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Recruitment of *Xenopus* Scc2 and cohesin to chromatin requires the pre-replication complex

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Cohesin is a multi-subunit, ring-shaped protein complex that holds sister chromatids together from the time of their synthesis in S phase until they are segregated in anaphase[1–7]. In yeast, the loading of cohesin onto chromosomes requires the Scc2 protein[8–10]. In vertebrates, cohesins first bind to chromosomes as cells exit mitosis, but the mechanism is unknown[3,11,12]. Concurrent with cohesin binding, pre-replication complexes (pre-RCs) are assembled at origins of DNA replication through the sequential loading of the initiation factors ORC, Cdc6, Cdt1 and MCM2-7 (the 'licensing' reaction)[13]. In S phase, the protein kinase Cdk2 activates pre-RCs, causing origin unwinding and DNA replication. Here, we use *Xenopus* egg extracts to show that the recruitment of cohesins to chromosomes requires fully licensed chromatin and is dependent on ORC, Cdc6, Cdt1 and MCM2-7, but is independent of Cdk2. We further show that *Xenopus* Scc2 is required for cohesin loading and that binding of XScc2 to chromatin is MCM2-7 dependent. Our results define a novel pre-RC-dependent pathway for cohesin recruitment to chromosomes in a vertebrate model system.

To study chromosome duplication, we have used a cell-free system derived from *Xenopus* eggs, in which de-membranated sperm chromatin is replicated by sequential exposure to two extracts[14] (Fig. 1a). The first extract, a high speed supernatant (HSS) of egg cytoplasm, supports formation of pre-RCs, which are assembled by sequential chromatin loading of the replication initiation factors ORC, Cdc6, Cdt1 and MCM2-7. The second extract, a concentrated nucleoplasmic extract (NPE) isolated from synthetic nuclei, supplies high levels of Cdk2–Cyclin-E activity and thus promotes loading of the initiation factor Cdc45, origin unwinding and a complete round of DNA replication. Origin unwinding can be detected by the chromatin-loading of the single-stranded DNA (ssDNA)-binding protein RPA. DNA replication in this system is limited to a single round, because NPE contains high concentrations of geminin, an inhibitor of Cdt1 that blocks *de novo* pre-RC assembly[14–17].

To identify novel factors whose recruitment to chromatin at the onset of S phase is dependent on pre-RCs, sperm chromatin was incubated in HSS in the presence or absence of geminin. NPE was then added to initiate DNA replication, and chromatin was isolated. To prevent displacement of replication proteins from chromatin as a result of DNA replication, we inhibited DNA synthesis by supplementing NPE with the DNA polymerase inhibitor aphidicolin. Two-dimensional (2D) gel electrophoresis was used to resolve the chromatin-bound proteins, some of which were absent in the geminin-containing sample (Fig. 1b, c). Protein sequencing by tandem mass spectrometry identified five of the six subunits of the MCM2-7 complex among the geminin-sensitive spots (Fig. 1b; and data not shown). In addition, two proteins with relative molecular masses (M_rs) of 140,000 and 160,000 were absent from chromatin assembled in the presence of geminin. Mass spectrometery identified 23 peptides corresponding to XSmc3 within p140, and seven peptides corresponding to XSmc1 within p160 (data not shown).

To confirm that x-cohesin loading is geminin-sensitive, we examined chromatin loading of all x-cohesin subunits by immunoblotting. In *Xenopus* egg extracts, the cohesin holo-complex exists in two forms, 12.5S and 14S, both of which contain XSmc1, XSmc3 and XRad21 (Scc1 in *S. cerevisiae*)[3,18]. The 12.5S complex also contains XSA-2, whereas the 14S complex contains XSA-1. Both XSA-1 and XSA-2 are homologues of Scc3 in *S.cerevisiae*. In HSS, we detected weak loading of cohesin subunits onto chromatin, which was abolished by geminin, as was MCM2-7 loading (Fig. 1d; lanes 2 and 3). Loading of all cohesin subunits was stimulated by the subsequent addition of NPE, and was inhibited when geminin was added to HSS (Fig. 1d; lanes 5 and 6), confirming the 2D gel results. Therefore, chromatin loading of 12.5S and 14S x-cohesin is geminin-sensitive. XRad21, XSA-1 and XSA-2, as well as Mcm6, were not detected on the 2D gel, most probably because they are too acidic to be resolved by the first-dimension pH gradient. To determine whether the inhibition of cohesin loading by geminin resulted from global effects of geminin on recruitment of proteins to chromatin, we used 2D gels to examine the range of proteins associated with a large quantity of sperm chromatin incubated in HSS that contained or lacked geminin. We found that among roughly 260 chromatin-bound proteins, geminin affected binding of only about 19 (including MCMs; data not shown).

