associated Smc3 was immunopurified and blotted for acetylated lysine or total Smc3. Wildtype (WT) and non-acetylatable (AA) Smc3 variants were included as controls. Soluble and chromatin fractions from S-phase cells were analyzed by immunoblotting. 

Fork velocities were determined in cells from normal donors and RBS patients, and in cells expressing Smc3\textsuperscript{WT} or Smc3\textsuperscript{AA} from a doxycycline-inducible promoter.

**Figure 4. Processive fork movement requires acetylation-mediated dissociation of Pds5a and Wapl.**

\textbf{a}, Smc3 variants were immunopurified and examined for Wapl and Pds5a binding. \textbf{b}, Cells were synchronized by serum withdrawal (G1 phase) or thymidine block and release (S and G2). Acetylation of chromatin-bound Smc3 (top) and association with Wapl and Pds5a (bottom) were determined as in Fig. 3c and 4a. 

\textbf{c and d}, Wapl depletion rescues fork progression in Smc3\textsuperscript{AA}-expressing cells. \textbf{e}, Downregulating Wapl or Pds5a reverses fork slowing in Esco1- and Esco2-depleted cells. \textbf{f and g}, Pds5a depletion rescues fork progression in the absence of RFC\textsuperscript{Ctf18}.

**Online Methods**

**Gene targeting and RNA interference.** Sequences surrounding exon 2 of human DCC1 were amplified from BAC clone RP11-188E9 with PfuTurbo (Stratagene) and cloned either side of a central FRT-neo\textsuperscript{R}-FRT-loxP cassette. A second loxP site was introduced upstream of exon 2, and the entire insert was then moved into pAAV as a Not I fragment. The final construct was fully sequenced to verify its integrity. Preparation of infectious AAV particles, gene-targeting procedures, and Southern blotting were performed as described\textsuperscript{31,32}. Excision of the FRT-flanked neo\textsuperscript{R} cassette was accomplished by
transfection with pCAGGS-FLPe (Open Biosystems), followed by limiting dilution to recover G418-sensitive colonies. During the second round of gene targeting, we identified polymorphisms within the 5’ and 3’ homology arms that biased recombination towards the already targeted allele. A vector isogenic to untargeted allele was constructed and used to delete exon 2. Synthetic 21-nt siRNA duplexes (GFP, target GGCTACGTCCAGGAGCGCA; Wapl, target CGGACTACCTTAGCACAA; Esco1, targets GGACAAAGCTACATGATAG (Esco1 #1) and GAGAATAAATTCCAGGTT (Esco1 #2); Esco2, target TAAGTCCACTGTCTATCCA; Scc1, target TGGAAAGATCTCCTAACTAA; Pds5a, target TTCTTCCTCAGGAAACCAtt) were obtained from Dharmacon and transfected using Oligofectamine. Mock RNAi was performed by omitting siRNAs from the transfection mixture.

**Cell culture and drug treatments.** hTERT-RPE1 cells were grown in Dulbecco's modified essential medium (DMEM):F-12 supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. HeLa and HEK293 Flp T-Rex cells were grown in DMEM with 10% FBS and 1% penicillin-streptomycin. FLAG-Smc3 expression was induced with 1 μg/mL doxycycline. Human lymphoblastoid cell lines (GM07524 and GM20466) and primary fetal fibroblasts (GM06112 and GM21872) were grown in RPMI 1640 with 15% FBS and 1% penicillin-streptomycin and DMEM with 15% FBS and 1% penicillin-streptomycin, respectively. Nocodazole (0.2 μg/ml), hydroxyurea (1 mM), adriamycin (0.5 μM), thymidine (2.5 mM), caffeine (5 mM) and aphidicolin (0.3-30 μM) were also used.
**Retroviral transduction.** Epitope-tagged forms of Dcc1 were cloned into pQCXIN (Clontech). A pLXSN clone containing human papillomavirus serotype 16 E6 and E7 was obtained from D. Galloway (Fred Hutchinson Cancer Research Center). Vectors were transfected into Phoenix cells together with a VSV-G envelope expression plasmid. Virion-containing supernatants were supplemented with 10 μg/ml polybrene and applied to target cells for 15 hours. Transductants were selected with 0.4 mg/ml G418.

