DAPI for DNA (blue) and with a polyclonal antibody recognizing ScII/SMC2 (red). Merged images are shown in (A) and (D), with single channels shown for ScII/SMC2 (B and E) and for DNA (C and F).

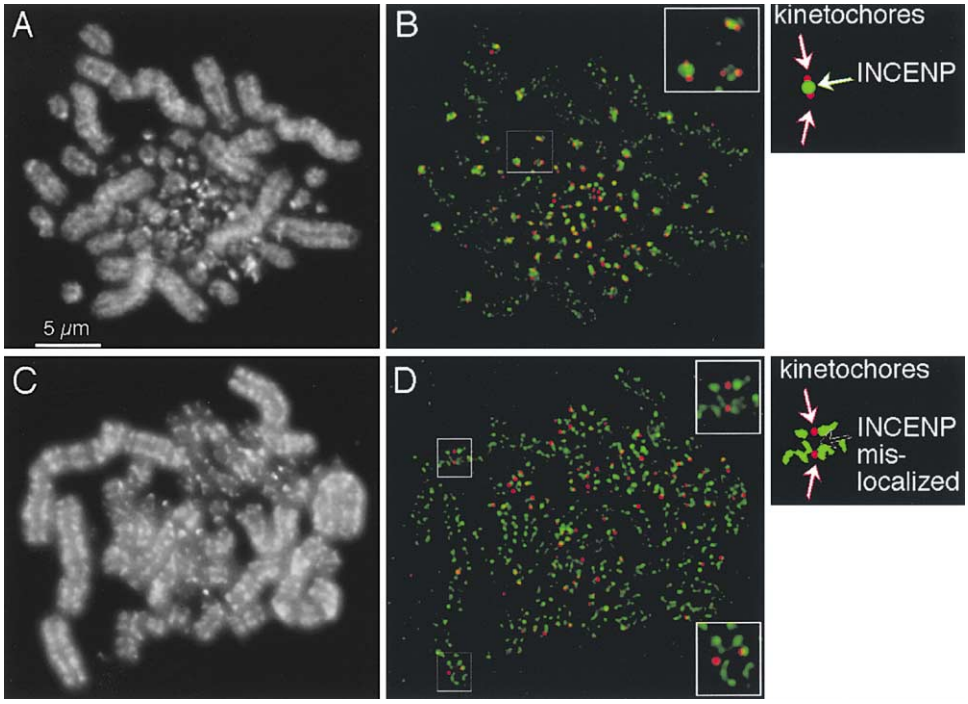

Figure 6. Defective Accumulation of INCENP at Kinetochores in Scc1-Deficient Cells

Chromosome spreads from asynchronous populations of colcemid-blocked *SCC1*$^{-/-/-}$Scc1^{+} cells (A and B) or colcemid-blocked *SCC1*$^{-/-/-}$Scc1^{-} cells that had been treated with tet for 24 hr (C and D) were stained with DAPI (A and C) and antibodies to CENP-C (red) and INCENP (green) (B and D). Boxes show close-ups of chromosomal regions in which the loss of INCENP from the inner centromere can be seen more clearly, as diagrammed in the panels at right.

Discussion

Sister Chromatid Cohesion in Mitosis and in Interphase Requires Scc1 in Vertebrate Cells

To confirm that the loss of Scc1 did indeed affect sister chromatid cohesion in our system, we monitored the separation between sister chromatids in interphase and mitosis in cells from a population that had been synchronized at G1/S during the depletion of Scc1. The significantly increased distance between sisters seen in interphase by FISH and in mitosis by Giemsa staining and light or atomic force microscopy shows that Scc1 is necessary for sister chromatids to remain closely connected in vertebrate cells. Interestingly, we have found that even in the absence of functional Scc1, sister chromatids remain in proximity in most cells. The mechanisms underlying sister chromatid association remain unknown. This could be due to an alternative system for the linking of sister chromatids, or to the persistence of low levels of residual Scc1 that are not detected by the methods used.

