Cohesin acetylation and Wapl-Pds5 oppositely regulate the translocation of the cohesin along DNA

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Appendix

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Appendix Supplementary Methods

TEV cleavage assay

Scc2-Scc4 (7.5 nM) and cohesin (7.5 nM) was loaded onto DNA under the same conditions but in the presence of 1 mM $CaCl_2$ instead of $MgCl_2$. After the cohesin loading reaction, the flow cell was washed by TW buffer (Ca) [35 mM Tris-HCl (pH 7.4), 300 mM NaCl, 0.5 mM TCEP, 1 mM $CaCl_2$, 0.25 mM ATP, 0.1% Triton X-100] followed by TC buffer (Ca) [35 mM Tris-HCl (pH 7.4), 100 mM NaCl, 0.5 mM TCEP, 1 mM $CaCl_2$, 0.25 mM ATP, 0.05% Tween20]. The AcTEV™ protease (1.25 U; Invitrogen) in 50 μl of TC buffer (Ca) was introduced into the flow cell and incubated for 45 min, twice (total 1.5 h). After the reaction, the flow cell was washed by TC buffer (Ca) containing 300 mM NaCl, and then blocking buffer [20 mM Tris-HCl (pH 7.4), 100 mM NaCl, 2 mM EDTA, 1 mM ATP] containing TO-PRO®-3 was flowed and cohesin particles were observed.

Purification and labeling of Halo-tagged human and *Xenopus* cohesin tetramer complex

Sf21 cells expressing Halo-tagged human and *Xenopus* cohesin were lysed in Cytobuster™ Protein Extraction Reagent (Novagen) containing 0.5% Tween-20 and cOmplete™ EDTA-free protease inhibitor cocktail (Roche), with gentle rotation at 4°C for 30 min. The cell extract was centrifuged at 15,000 rpm for 10 min, and the supernatant was mixed with anti-FLAG M2 agarose beads (Sigma) and rotated at 4°C for 1.5 ~ 5 h. Then the beads were washed with buffer AT [25 mM Tris-HCl (pH 8.0), 100 mM NaCl, 10% glycerol, 0.5% Tween-20] and incubated in buffer AT containing 0.1 mg/ml 3xFLAG peptide for 2 ~ 5 h at 4°C. The cOmplete His-Tag Purification Resin (Roche) and 1/25 volume of Nickel buffer B [25 mM KPi (pH 7.2), 150 mM KCl, 20 mM β-mercaptoethanol, 10% glycerol, 500 mM imidazole] were added to the elution and incubated at 4°C for 2 h with constant rotation. After washing with buffer AT, bead-bound proteins were labeled by Halo-Tag Alexa Fluor 488 or TMR ligand (Promega) for 30 min at room temperature. Then the beads were

washed with buffer AT and the bead-bound proteins were eluted by Nickel buffer B. The purified cohesin complex was dialyzed against buffer A [25 mM Tris-HCl (pH 8.0), 100 mM NaCl, 10% glycerol] or ELB-glycerol [10 mM HEPES-KOH (pH 7.7), 50 mM KCl, 2.5 mM $MgCl_2$, 10% glycerol].

Purification of Wapl-Pds5 complex

FLAG3-hWapl and Pds5A-His6 were expressed in Sf21 cells, and the FLAG3-hWapl-Pds5-His6 complex was purified as described for the cohesin complex purification (above), except that the complex was eluted from cOmplete His-Tag Purification Resin without fluorescence labeling.

