SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Protein Expression and Purification

Recombinant human Pds5B proteins were expressed in insect cells using the Bac-to-Bac system (Invitrogen) and purified with a combination of affinity and conventional chromatography. Briefly, baculoviruses encoding $Pds5B_{1–1120}$ and $Pds5B_{21–1120}$ proteins with an N-terminal His_6 tag were prepared with standard protocols. Hi5 cells were harvested after being infected with the Pds5B baculovirus for 56 h. Cell pellet was resuspended with the lysis buffer (50 mM Tris-HCl pH 7.7, 150 mM KCl, 0.1% Triton X100, 20 mM imidazole, 1X protease inhibitor cocktail, 10 mM β-mercaptoethanol) and sonicated. After centrifugation, the supernatant was applied to Ni^{2+}-NTA beads equilibrated with the lysis buffer. After successive washes with the wash buffer I (50 mM Tris-HCl pH 7.7, 1.2 M KCl, 20 mM imidazole) and wash buffer II (50 mM Tris-HCl pH 7.7, 300 mM KCl, 20 mM imidazole), the bound Pds5B protein was eluted with the elution buffer (50 mM Tris-HCl pH 7.7, 300 mM KCl, 500 mM imidazole). The fractions containing Pds5B were pooled, supplemented with 1 mM EDTA, and digested with the tobacco etch virus (TEV) protease at 4˚C overnight to remove the His_6 tag. The cleaved Pds5B protein was further purified with Resource Q and Superdex 200 columns (GE Healthcare). The protein was stored in the storage buffer (20 mM Tris-HCl, pH 8.5, 200 mM NaCl, 5 mM TCEP).

For preparation of the selenomethionine-containing $Pds5B_{21–1120}$ protein, Hi5 cells grown in the ESF921 medium (Expression Systems) were infected with the Pds5B baculovirus, and pelleted at 20 h post-infection. The cell pellet was washed and resuspended with the ESF921 medium without methionine. The cells were incubated with shaking for 6 h to deplete the remaining L-methionine, and then pelleted again and resuspended into the ESF921 medium without methionine that had been supplemented with 100 mg/ml L-selenomethionine. After 30 h incubation, the cells were harvested. Selenomethionine-containing $Pds5B_{21–1120}$ was purified following the same procedure described for the native protein.

Crystallization, Data Collection, and Structure Determination

The $Pds5B_{21–1120}$ protein was concentrated to 11.2 mg/ml, and a synthetic $Wapl_{1–33}$ peptide was added to a 1:5 molar ratio. Crystal screening trays were set up with the sitting drop vapor diffusion method at 20˚C. Initially crystals were observed in 4 similar conditions: 0.2 M sodium citrate and 20% PEG3350 (v/v) without buffer or with 0.1 M Bis-Tris propane buffers at pH 6.5, pH 7.5, or pH 8.5. Single crystals were obtained by the seeding method. Crystals of selenomethionine-containing Pds5B were obtained by seeding using the native protein crystals as seeds. The crystals were cryoprotected with the reservoir solution supplemented with 18% (v/v) glycerol and then flash-cooled in liquid nitrogen. The space group of the crystal is $P2_12_12_1$, with cell dimensions of a = 121 Å, b = 162 Å, and c = 173 Å. There are two molecules in the asymmetric unit with a 63% solvent content.

Diffraction data on a selenomethionine-derivatized crystal of Pds5B–Wapl to a d_{min} of 2.7 Å were collected at beamline 19-ID (Structural Biology Center Collaborative Access Team) at the Advanced Photon Source (Argonne National Laboratory, Argonne, IL) and processed with HKL3000 (Minor et al., 2006), with applied corrections for effects resulting from absorption in a crystal and for radiation damage (Borek et al., 2003; Otwinowski et al., 2003), the calculation of an optimal error model, and corrections to compensate the phasing signal for a radiation-induced increase of non-isomorphism within the crystal (Borek et al., 2010; Borek et al., 2013). These corrections were crucial for successful phasing. Selenium heavy atom positions were located within the program SHELXD (Schneider and Sheldrick, 2002); 73 of 66 possible Se positions were located due to statistical disorder of some SeMet residues. Phases obtained from a single-wavelength selenium anomalous dispersion experiment were refined, and the phases improved via density modification and 2-fold non-crystallographic symmetry averaging within the program PHENIX (Adams et al., 2010).