Scc1 Is Dispensable for DNA Replication and Chromosome Condensation in Vertebrate Cells

Sister chromatid cohesion is established during S phase (Skibbens et al., 1999; Toth et al., 1999; Uhlmann and Nasmyth, 1998). Budding yeast mutants deficient in Scc1 experience a slight delay in S phase progression during the mitotic cell cycle (Guacci et al., 1997). Similarly, temperature-sensitive Rad21-K1 mutants of fission yeast experience a slight delay in S phase progression and lose viability during the S phase (Tomonaga et al., 2000). However, normal in vitro DNA replication occurs in Scc1-depleted *Xenopus* oocytes (Losada et al., 1998), and S phase progression appeared normal in Scc1^{-} DT40 cells. FISH analysis of interphase nuclei from Scc1^{+} and Scc1^{-} DT40 cells revealed a significant separation of sister chromatids during or after replication in the absence of Scc1. However, this separation did not result in a significant S or G2 phase cell cycle arrest, suggesting that no extensive DNA damage occurred during replication in the absence of cohesion. Furthermore, the level of spontaneously occurring chromosomal aberrations was elevated only slightly in the absence of Scc1, confirming that Scc1 is dispensable for DNA replication.

Scc1 deficiency causes chromosome condensation defects in budding yeast (Guacci et al., 1997), but not in the in vitro system using *Xenopus* nuclei or chromatin (Losada et al., 1998). Here we found that chromosome condensation in vivo, as monitored both by the chromosomal morphology and by the association of the condensin subunit ScII/SMC2 (Saitoh et al., 1994; reviewed in Hirano, 2000) with sister chromatids, appeared to be normal in Scc1^{-} cells. Also different between yeast and vertebrate cells is the behavior of cohesins during prometaphase. Cohesins dissociate from prometaphase chromosome arms in higher eukaryotes, but not in budding yeast (Losada et al., 1998; Sumara et al., 2000; Uhlmann and Nasmyth, 1998; Uhlmann et al., 2000; Waizenegger et al., 2000). These differences between yeast and vertebrates may reflect differing requirements for interactions between the sisters during the compaction of

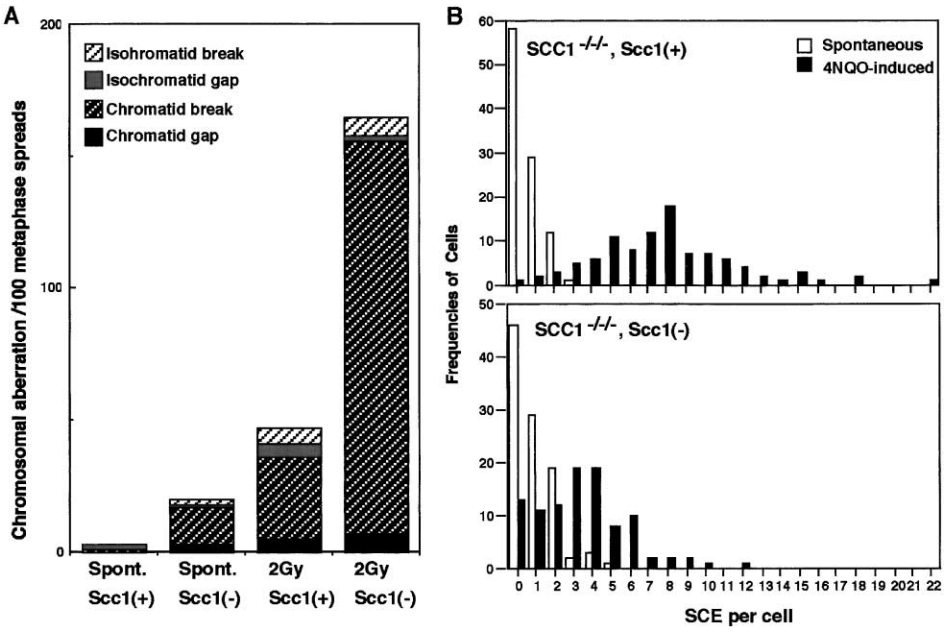

Figure 7. Defective Homologous Recombinational DNA Repair in the Absence of Cohesin

(A) Chromosome aberrations occurring in the macrochromosomes of synchronized cells 7 hr after release from G1/S block were stained and counted as described (Sonoda et al., 1998). Cells were treated with colcemid for the last 3 hr and, where indicated, were treated with two Gy γ irradiations 4 hr after release from synchronization. Data are presented as the number of aberrations per 100 cells.