Purification of hScc2/FLAG3-hScc4 from HeLa Cells

HeLa Kyoto cells transfected with pIRESpuro3-FLAG3-hScc4 were collected (50 ml packed volume) and washed twice with ice-cold PBS. The cells were incubated in 100 ml of hypotonic buffer B [20 mM HEPES-NaOH (pH 7.9), 1.5 mM $MgCl_2$, 5 mM KCl, 1 mM DTT, 1 mM PMSF and cOmplete™ EDTA-free protease inhibitor cocktail (Roche)] on ice for 10 min. Then the cells were lysed by Dounce homogenization (20 strokes) and NaCl was added to the cell extract to a final concentration of 0.5 M. The extract was centrifuged at 43,200 *g* for 45 min at 4 °C and the supernatant was recovered. TURBO DNase (1,000 units; Ambion) and Benzonase (2,000 units; Novagen) were added to the supernatant, which was incubated for 4 h at 4°C with gentle mixing, then centrifuged at 43,200 *g* for 1 h at 4°C. Anti-FLAG M2 agarose beads (Sigma) were added to the supernatant and incubated overnight at 4°C with constant rotation. The beads were collected on a Poly-Prep column (Bio-Rad) and washed with FLAG IP buffer [25 mM HEPES-NaOH (pH 7.9), 0.5 M NaCl, 1 mM DTT, 1 mM EDTA, 10% glycerol, 0.05% Nonidet P-40, 1 mM PMSF, and cOmplete™ EDTA-free protease inhibitor cocktail (Roche)]. Then the beads were incubated with FLAG IP buffer containing 1 mg/ml 3xFLAG peptide for 2 h. The elution was diluted 5-fold with Heparin buffer [25 mM Tris-HCl (pH 7.4), 2.5 mM DTT, 1 mM

EDTA, 10% glycerol, 0.1% Nonidet P-40, 1 mM PMSF, and cOmplete™ EDTA-free protease inhibitor cocktail (Roche)] and loaded on a HiTrap Heparin HP Column (GE healthcare). The column was washed with Heparin buffer containing 0.3 M NaCl and the bead-bound hScc2/FLAG3-hScc4 was eluted with Heparin buffer containing 0.6 M NaCl. A total of 900 μl of the elution from the Heparin column was applied onto a 15–40% glycerol gradient [50 mM Tris-HCl (pH 7.4), 0.5 M NaCl, 1 mM DTT, 1 mM EDTA, 0.01% Nonidet P-40, 1 mM PMSF, and cOmplete™ EDTA-free protease inhibitor cocktail] and centrifuged at 43,000 rpm for 18 h at 4°C. Fractions were collected from the bottom of the tube, and analyzed by SDS-PAGE followed by SYPRO-Ruby staining. The purified hScc2/FLAG3-hScc4 complex was dialyzed against buffer A [25 mM Tris-HCl (pH 8.0), 100 mM NaCl, 10% glycerol].

Purification of hEsco1

To purify hEsco1, His6-hEsco1 was expressed in Sf21 cells. The cell pellet was suspended in buffer B [25 mM NaPi (pH 8.0), 150 mM NaCl, 20 mM β-mercaptoethanol, 10% glycerol, 15 mM imidazole] containing 5 mM PMSF, 0.5% Tween20, and cOmplete™ EDTA-free protease inhibitor cocktail (Roche) and lysed by Dounce homogenization (25 strokes). The extract was centrifuged at 45,000 rpm for 30 min at 4 °C and the supernatant was recovered. The cOmplete His-Tag Purification Resin (Roche) was added to the supernatant and incubated at 4°C for 3 h with gentle mixing. After washing with buffer B, the bead-bound proteins were eluted by buffer B containing 50 ~ 500 mM imidazole. The purified hEsco1 was dialyzed against buffer A [25 mM Tris-HCl (pH 8.0), 100 mM NaCl, 10% glycerol].

Purification of xFen1D179A-PAGFP

E.coli cells expressing xFen1D179A-PAGFP were incubated in lysis buffer [20 mM HEPES-NaOH (pH 7.9), 100 mM NaCl, 0.1% Nonidet P-40, 2 mM β-mercaptoethanol, 10% glycerol, 1 mM PMSF, cOmplete™ EDTA-free protease inhibitor cocktail (Roche),

and 1 mg/ml lysozyme] for 1 h at 4°C. Then NaCl was added to a final concentration of 0.5 M and the cells were sonicated. The cell extract was centrifuged at 28,000 rpm for 30 min at 4 °C and the supernatant was recovered. Imidazole was added to a final concentration of 7.5 mM and the cell extract was applied to a HisTrap HP column (GE healthcare) equilibrated in elution buffer [20 mM HEPES-NaOH (pH 8.0), 500 mM NaCl, 0.1% Nonidet P-40, 2 mM β-mercaptoethanol, 7.5 mM imidazole, 10% glycerol]. The bead-bound proteins were eluted by elution buffer containing 100, 200 and 500 mM imidazole and the purified xFen1D179A-PAGFP was dialyzed against ELB-glycerol containing 300 mM KCl [10 mM HEPES-KOH (pH 7.7), 300 mM KCl, 2.5 mM $MgCl_2$, 10% glycerol].