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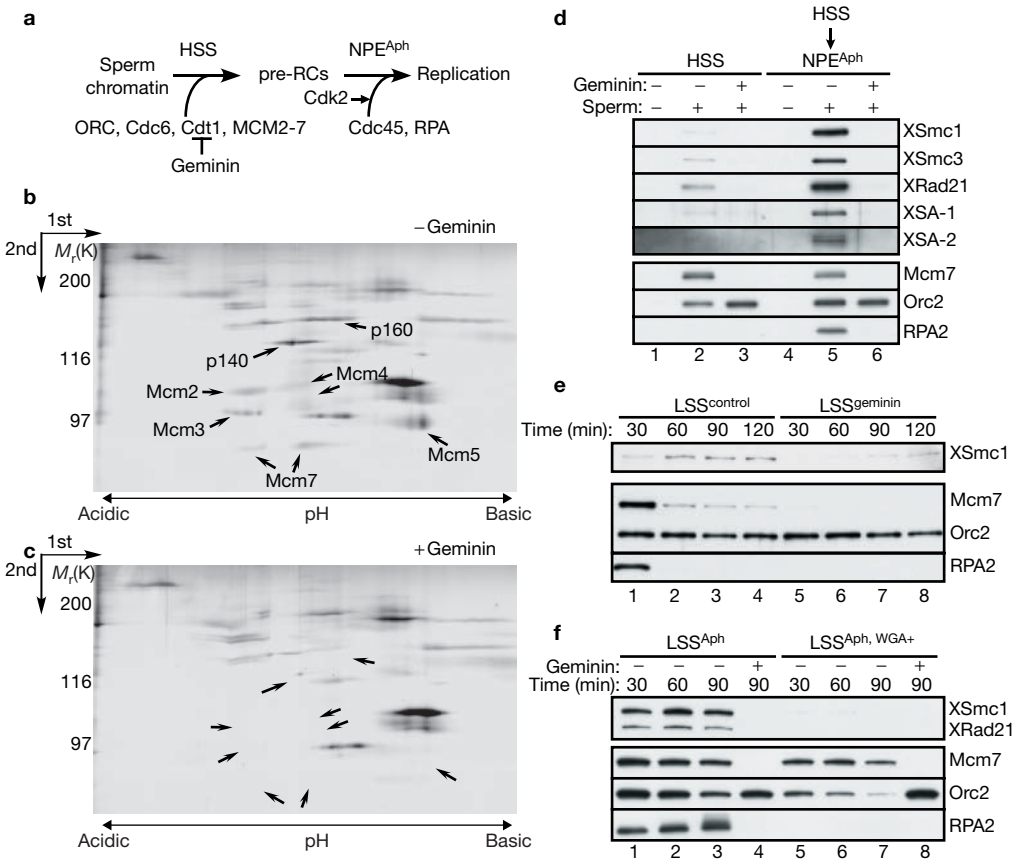

Figure 1 Chromatin loading of x-cohesin is inhibited by geminin. (**a**) The nucleus-free DNA replication system. (**b**, **c**) Sperm chromatin was incubated in HSS in the absence (**b**) or presence (**c**) of 500 nM geminin for 30 min and then supplemented with NPE containing 50 μg ml^{-1} aphidicolin. After 20 min, chromatin was isolated and bound proteins were analyzed on a two-dimensional gel, a portion of which is shown. Geminin-sensitive spots are indicated by arrows. (**d**) Western blot analysis of chromatin-bound proteins in the nucleus-free replication system. Sperm chromatin was incubated in HSS in the presence or absence of geminin and isolated after 30 min (lanes 1–3), or 15 min after the further addition of NPE containing aphidicolin (lanes 4–6). (**e**) Sperm chromatin was isolated at the indicated time points after incubation in LSS containing or lacking geminin. (**f**) Effect of nuclear transport on cohesin loading. Sperm chromatin was isolated after incubation in LSS containing or lacking 0.2 mg ml^{-1} wheat germ agglutinin.