(B) Reduced levels of SCE in Scc1$^+$ cells compared to controls. After doxycycline addition, the cells were labeled with BrdU during two cell cycles with or without 4NQO treatment (0.2 ng/ml) for the last 8 hr. Spontaneous and 4NQO-induced SCEs in the major chromosomes of 100 metaphase cells were scored. Histograms show the frequency of cells with the indicated numbers of SCEs per cell.

the genome for mitosis, perhaps dependent on the size of the genome to be compacted.

Sister Chromatid Cohesion in DNA Repair

Scc1$^-$ cells showed a moderate defect in their ability to repair spontaneous chromatid breaks as well as those induced by ionizing radiation. The level of this deficiency is comparable to that of *Rad54*$^{-/-}$ DT40 cells defective in DSB repair by homologous recombination (Takata et al., 1998). The simplest explanation for this is that the increased distance between sister chromatids in the absence of cohesion reduces the efficiency of the search for homologous sequences for repair. The reduced frequency of 4NQO-induced SCE further suggests that close proximity of sister chromatids is important for recombinational repair between sisters (Sonoda et al., 1999). Similarly, Scc1/Rad21 is involved in DNA double-strand break repair in fission yeast (Birkenbihl and Subramani, 1992) and in budding yeast (Sjogren and Nasmyth, 2001), suggesting that this feature of cohesion has been conserved throughout evolution.

Scc1 Function Is Required for Chromosome Alignment at the Metaphase Plate: A Role for Cohesion in Kinetochore Function?

Seven hours after release from the nocodazole-mimosine G1/S block, most Scc1$^+$ cells had already passed the metaphase to anaphase transition, whereas Scc1$^-$ cells remained in prometaphase or metaphase. The prometaphase cells showed a substantial fraction of chromosomes located between or around the spindle poles. The few anaphases we did observe showed significantly aberrant segregation of sister chromatids. This could be a consequence of defects in chromosome congression.

One obvious explanation for defects in chromosome alignment and mitotic progression would be that depletion of Scc1 caused abnormalities in kinetochore structure. However, kinetochore assembly in the absence of Scc1 appears to be normal, as judged by the binding of constitutive kinetochore proteins CENP-H (Fukagawa et al., 2001; Sugata et al., 1999) and CENP-C (Fukagawa et al., 1999; Saitoh et al., 1992) and the signaling component Mad2 (Chen et al., 1996; Fang et al., 1998). To date, the only centromeric component whose distribution appears to be abnormal in Scc1-depleted cells is the chromosomal passenger protein INCENP, which binds to chromosomes but fails to target normally to the inner centromere. This observation is in agreement with Rad21/Scc1-depleted *S. pombe*, which exhibited defective targeting of other chromosomal passengers, Bir1/Cut17 and the Aurora kinase (Terada et al., 1998), to mitotic chromosomes (Morishita et al., 2001). INCENP is necessary for chromosome alignment and segregation in human cells, *Drosophila melanogaster*, *Caenorhabditis elegans*, and mouse (Adams et al., 2000; Cutts et al., 1999; Kaitna et al., 2000; Mackay et al., 1998; Oegema et al., 2001) and is also required for the targeting of the Aurora B kinase to mitotic chromosomes (Adams et al., 2000, 2001). Loss of INCENP does not appear to affect assembly of the constitutive kinetochore proteins CENP-C or CENP-A (Adams et al., 2000, 2001; Oegema et al., 2001), so that its absence seems likely to have

an impact on the function, rather than the assembly, of the kinetochore.

Interestingly, the prometaphase phenotype seen in Scc1-depleted cells, with chromosomes both at a metaphase plate and clustered around the spindle poles, is remarkably similar to that seen upon expression of a dominant-negative mutant that interferes with INCENP targeting to chromosomes (Mackay et al., 1998). Thus, the prometaphase phenotype seen following Scc1 depletion could be explained by the requirement of Scc1 for proper targeting of INCENP to centromeres. Importantly, the defect in INCENP targeting to the inner centromere cannot be ascribed solely to the defects in sister chromatid cohesion that result from loss of Scc1; we also observed INCENP incorrectly targeted in mitotic chromosomes in which sister chromatid separation had not yet gone to completion. It has recently been shown that Aurora B kinase transfers normally to the spindle midzone at anaphase in cells expressing an uncleavable Scc1 (Hauf et al., 2001). It was not expected that transfer of the chromosomal passengers would require cleavage of Scc1, as we have previously shown that INCENP transfers from the chromosomes to the central spindle during late metaphase, at which time Scc1 would be expected to be intact (Earnshaw and Cooke, 1991). Thus, it is likely that the role of Scc1 in INCENP targeting involves some aspect of Scc1 function, rather than its destruction.