A partial model built with phases obtained from this dataset was refined against the SeMet diffraction data, with I+ and I– intensities merged in the Reflection File Editor tool of PHENIX. After the initial round of refinement, chain A of Pds5B was overlapped on chain B and vice versa to add parts of the model that had been automatically built in one chain but not the other. Alternate rounds of refinement in PHENIX with rebuilding guided by electron density map inspection in COOT (Emsley et al., 2010) led to the interpretation of ordered densities for the Pds5B polypeptide chain and bound inositol hexakisphosphate (IP_6). Electron density for chain A of Pds5B was stronger and more complete than density for chain B, and chain A coordinates exhibited lower refined atomic displacement parameters. Towards the final rounds of refinement, density for a 5-residue peptide bound near residue D189 in chain A of Pds5B became better defined, and was modeled as residues KTYSR of the Wapl peptide. The parameters of data collection, phasing, and refinement statistics of the final model are shown in Table S1.

Protein Binding Assays

For assaying the binding of Pds5 to Wapl, Sororin, and Scc1 *in vitro*, various truncation or point mutants of human Wapl, Sororin, and Scc1 were constructed with the pGEX6p1 vector to produce GST fusions of these proteins. These GST-Wapl, GST-Sororin, and GST-Scc1 proteins were expressed in *E. coli* and purified with the glutathione-Sepharose 4B resin (GE Healthcare). Beads bound to GST-Wapl, GST-Sororin, or GST-Scc1 proteins were incubated with purified recombinant $Pds5B_{1–1120}$/$Pds5B_{21–1120}$ wild type and mutant proteins or ^{35}S-labeled full-length Pds5B wild type and mutant proteins obtained through the TNT Quick Coupled Transcription/Translation System (Promega). (In the competition assay, beads

bound to GST-Wapl were incubated with purified Pds5B, in the absence or presence of increasing amount of a synthetic Sororin$_{132–171}$ peptide.) The beads were washed four times with TBS supplemented with 0.1% Tween 20 (TBST) for binding assays with GST-Wapl and GST-Sororin, and with the wash buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 2 mM TCEP) for the GST-Scc1 assays. The bound proteins were separated on SDS-PAGE gels, which were stained with Coomassie or analyzed with a phosphorimager (Fujifilm) or both. The intensities of bound ^{35}S-Pds5B were quantified with Image J.

Human Smc3 head domain (HD) (residues 1–251 connected to residues 956–1217 of human Smc3 with a five-glycine linker) was expressed as a GST fusion protein in *E. coli* and purified with Glutathione-Sepharose resin. The ^{35}S-labeled Scc1$_{1–210}$ and Scc1$_{21–210}$ (wild type and 4E) proteins were obtained through *in vitro* translation. For assaying the effects of Pds5B on the preformed DNA exit gate of cohesin, beads bound to GST or GST-Smc3 HD were incubated with ^{35}S-labeled Scc1$_{1–210}$ wild type or 4E for 1h at 4˚C, and washed with TBST. Purified recombinant Pds5B, Wapl, SA2, or their combinations were then added to the beads. After another 1 h incubation at 4˚C, the beads were washed with TBST, and the bound proteins were separated with SDS-PAGE and analyzed with a phosphoimager.

For assaying the effects of Pds5B on the formation of the DNA exit gate, ^{35}S-labeled Scc1$_{1–210}$ or Scc1$_{21–210}$ (wild type or 4E) were first incubated with Pds5B, Wapl, SA2 or combinations of these proteins at room temperature for 30 min. The protein mixture was then added to beads bound to GST or GST-Smc3 HD. After another 1 h incubation at 4˚C, the beads were washed four times with TBST, and the bound proteins were analyzed with a phosphorimager.