As DNA replication and the establishment of sister chromatid cohesion normally occur within nuclei, we examined whether cohesin loading is also geminin-sensitive in conventional nuclear assembly extracts. In this system, sperm chromatin is added to a low speed supernatant (LSS) of egg cytoplasm[19]. Pre-RCs assemble rapidly on sperm chromatin, and subsequent nuclear envelope assembly promotes initiation of DNA replication. In LSS, geminin also inhibited cohesin loading (Fig. 1e). Cohesin loading was also inhibited when metaphase-arrested egg extracts[20] were released into interphase in the presence of added geminin (data not shown). Consistent with the fact that NPE greatly stimulated cohesin loading in the nucleus-free system (Fig. 1d), cohesin loading in LSS was inhibited when nuclear transport was blocked with wheat germ agglutinin (WGA) (Fig. 1F), or a mutant of the Ran-GTPase, RanT24N (data not shown). Taken together, the results show that geminin inhibits chromatin loading of cohesin in *Xenopus* egg extracts, and that the nuclear environment stimulates cohesin loading.

The fact that geminin inhibits cohesin loading onto chromosomes suggests that this process requires Cdt1. To test this prediction, Cdt1 was removed from LSS with anti-Cdt1 antibodies, which resulted in complete inhibition of DNA replication (Fig. 2b) and MCM2-7 loading (Fig. 2a). Cdt1-depleted extracts were also severely impaired for cohesin loading (Fig. 2a). Addition of recombinant Cdt1 to the depleted extracts rescued DNA replication, MCM2-7 loading and cohesin loading (Fig. 2a, b). Cdt1 is required to recruit the MCM2-7 complex to chromatin in *Xenopus* egg extracts[21], raising the possibility that MCM2-7 might also be required for cohesin recruitment. Therefore, we removed the MCM2-7 complex from LSS using anti-Mcm7 antibodies. Depletion of MCM2-7 completely inhibited DNA replication (Fig. 2d) and cohesin loading (Fig. 2c). The fact that MCM2-7 depletion and geminin-addition both abolish cohesin loading strongly argues that chromatin-bound MCM2-7 is required for cohesin loading.

The requirement for Cdt1 and MCM2-7 in cohesin loading predicts that this process should also be dependent on ORC. As expected, we found that when extracts were sufficiently depleted of ORC to cause at least a 90–95% inhibition of DNA replication (Fig. 2f), clear defects in cohesin loading were detected (Fig. 2e). Moreover, we found that immunodepletion of Cdt1 or ORC from HSS, which can be subjected to more stringent immunodepletion conditions, completely blocked cohesin loading after NPE addition (data not shown). Finally, we examined Cdc6 because this protein was shown to be dispensable for cohesin loading in budding yeast[2]. Immunodepletion of Cdc6 from LSS was incomplete and only reduced DNA replication to about 40%; under these conditions, cohesin loading was unaffected (data not shown). However, Cdc6 depletion from HSS totally

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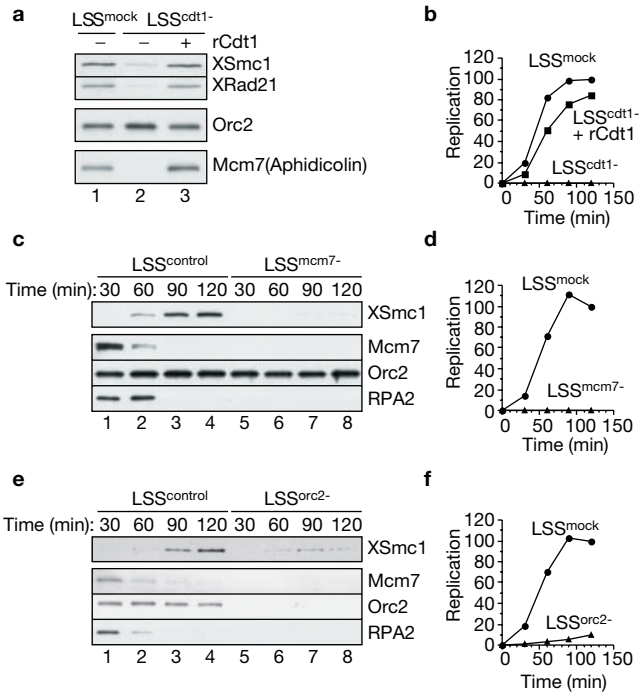

Figure 2 x-cohesin loading is dependent on pre-RC formation. (**a**) Cdt1-depletion inhibits x-cohesin loading. Sperm chromatin was isolated after 120 min incubation in LSS that was mock-depleted, Cdt1-depleted or Cdt1-depleted and supplemented with 4 ng μl^{-1} recombinant XCdt1. To examine Mcm7 loading, the same extracts were supplemented with aphidicolin and chromatin was isolated after 60 min. (**b**) Replication efficiency of the reactions described in **a** (units are arbitrary, here and throughout). (**c**) MCM2-7 is required for x-cohesin loading. Sperm chromatin was isolated after incubation in mock-depleted or Mcm7-depleted LSS. (**d**) Replication efficiency of the reactions described in **c**. (**e**) ORC is required for x-cohesin loading. Sperm chromatin was isolated after incubation in mock-depleted or ORC-depleted LSS. (**f**) Replication efficiency of the reaction described in **e**.