How might the loss of Scc1 affect metaphase chromosome alignment? The simple suggestion that the sister chromatids of the misaligned microchromosomes might have separated prematurely in the absence of cohesion is not consistent with the observation that some chromosomes located around the spindle poles exhibited paired CENP-H/GFP centromere signals (Figure 3R), indicating that their sister chromatids had not yet separated.

Another possibility is that Scc1 is required for chromosomes to make a proper bipolar attachment to the mitotic spindle. This is supported by the observation that the misaligned chromosomes appeared to be connected to microtubules from only one pole (Figures 3G and 3H). Significantly, budding yeast cohesin also facilitates the bipolar attachment of centromeres to the spindle microtubules (Tanaka et al., 2000).

We have shown here that Scc1 is required for the proper targeting of INCENP to the inner centromere, which may explain the misalignment of metaphase chromosomes in Scc1-deficient DT40 cells. Previous studies of INCENP depletion by RNAi in *Drosophila* cells found that the protein is absolutely required for chromosomes to achieve a stable bipolar attachment to the spindle (i.e., metaphase alignment; Adams et al., 2001). The role of INCENP in promoting bipolar attachment remains unknown, but may involve the action of Aurora B kinase, a binding partner of INCENP (Kaitna et al., 2000; Adams et al., 2001), which we have shown to be also required for INCENP targeting to centromeres in *Drosophila* cells (Adams et al., 2001). The present results thus raise the very intriguing possibility that the chromosomal passengers function downstream of Scc1 to regulate kinetochore activity, thereby suggesting that cohesins may function not only as structural links between sister chromatids, but also have other roles in mitotic regulation.

In budding yeast, Scc1 is cleaved and dissociates from chromosomes in anaphase, leading to sister chromatid separation (Nasmyth et al., 2000; Uhlmann et al., 1999, 2000). In contrast, in vertebrates, a cleavage-independent pathway removes cohesin from chromosome arms during prophase, whereas a separase-dependent pathway cleaves centromeric cohesin at the metaphase-anaphase transition (Losada et al., 1998; Sumara et al., 2000; Waizenegger et al., 2000). The latter event appears to be responsible for sister chromatid separation in anaphase (Hauf et al., 2001), as demonstrated also in fission yeast (Tomonaga et al., 2000). We have recently shown that the binding of INCENP to the inactive centromere of a stable dicentric chromosome correlates strongly with the degree of cohesion between the sister chromatids (Vagnarelli and Earnshaw, 2001). It will be interesting in future experiments to test whether the process of Scc1 dissociation from chromosomes during prometaphase modulates chromosome passenger behavior, or whether cohesin might have an additional, possibly catalytic capacity of its own.

Experimental Procedures

Construction of Targeting and Expression Vectors

A chicken SCC1 (*GdSCC1*) partial cDNA fragment was amplified from chicken testis cDNA by RT-PCR with primers (5′-CCGCCAACCAA GAAGCTTATGATG-3′, 5′-AGCTCGCTGAAGACCATGAAGCAT-3′) and 5′ and 3′ RACE on chicken testis cDNA used to isolate the entire open reading frame of *GdSCC1.* To construct the *GdSCC1* expression vector, chicken *SCC1* cDNA was inserted into an expression vector containing a tet-repressible promoter, pUHG10-3 (a gift from Prof. H. Bujard, Heidelberg, Germany). Twelve kilobases of partial chicken genomic *GdSCC1* locus was isolated from DT40 genomic DNA by long-range PCR. Chicken *SCC1* disruption constructs were made by replacing ~2 kb of genomic sequence containing the sequence encoding amino acids 81–91 with his, bsr, or puro selection marker cassettes. To construct a GFP-tagged chicken CENP-H knockin vector, an exon coding the C terminus of the *CENP-H* locus was replaced with GFP coding sequences and a neo selection marker (Fukagawa et al., 2001). For Mad2 expression vector, a human MAD2 cDNA (a gift from Dr. Hirota, Kumamoto, Japan) was inserted into a pDsRED1-C1 plasmid (Clontech).