**Pulse-labeling of replication forks and DNA fiber analysis.** Exponentially growing cells were labeled with 50 μM IdU for 20 minutes, then 50 μM CldU for 20 minutes. Extended DNA fibers were prepared using a modification of the procedure described by Jackson and Pombo\(^\text{30}\). Briefly, labeled cells were trypsinized and resuspended in ice-cold PBS at 1x10\(^6\) cells/ml. 2 μl of this suspension was spotted onto a clean glass slide and lysed with 10 μl of spreading buffer (0.5% SDS in 200 mM Tris-HCl (pH 7.4) and 50 mM EDTA). After 6 minutes, the slides were tilted at 15° to horizontal, allowing the DNA to spread. Slides were air-dried, fixed in methanol and acetic acid (3:1) for 2 minutes, and refrigerated overnight before immunolabeling. DNA was denatured with 2.5 M HCl for 30 minutes at room temperature. Slides were rinsed three times in PBS and blocked in PBS + 0.1% Triton X-100 (PBS-T) + 10% goat serum for 1 hour at room temperature. Rat anti-BrdU (Abcam ab6326, 1:100) and mouse anti-BrdU (Becton Dickinson 347580, 1:100) were then applied to detect CldU and IdU, respectively. After a 1-hour incubation, slides were washed three times in PBS and stained with Alexa Fluor 488-labeled goat anti-mouse IgG1 antibody and Alexa Fluor 594-labeled goat anti-rat antibody (Invitrogen, 1:350 each). Slides were mounted in Prolong Plus (Invitrogen) and
held at 4°C overnight. Replication tracks were imaged on a Nikon TE2000 microscope fitted with a 100X 1.4 NA oil objective and measured using NIS Elements software. Restart assays were conducted exactly as above, except that cells were treated with 10 μM aphidicolin for 2 hours after the IdU pulse. The drug was then washed out and the CldU pulse was administered. Continuity of DNA fibers was verified by staining with an anti-DNA antibody (Millipore MAB3034, 1:300) and an appropriate secondary antibody (Alexa 647-labeled goat anti-mouse IgG2a (Invitrogen), 1:350). Fork-velocity datasets were evaluated for statistical significance using one-way analysis of variance (ANOVA) and Tukey’s post hoc test.

**Population doubling assays and immunofluorescence microscopy.** Cells (15-20% confluent) were infected with AdCre or Adβgal at a multiplicity of infection (MOI) of 200. On day 3 post-infection, cells were counted with a hemocytometer and replated at a 1:16 ratio. This point was defined as passage 0. On day 4, cells were treated with hydroxyurea or aphidicolin for 15 hours (if indicated). On day 6, cells were again harvested and counted. This point was defined as passage 1. Two further rounds of subculturing were used to obtain cells at passages 2 and 3. Senescence-associated β-galactosidase staining was performed as described³³. For immunofluorescence, cells were grown in chamber slides and fixed with 4% paraformaldehyde at room temperature or 100% methanol at -20°C for 20 minutes. After permeabilization in PBS-T for 10 minutes, cells were blocked in PBS-T + 10% goat serum for 30 minutes, incubated with primary and Alexa 488- and 594-conjugated secondary antibodies for 1 to 2 hours each, counterstained with DAPI, and mounted in Prolong Plus (Invitrogen).
**DSP crosslinking, immunoprecipitation, and chromatin fractionation.** To stabilize labile protein complexes, dithiobis(succinimidyl propionate) (DSP) was added to the culture medium at 1 mg/ml for 10 minutes at 37°C. Extracts were prepared by lysing pellets on ice for 30 minutes in HB2 buffer (50 mM HEPES, pH 7.5, 0.5% NP-40, 10% glycerol, 100 mM NaCl, 10 mM Na pyrophosphate, 5 mM β-glycerophosphate, 50 mM NaF, 0.3 mM Na3VO4, 1 mM PMSF, and 1× complete protease inhibitor cocktail), followed by sonication and centrifugation at 13,000 × g for 30 minutes at 4°C. 3 mg of extract was immunoprecipitated with FLAG M2 antibody-agarose beads. Where indicated, a 50-fold excess of FLAG peptide was added as a competitive inhibitor. Beads were washed three times with HB2 prior to elution and reversal of crosslinks with Laemmli buffer.