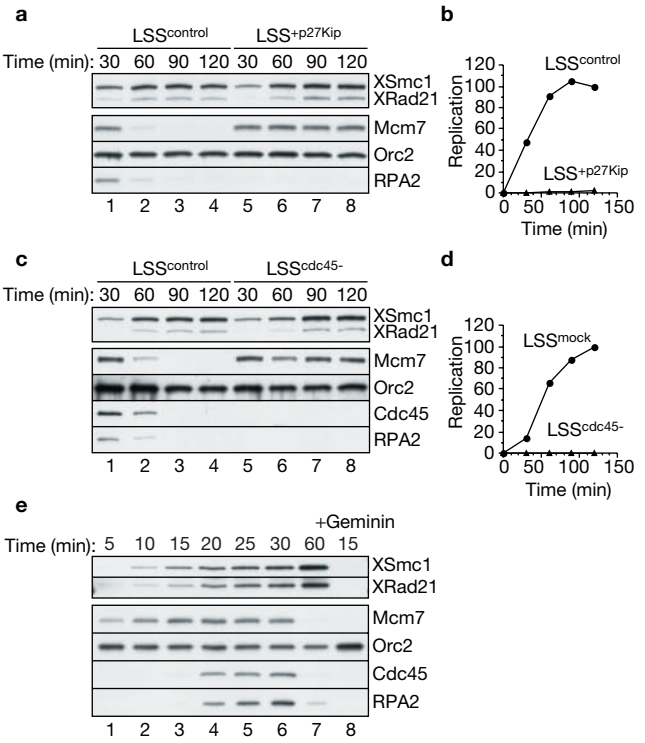

Figure 3 x-cohesin loading is independent of Cdk2 and origin unwinding. (**a**) x-cohesin loading is independent of Cdk2 activity. Sperm chromatin was isolated after incubation in LSS containing or lacking 50 $\mu g\ ml^{-1}$ $p27^{Kip}$. (**b**) Replication efficiency of the reactions described in **a**. (**c**) Cdc45 is not required for x-cohesin loading. Sperm chromatin was isolated after incubation in mock-depleted or Cdc45-depleted LSS. (**d**) Replication efficiency of the reactions described in **c**. (**e**) x-cohesin loading time course in LSS. Sperm chromatin was isolated after incubation in LSS in the presence or absence of geminin.

abolished DNA replication after addition of NPE (see Supplementary Information, Fig. S1b). In the absence of Cdc6, both the low level of cohesin loading seen in HSS, as well as the higher level induced by NPE addition were severely reduced, and the defect was fully rescued with recombinant Cdc6 (see Supplementary Information, Fig. S1a). Taken together, the results demonstrate that a fully formed pre-RC is essential for the association of x-cohesin with chromatin.

The dependence of cohesin loading on pre-RC formation and nuclear transport, as well as its stimulation by NPE, prompted us to examine whether cohesin loading requires replication initiation. As cohesin loading is insensitive to aphidicolin and therefore does not require DNA synthesis (Fig. 1)[18], we focused on an earlier initiation step: the Cdk2- and Cdc45-dependent origin unwinding. The addition of $p27^{Kip}$, a specific inhibitor of Cdk2, inhibited DNA replication (Fig. 3b) and RPA chromatin association (Fig. 3a), but it had no effect on cohesin recruitment in LSS (Fig. 3a). To independently confirm that origin unwinding is not required for cohesin loading, we removed Cdc45. Depletion of Cdc45 from LSS completely inhibited DNA replication and RPA loading; however, cohesin loaded normally (Fig. 3c, d). Therefore, Cdk2, Cdc45 and origin unwinding are not required for chromatin association of x-cohesin. Consistent with it being pre-RC-dependent and initiation-independent, the chromatin binding of x-cohesin was detected after MCM2-7 but before Cdc45 and RPA (Fig. 3e). The continual increase in x-cohesin loading over time in LSS is most probably due to nuclear transport, because x-cohesin binding reaches a maximum only 5 min after NPE addition in the nucleus-free system (data not shown).