Appendix Supplementary References

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Appendix Figure Legends

Appendix Figure S1. Characterization of cohesin translocation (related to Fig 1).

(A) λ DNAs tethered at both ends were observed under TIRFM. DNA was stained with SYTOX. Scale bar, 10 μm.

(B) Histogram of EMCCD count distribution of human cohesin tetramer-Alexa 488 particles on DNA. Magenta arrowhead indicates a peak corresponding to single particle intensity.

(C) EMCCD count of a cohesin particle corresponding to single molecule was monitored during photo-bleach. It was bleached in one-step.

(D) TEV cleavage assay of Alexa 488-labeled cohesin complex harboring TEV-cleavable Scc1-Halo. The asterisk indicates non-specific signals.

(E) Cohesin^{Halo488} was introduced into flow cell and incubated with DNA in the presence of 50 or 100 mM NaCl. No cohesin was associated with DNA in the presence of 100 mM NaCl. DNA was counterstained with SYTOX. Scale bar, 5 μm.

(F) Kymograph of TetR-GFP signals on DNA. 22 kb of DNA possessing a *tetO* sequence at each end (indicated as triangles) were tethered onto coverslips and incubated with TetR-GFP. TetR-GFP signals did not undergo any displacement from the place where they initially bound at least for 20 sec.

(G) Cohesin^{Halo488} was topologically loaded in the presence of ATP and Scc2-Scc4. After high-salt washing, the movement of DNA-bound cohesin was observed. Note that particles which fluorescence intensities were around 2000 (A.U.) corresponding to single molecules exhibited higher diffusion coefficient. Whereas, aggregated cohesins which intensities were more than 4000 (A.U.) were immobile.

(H) Cohesin^{Halo488} was topologically loaded in the presence of ATP and Scc2-Scc4. After high-salt washing, DNA-bound cohesin was treated with the SB buffer containing 400 mM KCl and ATP or AMP-PMP. Cohesin intensities were measured after high-salt washing (wash) and SB buffer 400 mM KCl washing (400 mM), and intensities after high-salt

washing were normalized to 1 (n = 100, mean ± s.e.m.).

Appendix Figure S2. Wapl-Pds5 and Sororin affect translocation activity of cohesin (related to Fig 2).

(A) SYPRO-Ruby stain for Wapl-Pds5 heterodimer purified from insect cells.

(B) Wapl-Pds5 is colocalized with cohesin on DNA. Cohesin^{Halo488} was topologically loaded and Wapl-Pds5 was introduced after high-salt washing. Wapl was detected by immunofluorescence staining for anti-Wapl antibodies. Line scan was performed by NIS Elements software (Nikon). Scale bar, 5 μm.

(C) Cohesin^{Halo488} was loaded onto DNA in the presence of Scc2-Scc4, followed by further incubation with buffer or Wapl-Pds5. After high-salt washing, cohesin signals on DNA were observed. DNA was counterstained with SYTOX Orange. Scale bar, 10 μm.

(D) Topologically loaded cohesin^{Halo488} was incubated with Wapl-Pds5. Cohesin particles were observed in the presence of 100 mM KCl and ATP or AMP-PCP. MSD versus time is shown. D indicates the diffusion coefficient (n = 45, mean ± s.e.m.).

(E) CBB staining of His-tagged human Sororin purified from *E.coli*.

(F) Topologically loaded cohesin^{Halo488} was sequentially incubated with Wapl-Pds5 and Sororin. Sororin was detected by immunostaining. Scale bar, 2 μm.

(G) Topologically loaded cohesin^{Halo488} was incubated with Wapl-Pds5, or Wapl-Pds5 plus Sororin, washed in high-salt buffer, and cohesin intensities were measured. As a control, Wapl-Pds5-treated cohesin without high-salt washing is shown. Red bars denote the median, lower, and upper quartile values (n = 100, $*p < 0.0001$, two-tailed Mann-Whitney test).