To analyze acetylation of chromatin-bound Smc3, DSP was omitted, and cells were lysed in NETN buffer (ref. 8) supplemented with 10 mM sodium butyrate. After centrifugation the chromatin pellet was resuspended in the same buffer, sonicated, and recentrifuged. Soluble chromatin was immunoprecipitated with antibodies to Smc3 or FLAG as indicated. For quantification, Western blots were incubated with species-specific secondary antibodies conjugated to IRDye 680 or IR Dye800 and scanned on an Odyssey Infrared Imaging System (Li-Cor Biosciences). Chromatin fractionation in Fig. 3d was performed using the procedure of Mendez and Stillman34.
Antibodies. The following antibodies were used: rabbit α-Dcc1 and α-Ctf18 (gift of J. Hurwitz, MSKCC); rabbit α-Esco1 and α-Esco2 (gift of H. Zou, UT Southwestern); rabbit α-Nbs1 (gift of J. Petrini, MSKCC); rat α-BrdU (Abcam) and mouse α-BrdU (Becton Dickinson); mouse α-FLAG (Sigma); mouse α-Rad17, α-p53 and α-Chk1, rabbit α-RFC1 and α-PCNA (Santa Cruz), goat α-RFC2, rabbit α-RFC3, α-RFC4, α-RFC5, α-Pds5a, α-Scc1, α-Smc3, α-Wapl, and α-APC4 (Bethyl); rabbit α-acetyl-lysine (Abcam); mouse α-topo II (Millipore); rabbit α-pS345-Chk1 (Cell Signaling); rat α-tubulin (Chemicon). A polyclonal antibody to Esco1 was prepared by immunizing rabbits with a recombinant fragment (amino acids 1-454) and affinity purified prior to use.

Insulator assays and microarrays. Insulator assays were performed as described\(^5\). Briefly, HeLa cells were transfected with Scc1, Esco1, Esco2 or control siRNAs and synchronized by a double thymidine block (14 hr thymidine 2.5 mM, 8 hr release, 16 hr thymidine 2.5 mM, 6 hr release). During the release from the first thymidine arrest the firefly luciferase reporter plasmid (pIHLIE or pIHLME) and a Renilla luciferase control plasmid were transfected in a ratio of 1:20 using Lipofectamine Plus (Invitrogen). After harvesting and lysis of the cells, the activities of both luciferases were detected using a Dual-Luciferase Reporter Assay System kit (Promega) and a Glomax 96 microplate luminometer (Promega). To control for transfection efficiency, the firefly luciferase activity was normalized against the Renilla luciferase activity. Each condition was analyzed in triplicate. The reporter plasmids pIHLIE and pIHLME have been described\(^3\).
For expression profiling of endogenous cohesin-regulated genes, total RNA was isolated from Smc3\(^{AA}\) and Smc3\(^{WT}\) cells. Two biological replicates were examined per condition. Quality of RNA was ensured before labeling by analyzing 20–50 ng of each sample using the RNA 6000 NanoAssay and a Bioanalyzer 2100 (Agilent). Samples with a 28S/18S ribosomal peak ratio of 1.8–2.0 were considered suitable for labeling. For samples meeting this standard, 200 ng of total RNA were labeled using the Illumina TotalPrep RNA Amplification kit (Ambion), according to manufacturer’s instructions. 3 µg of labeled and fragmented cRNA were hybridized to HumanRef8 arrays (Illumina). Microarray bioinformatics analyses were conducted with GeneSpring GX v.10.0 software (Agilent Technologies, Santa Clara, CA). A collection of 36 genes proximal to cohesin-binding sites and known to be transcriptionally regulated by cohesin\(^5\) was interrogated. Relative expression of these genes in Smc3\(^{AA}\) and Smc3\(^{WT}\) cells was evaluated using unpaired t-test and Benjamini-Hochhberg FDR correction.

**Online Methods References**

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