In budding and fission yeast, cohesin loading requires Scc2/Mis4, a HEAT-repeat-containing protein[8–10]. It was recently shown that *Scc2/NIPBL* encodes a 300K protein that is implicated in Cornelia de Lange syndrome[22,23]. We identified a human sequence in the database with homology to yeast Scc2 and used this sequence to identify expressed sequence tags (ESTs) derived from the *Xenopus* gene. Using these EST sequences, we cloned a C-terminal 60K fragment of *Xenopus* Scc2 (XScc2). Within this fragment, the predicted amino-acid sequences encoded by the *Xenopus* and human genes are 83% identical (93% similar). In budding yeast, Scc2 forms a complex with Scc4 (ref. 10), but we were unable to find a vertebrate orthologue of Scc4 in the database. The XScc2 fragment was expressed in bacteria and used to immunize two rabbits. Both antisera, but not the corresponding pre-immune sera, readily detected about 0.2 ng of the antigen (see Supplementary Information, Fig. S2a), and both sera detected the same roughly 300K protein in NPE (see Supplementary Information, Fig. S2b). XScc2 was much more abundant in NPE than in LSS, showing that the protein localizes to the nucleus in *Xenopus* egg extracts (see Supplementary Information, Fig. S2c). When we used XScc2 antibodies to immunodeplete LSS, we repeatedly saw a modest, roughly two-to-threefold reduction in cohesin loading compared to mock-depletion using pre-immune serum (data not shown). However, because XScc2 is difficult to detect in LSS (see Supplementary

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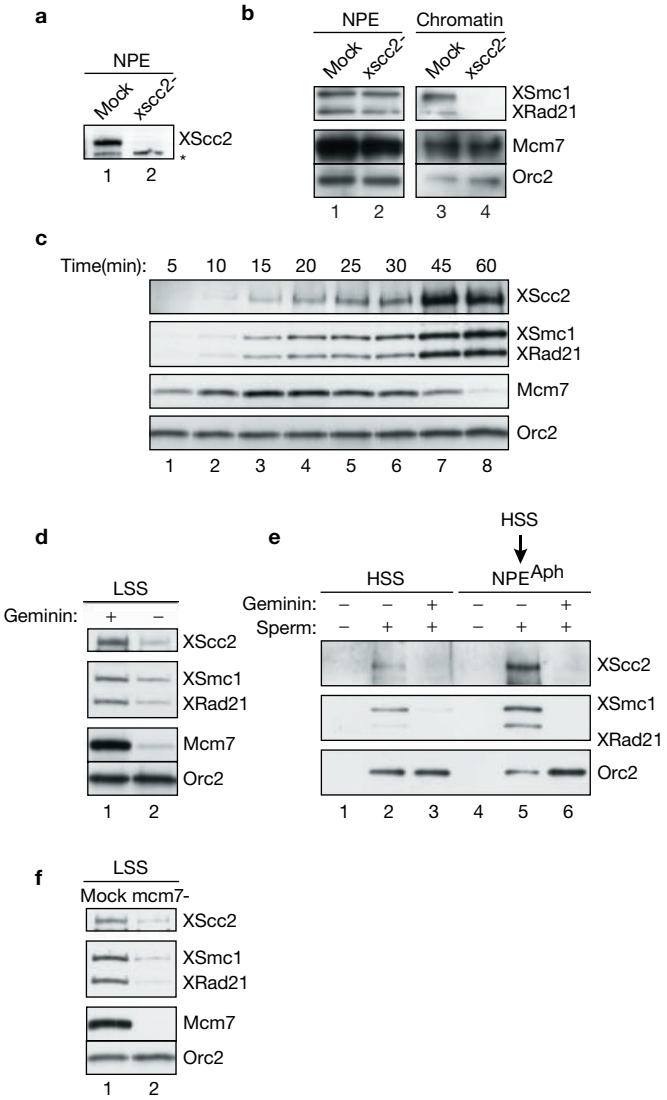

Figure 4 XScc2, which is required for cohesin loading, binds to chromatin in a pre-RC-dependent manner. (**a**) 0.2 μl of mock-depleted or XScc2-depleted NPE was blotted with anti-XScc2 antibody. Asterisks, background band used as a loading control. (**b**) Sperm chromatin was incubated sequentially with HSS and NPE that were both either depleted with pre-immune serum (lane 3) or anti-XScc2-serum (lane 4). After 15 min in NPE, the chromatin was isolated. 0.2 μl of the mock-depleted (lane 1) or XScc2-depleted (lane 2) extract was also blotted. (**c**) Sperm chromatin was incubated in LSS and isolated at various times. (**d**) Sperm chromatin was isolated after 45 min incubation in LSS in the presence or absence of geminin. (**e**) Sperm chromatin was incubated in HSS in the presence or absence of geminin or sperm chromatin and isolated after 30 min (lanes 1–3), or 15 min after the further addition of NPE containing 50 μg ml^{-1} aphidicolin (lanes 4–6). (**f**) Sperm chromatin was incubated in mock-depleted or Mcm7-depleted LSS and isolated after 90 min. To detect MCM2-7 before it was displaced from chromatin, MCM7 was examined on chromatin 30 min after sperm addition to extract.