Appendix Figure S3. Effects of mitotic kinases on cohesin translocation activity (related to Fig 3).

(A) The cohesin tetramer was incubated with Plk1 in the presence of [γ−32P] ATP, separated by SDS-PAGE, and incorporation of radiolabelled phosphate assessed by autoradiography (autorad.). The same sample was immunoblotted for SA1.

(B) Cohesin tetramer was treated by Plk1 and the phosphorylation sites were analyzed by mass spectrometry. The phosphorylated peptides detected in SA1 are shown.

(C) Cohesin^{Halo488} was topologically loaded onto DNA, sequentially incubated with Wapl-Pds5 and Sororin, and treated by kinase buffer (control), Plk1, Aurora B, or CDK1. After the kinase reaction, cohesin was treated in SB buffer containing increasing concentration of KCl and cohesin intensities were measured. The intensities measured before kinase treatment were normalized to 1 (n = 100, mean ± s.e.m.).

(D) Topologically loaded cohesin^{Halo488} was incubated as in (C). Wapl was detected by immunostaining. Scale bar, 10 μm.

(E) Aurora B phosphorylates Sororin *in vitro*. 1.5 μg of Sororin or 2 μg of Histone H3 was incubated with 150 ng (1x) or 300 ng (2x) of Aurora B at 25 ºC for 30 min in the presence of [γ−32P] ATP. Resulting reaction mixtures were separated by SDS-PAGE and stained with CBB (left). Incorporation of radiolabelled phosphate assessed by autoradiography (right).

(F) Plk1 suppresses cohesin translocation in the absence of Wapl-Pds5 and Sororin. Cohesin was loaded on DNA in the presence of Scc2-Scc4 and washed in high-salt buffer. The DNA-bound cohesin was further incubated with Plk1 in the presence or absence of BI4834. Cohesin^{Halo488} particles were observed in the presence of ATP and 100 mM KCl. MSD versus time is shown. D indicates the diffusion coefficient (n = 45, mean ± s.e.m.).

Appendix Figure S4. Human Cohesin^{Halo488} is loaded onto chromatin in *Xenopus* egg extracts (related to Fig 4).

(A and B) Alexa 488-labeled wild-type (WT) or KA mutant (KA) of human cohesin was incubated with HSS in the presence or absence of Scc2-Scc4, and introduced into the flow cell to bind to DNA. Cohesin loading efficiency (number of particles/μm DNA) is shown in (A) ($n \geq 3$; $*p < 0.01$, two-tailed Mann-Whitney test) and the normalized distance between two particles was calculated (B).

(C) Tethered DNA was treated with HSS and immunostaining of histone H2A.X-F (green)

and H3 (magenta) was performed. Two representative regions are shown. H2A.X-F and H3 was frequently colocalized (Pearson correlation coefficient; PCC > 0.8). Scale bar, 2 μm.

Appendix Figure S5. XEco2 acetylates *Xenopus* cohesin^{HaloTMR} in *Xenopus* interphase egg extracts (related to Fig 5).

(A) CBB staining of purified TMR-labeled Halo-tagged *Xenopus* cohesin complex (xCohesin^{HaloTMR}).

(B) Tethered DNAs were incubated in xCohesin^{HaloTMR}-containing HSS in the presence of ATP for 30 min to allow cohesin loading on DNA. HSS was replaced to the fresh HSS containing ATP or AMP-PNP and incubated for another 20 min. After washing out the HSS, acetylated Smc3 were detected by immunostaining and the number of acetylated cohesin or non-acetylated cohesins were counted (n = 3, mean ± s.d.).