Transfection, Cell Culture, and Synchronization

DT40 cells were cultured and transfected as described previously (Sonoda et al., 1998) and were synchronized at the G1/S phase transition by sequential nocodazole and mimosine blocks. Briefly, cells were cultured in medium with or without doxycycline (1 ng/μl). After 8 hr, nocodazole was added to a final concentration of 0.5 μg/ml and incubated for 4 hr. Cells were then washed three times and incubated in medium containing 0.8 mM mimosine with or without doxycycline for 15 hr. At 27 hr after the addition of doxycycline, cells were washed three times and incubated in fresh medium. At various time points, cells were harvested, fixed with formaldehyde, and stained in 0.1 μg/ml DAPI solution. A portion of harvested cells was fixed by 70% ethanol and stained in propidium iodide solution. Cell cycle analysis was performed on a FACScan (Becton Dickinson) using CellQuest software.

Western Blot Analysis

Total protein from 10^6 cells was separated by 7.5% SDS-PAGE. After transfer to a membrane, proteins were detected by immunoblotting with polyclonal rabbit anti-human Scc1 serum at 1:2000 (Waizenegger et al., 2000) and horseradish peroxidase-conjugated goat anti-rabbit Ig (Amersham) using Super Signal System (Pierce).

Karyotype Analysis and Measurement of SCE Levels

Karyotype analysis was carried out as previously described (Sonoda et al., 1998). For SCE analysis, cells were cultured in the presence of 10 μM bromodeoxyuridine and 1 ng/ml doxycycline for 16–18 hr

(two cell cycle periods) and pulsed with 0.1 μg/ml of colcemid for the last 2.5 hr. 4NQO (0.2 ng/ml) was added 8 hr before harvest. Staining for SCE was as described previously (Sonoda et al., 1999).

Fluorescent In Situ Hybridization (FISH)

FISH was performed by standard methods. The plasmid probe for the *OVALBUMIN* locus was biotin labeled by nick translation (Boehringer). Nuclei were visualized using an MRC-1024 confocal microscope (Bio-Rad).

Indirect Immunofluorescence Microscopy

Mitotic cells were fixed with 2% paraformaldehyde for 10 min, washed twice with PBS, and incubated with FITC-labeled polyclonal anti-chicken tubulin (Sigma) at 1:50 in 1% BSA/PBS for 30 min at room temperature. After washing twice with PBS, the cells were incubated with PBS containing 0.1 μg/ml DAPI and were analyzed by fluorescence microscopy. Serial optical section data for DNA, GFP, or anti-tubulin were collected, processed using a three-dimensional blind deconvolution method (AutoDeblur; AutoQuant Imaging), and projected onto a single plane. For chromosome spreads, cells were swollen in 75 mM KCl for 10 min, fixed in ice-cold 3:1 methanol:acetic acid, dropped onto slides, and washed with TEEN (1 mM triethanolamine [pH 8.5], 0.2 mM Na-EDTA, 0.25 mM NaCl), 0.1% Triton X-100, 0.1% BSA. Rabbit polyclonal antibodies to CENP-C (Fukagawa et al., 1999) and ScII (Saitoh et al., 1994) and the mouse 3D3 anti-INCENP monoclonal (Cooke et al., 1987) were used at 1:1000, 1:200, and 1:500, respectively, in TEEN, 0.1% Triton X-100, 0.1% BSA. After incubation at 37°C, slides were washed with KB^- (10 mM Tris-Cl [pH 7.7], 0.15 M NaCl, 0.1% BSA) and fluorescence-labeled secondary antibodies applied at 1:200 in KB^-. Three-dimensional data sets of chromosome spreads were collected using a DeltaVision microscope (Applied Precision). Data sets were deconvolved, and projected onto a single plane.

Atomic Force Microscopy (AFM)

AFM samples were prepared as described previously (Ushiki et al., 1996) with the following modification. Chromosome spreads were made by dropping the fixed cell suspension onto glass slides, followed by air drying. AFM imaging was carried out using an SPI 300 or 400 scanning probe microscope controlled by an SPI 3800 probe station (Seiko Instruments). All images were obtained by a noncontact mode AFM in air at room temperature and were graphically displayed as gradation images.

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