Information, Fig. S2), the extent of immunodepletion was difficult to verify. Therefore, the experiment was repeated in NPE, where the higher concentration of XScc2 allowed for verification that the protein had been efficiently depleted (Fig. 4a). When XScc2 was immunodepleted from HSS and NPE, cohesin loading was eliminated compared to mock-depleted extract, whereas the loading of other proteins such as ORC2 and MCM7 was normal (Fig. 4b; lanes 3 and 4). The cohesin-loading defect did not result from co-depletion of cohesin with XScc2 (Fig. 4b; lanes 1 and 2). The greatly increased efficiency of cohesin loading in NPE (Fig. 1d) and after nuclear transport (Fig. 1f) is most probably due to the fact that XScc2 and cohesin themselves are highly enriched in nuclei (see Supplementary Information, Fig. S2c; and data not shown). The data confirm experiments in yeast that showed that Scc2 is required for the loading of cohesin onto chromosomes[8–10].

We next examined whether XScc2 binds to chromatin in *Xenopus* egg extracts, and if so, whether the binding is pre-RC dependent. XScc2 bound to chromatin in LSS with similar kinetics to cohesin (Fig. 4c). XScc2 binding to chromatin was reduced in the presence of geminin (Fig. 4d), indicating that it was Cdt1-dependent. In the nucleus-free system, low levels of XScc2 were loaded onto chromatin in HSS, and loading increased after addition of NPE (Fig. 4e), as seen for cohesin (Fig. 1d). In both cases, addition of geminin blocked XScc2 and cohesin loading (Fig. 4e). Depletion of MCM2-7 from LSS inhibited XScc2 loading, demonstrating that this process requires fully assembled pre-RCs (Fig. 4f). Interestingly, like cohesin, XScc2 persisted on chromatin after the vast majority of MCM2-7 had dissociated (Fig. 4c; lane 8), suggesting that only the initial recruitment of XScc2 requires pre-RCs. As seen for cohesin, XScc2 loading was unaffected by the addition of aphidicolin or $p27^{Kip}$ (data not shown). Therefore, XScc2 binds to chromatin by a pre-RC-dependent, but initiation-independent mechanism. XScc2 was not detected on our 2D gels probably owing to its large size (Fig. 1b).

In summary, our results show that the efficient recruitment of x-cohesin onto chromatin requires pre-RCs, but that it is independent of replication initiation. In LSS, cohesins bind to chromatin on average about once every 20 kb, and immunodepletion of cohesins causes a loss of sister chromatin cohesion along chromosome arms[3]. Therefore, because of the large reduction in cohesin loading seen when pre-RCs are not formed, we suggest that this pathway of cohesin recruitment is critical to establish cohesion. We further show that the chromatin loading of cohesin requires XScc2, and that XScc2 also binds to chromatin in a pre-RC dependent manner. Therefore, a plausible mechanism for cohesin loading in *Xenopus* egg extracts is that XScc2 is recruited to the chromatin through the MCM2-7 complex, and perhaps other pre-RC components, forming a loading site for cohesin. We have not detected complexes between cohesin or XScc2 and pre-RC components in egg extracts in the absence of chromatin, so the biochemical mechanism of pre-RC dependent cohesin loading is uncertain. Whatever the precise mechanism, our results represent the first connection between the events underlying DNA replication and the recruitment of cohesins to chromosomes. We propose that the pre-RC dependence of cohesin loading ensures that cohesin-binding sites are established early in the cell cycle, and that they are regularly spaced along the chromatin, as are pre-RCs.

In tissue culture cells, cohesin loads onto chromatin in telophase[3,11,12], as does the MCM2-7 complex[24], suggesting that cohesin loading in this system might also be pre-RC dependent. In budding yeast, cohesins load onto chromatin in mid-G1-phase, but loading is Cdc6-, and therefore presumably pre-RC independent[2]. In contrast, cohesin loading in *Xenopus* egg extracts is Cdc6-dependent (Fig. S1). Importantly, Scc2 is required in both systems. Thus, if Scc2 is the molecular machine that leads to engagement of cohesin with chromatin, the fundamental mechanism of cohesin loading is highly conserved among eukaryotes. What differs is the pathway by which this machine is localized to chromosomes.