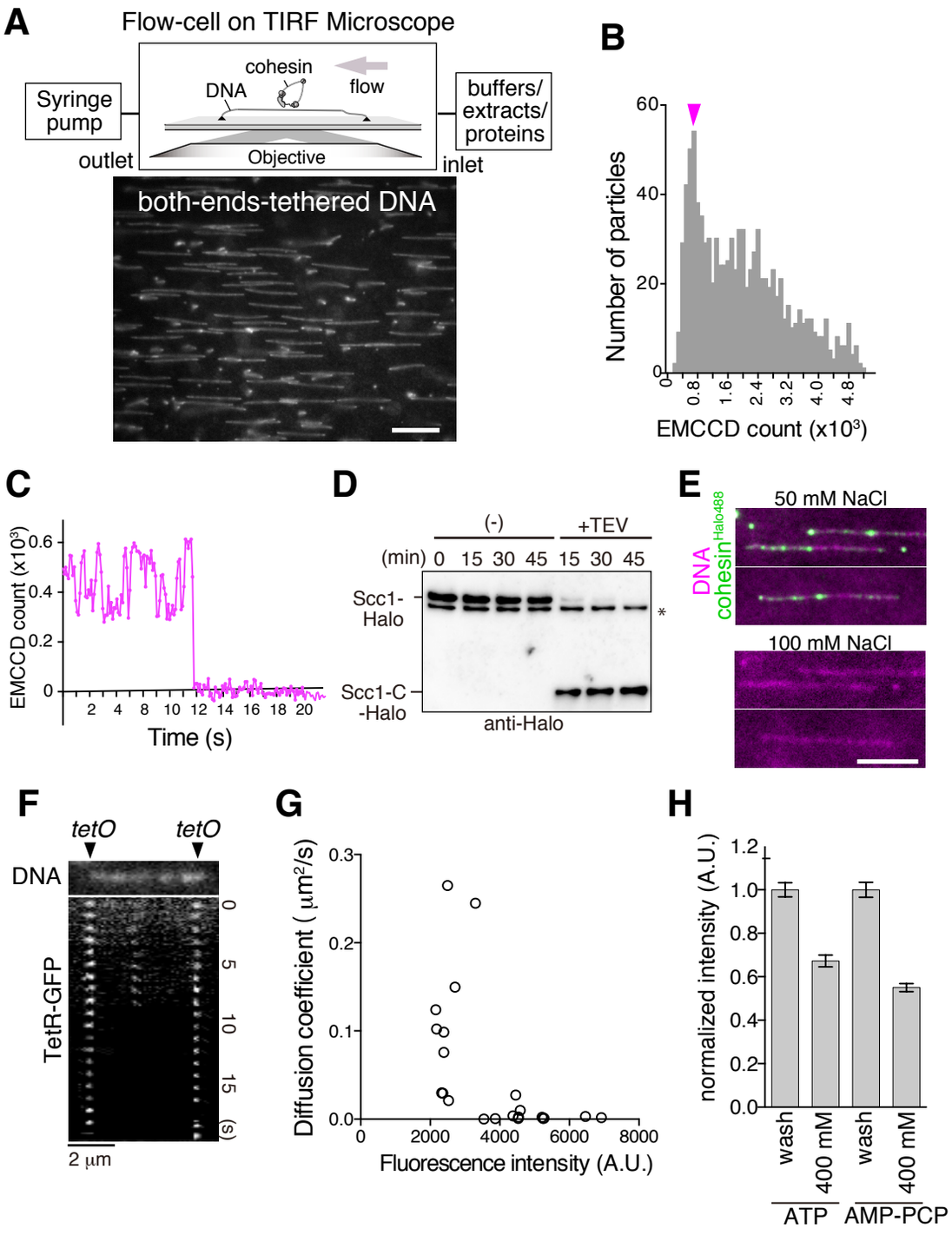
(C) Tethered DNAs were incubated in xCohesin^{HaloTMR}-containing HSS for 30 min to allow cohesin loading on DNA. After washing out the HSS, Wapl and acetylated Smc3 (Smc3-ac) were detected by immunostaining. Scale bar, 2 μm.

(D) CBB staining of His-tagged human Esco1 purified from insect cells.

Appendix Figure S6. Cohesin is incorporated in replicating DNA (related to Fig 6).

(A) CBB staining of His-tagged *Xenopus* Fen1^{D179A}-PAGFP purified from *E.coli*.

(B) Tethered DNAs were replicated in NPE in the presence of DIG-dUTP. After replication, Smc3 and DIG were detected by immunostaining. DIG staining indicates replicated region. Note that cohesin is localized on both replicated and unreplicated DNA. DNA was counterstained with SYTOX. Scale bar, 10 μm.



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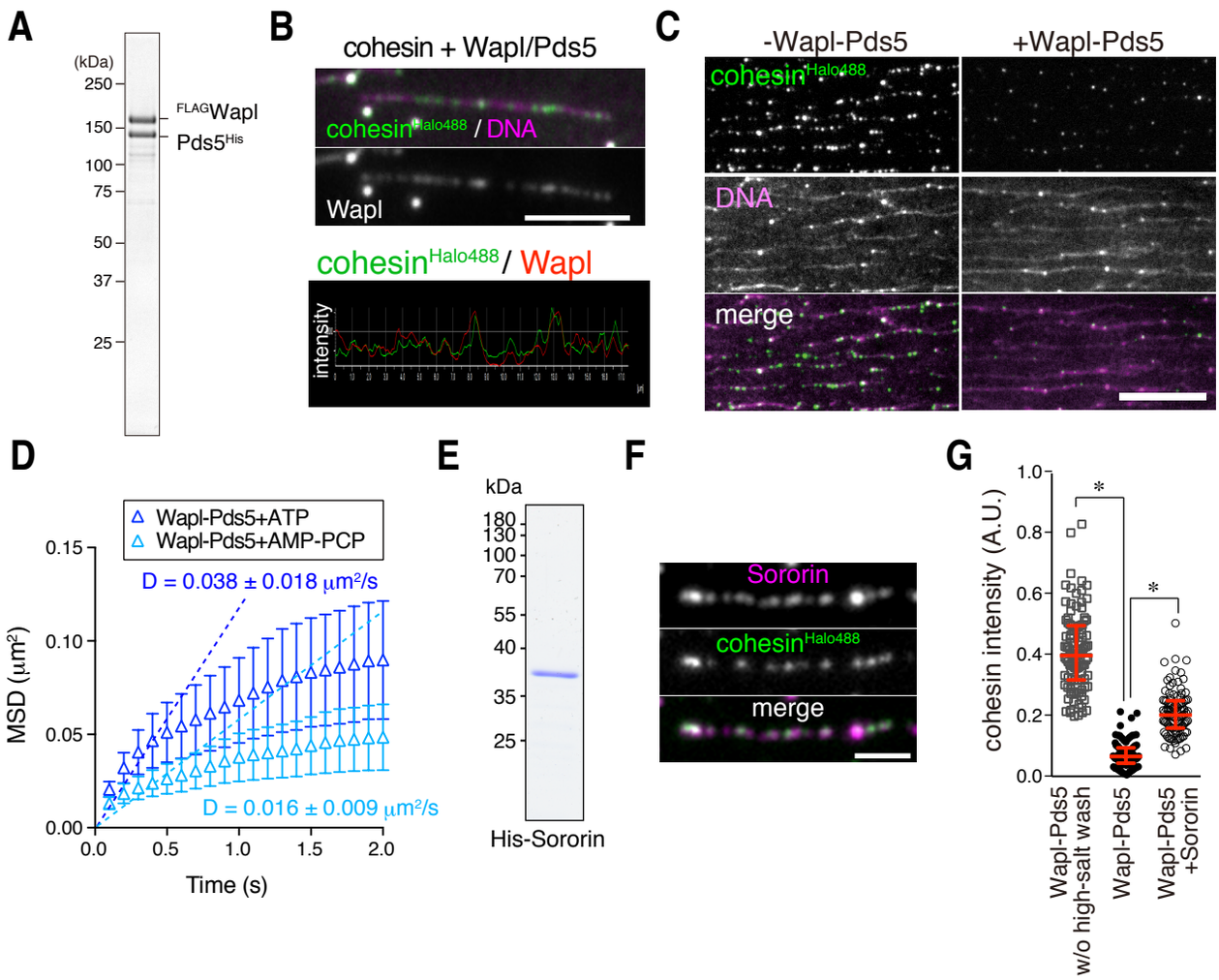
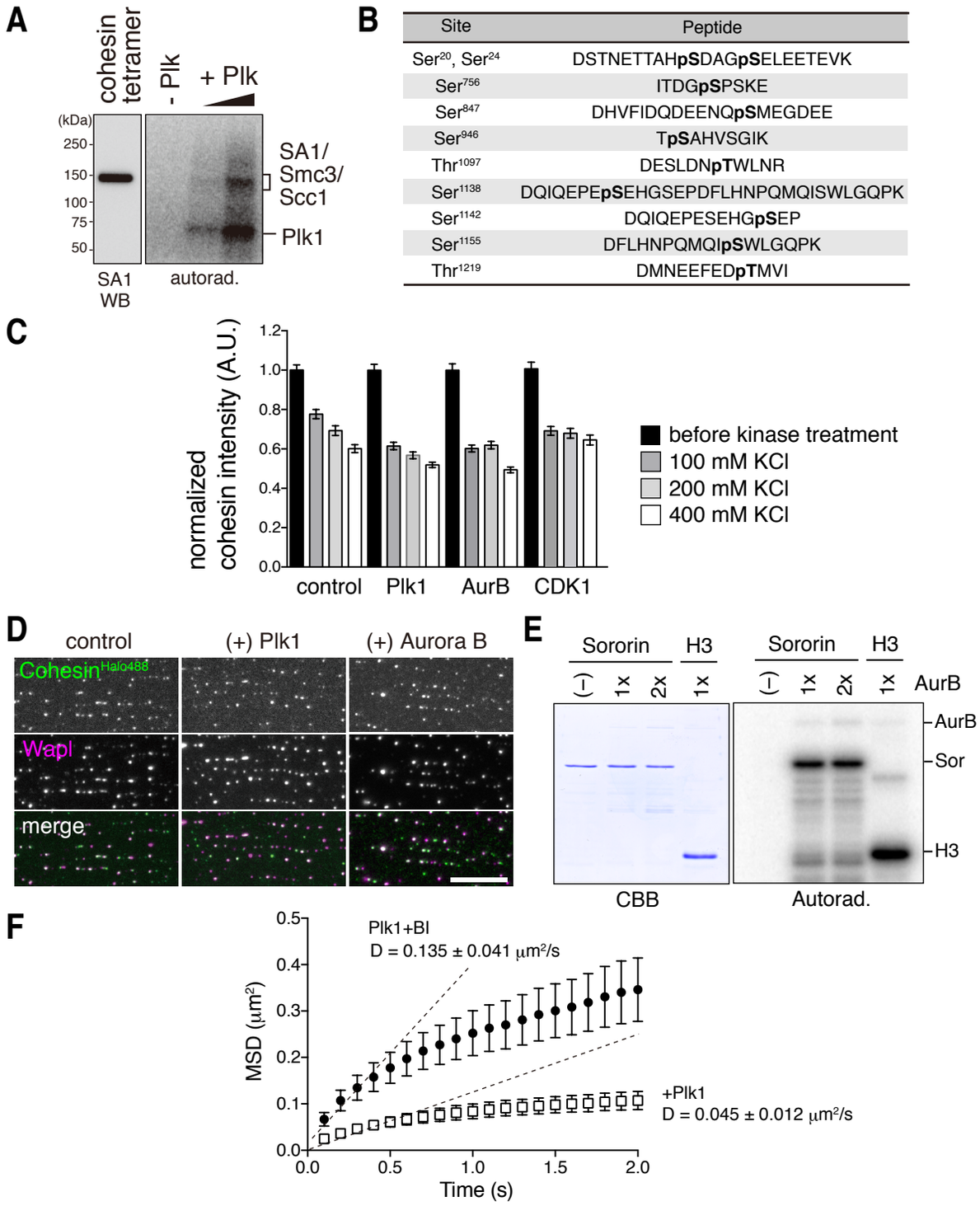