Note added in proof: Geminin-sensitivity of XScc2 and x-cohesin-chromatin binding was recently also reported by Gillespie et al. *(Gillespie P. J. and Hirano, T.* Curr. Biol. ***14,*** *1598–1603; 2004).* □

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METHODS

Immunological methods. To deplete LSS, 0.2 volumes of antibody-bound protein A-sepharose (Pharmacia, Sweden) was incubated with extract for 2 h at 4 °C and the procedure repeated. Depletion of HSS and NPE were performed as described[14].

DNA replication and chromatin-binding assays. Extract preparation, replication assays and chromatin binding assays in the nucleus-free replication system were performed as described[14,25]. Sperm nuclei were incubated in HSS at 10,000 nuclei μl^{-1}. Preparation of LSS was performed as described[26]. For chromatin binding assays in LSS, sperm nuclei were incubated in LSS at 3,000 nuclei μl^{-1}. Aliquots (5 µl) were mixed with 60 µl of cold egg lysis buffer (ELB; 10 mM HEPES–KOH at pH 7.7, 50 mM KCl, 2.5 mM $MgCl_2$ and 250 mM Sucrose), layered over 180 µl of ELB containing 500 mM sucrose in 5 × 44 mm microcentrifuge tubes (Beckman, Fullerton, CA) and centrifuged at 16,000*g* for 25 s at 4 °C in a horizontal microcentrifuge. The supernatant was aspirated, the chromatin pellet resuspended in 150 µl of ELB containing 0.6% Triton-X100 and the resuspended chromatin centrifuged through an identical sucrose cushion for 1 min. The chromatin pellet was resuspended in SDS sample buffer. To measure DNA synthesis, extracts were supplemented with 3,700 Bq μl^{-1} of ^{32}P-dATP and the incorporation of radioactivity into DNA was quantified in a phosphoimager (Molecular Dynamics, Sweden) after purification of DNA by agarose gel electrophoresis. In all experiments, the amount of DNA synthesis was calculated using the model described[27], and endogenous dNTP concentration was measured for each extract. In all cases where full DNA replication was expected, at least 80% of the input DNA was replicated.

Protein 2D gel electrophoresis and mass spectrometry. Protein 2D gel electrophoresis was performed as described[28]. Sperm chromatin was incubated in 10 µl of HSS (final concentration of 10,000 sperm μl^{-1}), and then supplemented with 20 µl of NPE containing 50 µg ml^{-1} aphidicolin and isolated. Chromatin bound proteins were eluted with 100 µl of Elution buffer (2% SDS and 20 mM Tris-HCl at pH 8.0) at 70 °C for 10 min, precipitated with four volumes of acetone and resuspended in 50 µl of 2D sample buffer (8 M Urea, 1.7% NP-40, 1.7% 3/10-ampholytes (BioRad) and 20 mM dithiothreitol). The sample was separated in a 120-mm × 1-mm diameter non-equilibrium pH-gradient tube gel[28] at 800 V for 4 h, followed by standard 160-mm × 160-mm × 1-mm SDS–PAGE electrophoresis. The gel was fixed with 50% methanol and stained with silver nitrate. Gel bands were analysed by nano-scale microcapillary liquid chromatography tandem mass spectrometry (LC-MS/MS) as described[29]. Database searches for peptide identifications were performed against the EST and protein databases from *Xenopus*.

Cloning of XScc2 fragment, protein expression and antibody preparation. A BLAST search using yeast Scc2 identified two human Scc2 orthologues, IDN3 and IDN3B, which differ at their extreme C termini. A BLAST search using IDN3B identified two *Xenopus* ESTs (TC168217 and TC159198), which correspond to the C-terminal region of human IDN3 and IDN3B. On the basis of these ESTs, we designed two primers (forward primer, 5′-GGGGACAAGTTTGTACAAAAAAGCAGGCTTAACCGTCGGAGCACTGTGTCGC-3′; reverse primer, 5′-GGGGACCACTTTGTACAAGAAAGCTGGGTCCTACCCTTTCCCTGATGTGCCCCCTGC-3′), and used them to amplify a 1.6-kb fragment from a *Xenopus* cDNA library that encoded a 537 amino-acid portion of the gene that is in common between IDN3 and IDN3B, and cloned it into pDONR201 vector (Life Science, Carlsbad, CA) using the Gateway system (Life Science). Sequencing of the cloned fragment confirmed that it shows 83% identity (93% similarity) to the corresponding fragment of human IDN3B. To express the *Xenopus* Scc2 fragment in *Escherichia coli*, the *Scc2* fragment was transferred into pDEST17 (Life Science) using the Gateway system, and the resulting plasmid was used for transformation of BL21(DE3). A 60K protein was specifically expressed after induction, and this protein was purified and used for immunization.