Kanke et al., Appendix Figure S2



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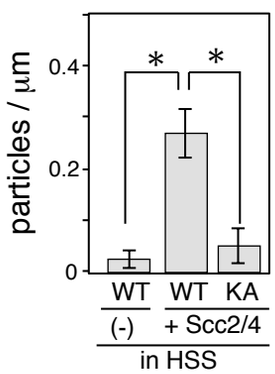
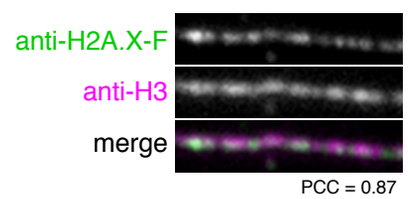
A

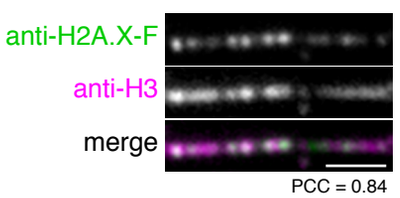


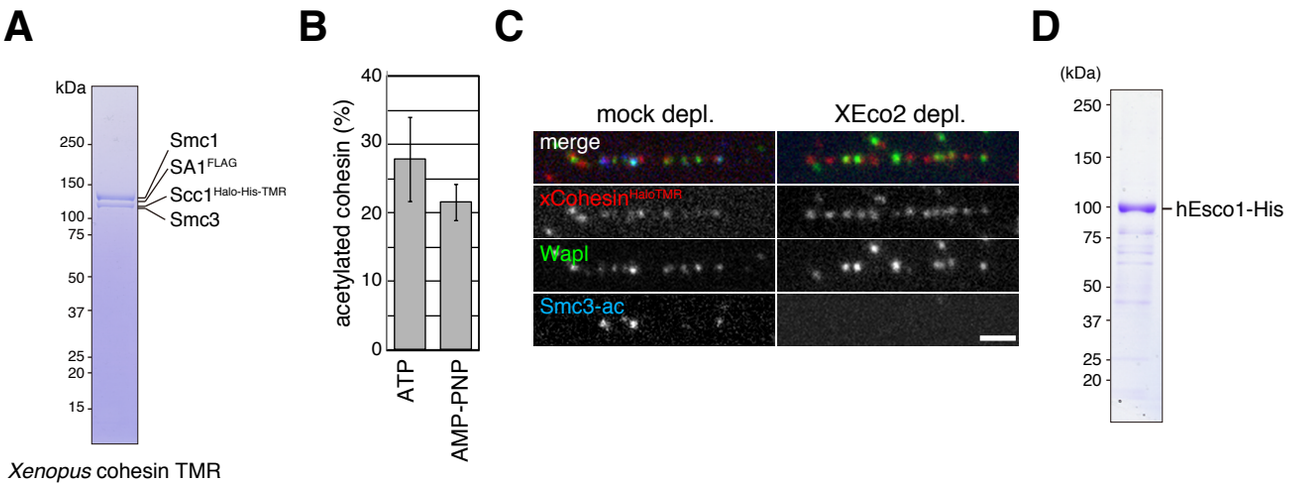
B

	Normalized distance between 2 particles (μm)
HSS	37.42
HSS+Scc2/4	3.69
HSS+Scc2/4+KA	19.52

C







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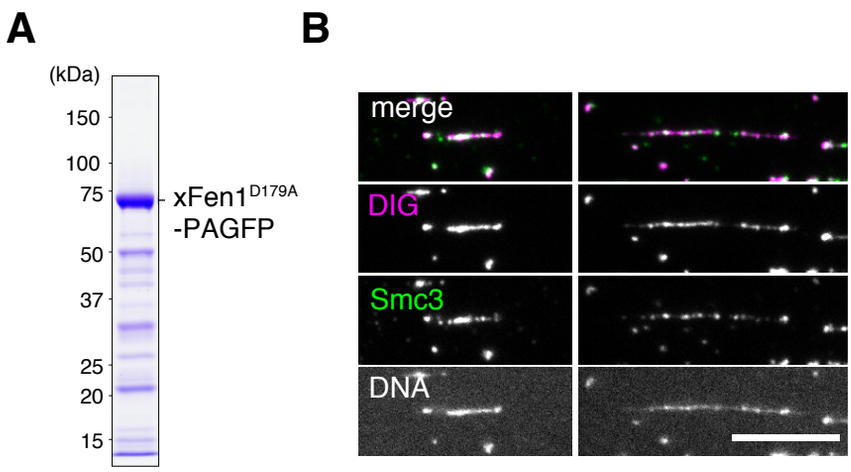

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