Antibodies. The following antibodies were used in this study: anti-cohesin antibodies were purchased from Bethyl laboratories (Montgomery, TX); anti-Smc1, Smc3, Rad21, SA-1 and SA-2 were used for immunoblotting at 1:2,000, 1:2,000, 1:2,000, 1:500 and 1:500 dilutions, respectively; and anti-Orc2 (ref. 30), anti-Mcm7 (ref. 25), anti-Cdc45 (ref. 25) and anti-RPA[25] antibodies were all used at 1:5,000 dilutions. The Expression and purification of XCdt1 will be described elsewhere (E. Arias and J.C.W., unpublished observations).

Accession number. The GenBank accession number for the sequence of *Xenopus* Scc2 reported in this paper is AY731711.

Note: Supplementary Information is available on the Nature Cell Biology website.

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COMPETING FINANCIAL INTERESTS

The authors declare that they have no competing financial interests.

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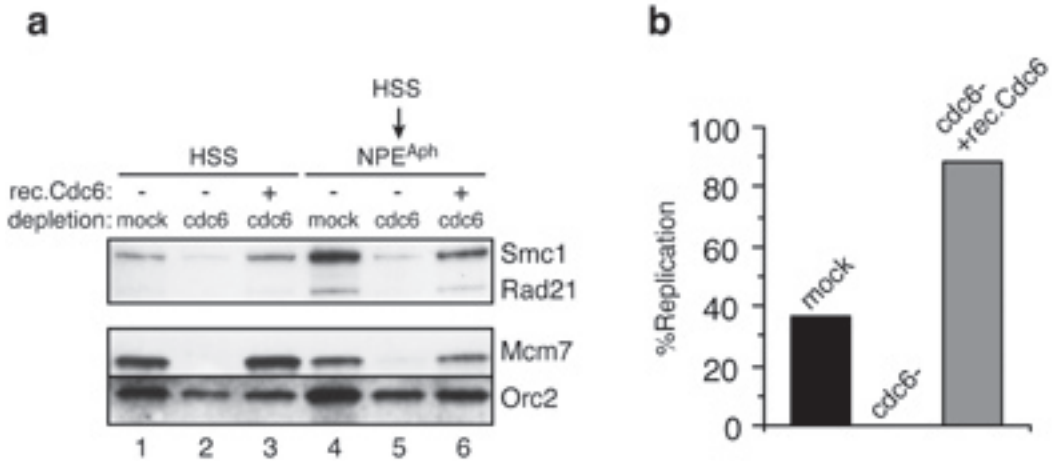

Figure S1 Cdc6 is required for cohesin loading. **a,** Sperm chromatin was incubated sequentially with HSS and NPE (containing aphidicolin) that were both either depleted with non-immune serum (lanes 1 and 4) or anti-Cdc6-serum (lanes 2, 3, 5, 6). In lanes 3 and 6, HSS was supplemented with 50 ng/µl recombinant his-Cdc6 [1]. After 15 minutes in NPE, the chromatin was isolated and immunoblotted. **b,** Replication efficiency of the reactions described in S1a, lanes 4-6 (lacking aphidicolin).

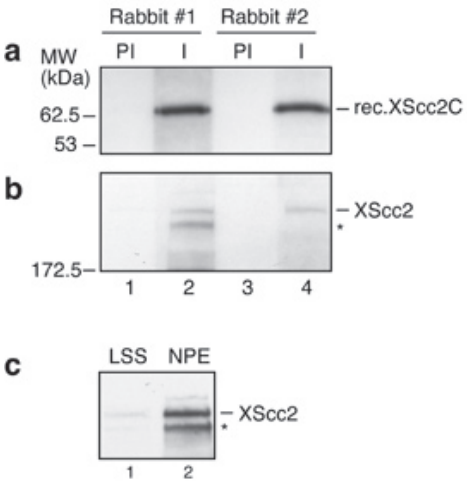

Figure S2 Characterization of XScc2 antibodies **a-b**, 0.2 ng of recombinant XScc2 fragment (a) or 1 µl NPE (b) was immunoblotted with a 1:2000 dilution of pre-immune (lanes 1 and 3) and immune serum (lanes 2 and 4) from two rabbits. * indicates a background band. **c,** 1 µl LSS (lane 1) or NPE (lane 2) was blotted with immune serum of rabbit #1. * indicates a background band.

SUPPLEMENTARY REFERENCE

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