Isothermal Titration Calorimetry (ITC)

The affinity between purified recombinant Pds5B$_{1–1120}$ and Sororin$_{91–252}$ proteins was measured with a MicroCal iTC200 instrument (GE Healthcare) at 20˚C. A 23 μM of Pds5B sample in the ITC buffer (20 mM Tris-HCl, pH 8.5, 200 mM NaCl) was titrated with 298 μM of Sororin in the same buffer. For measuring the affinity between Pds5B$_{1–1120}$ and Scc1$_{76–150}$, ITC was performed using a MicroCal VP-ITC instrument (GE Healthcare) at 20˚C. A 4.25 μM of Pds5B sample in the buffer containing 20 mM Tris-HCl, pH 8.5, 150 mM NaCl, 2 mM TCEP was titrated with 53.7 μM of Scc1 in the same buffer. In both cases, binding parameters were calculated with the NITPIC software.

Isolation and NMR Analysis of IP_6 from Recombinant Pds5B

The isolation of IP_6 from human Pds5B expressed and purified from insect cells was performed essentially as described (Sheard et al., 2010). Briefly, 13 mg of purified Pds5B$_{21–1120}$ at 1 mg/ml concentration in the storage buffer was mixed with equal volume of Tris-saturated phenol (Acros Organics). The mixture was inverted and incubated at room temperature for 30 min until phase separation. After centrifugation at 20,800 g for 5 min, the upper aqueous phase was collected and diluted with the Tris buffer (25 mM Tris-HCl, pH 8.0). The phenol phase was extracted again with the Tris buffer, and the aqueous phase was collected. The two extractions were then combined and further purified by gravity flow on Q sepharose anion-exchange resin (GE Healthecare). After the beads were washed with 10 column volume of 0.1 N formic acid, the bound ligand was eluted with 0–2 M step gradients of ammonium formate. The phosphate content of each fraction was analyzed by mixing with perchloric acid in Pyrex culture tubes. The inorganic phosphate was released by heating with continuous shaking to dryness, and dissolved in distilled water after the tubes cooled down to room temperature. Phosphate content was measured with the ATPase assay kit (Innova Biosciences). Peak fractions containing phosphate were collected and lyophilized. Typically, about 19 μg of IP_6 can be extracted from 13 mg of Pds5B.

IP_6 purchased from Sigma was dissolved into a buffer containing 20 mM sodium phosphate (pH 6.8), 89 mM KCl, and 1.8 mM deuterated EDTA (D16, 98%; Cambridge Isotope). The sample was lyophilized overnight and dissolved in fresh D_2O (D, 99.96%; Cambridge Isotope). The final sample concentration of this IP_6 standard was 10 mM. About 9 μg of IP_6 purified from Pds5B was dissolved into a buffer containing 20 mM sodium phosphate (pH 6.8), 50 mM KCl, and 1 mM deuterated EDTA. The sample was then lyophilized overnight and dissolved in fresh D_2O. The final sample concentration of IP_6 extracted from Pds5 was about 10 μM. The 1D ^{1}H NMR spectra were acquired on both samples at 30˚C using an Agilent DD2 600 MHz spectrometer equipped with four channels and pulsed-field gradients. The ^{2}H signal of D_2O was used as a field frequency lock. The spectra were obtained with a 2000 Hz spectral window, a 4.0 s acquisition time, and a 1.5 s relaxation delay. Data were processed and analyzed with the Agilent VnmrJ 4.2 software.

The spectra of the IP_6 standard and IP_6 isolated from Pds5B are highly similar, and contain a distinct double-triplet near 4.90 ppm, a two-proton quartet near 4.43 ppm, and overlapping three-proton resonances near 4.17 ppm. The 4.90 ppm double-triplet is from H-2, as this is the sole equatorial proton on the inositol ring, and is shifted downfield relative to the five other protons that are in axial positions. Two of the three resonances at 4.17 ppm show triple-triplet line shape, indicating that these arise from H-1 and H-3. These resonances are chemically equivalent due to the axis of symmetry through positions 2 and 5. The two-proton quartet at 4.43 ppm arises from chemically equivalent H-4 and H-6 resonances due to the symmetry axis. The remaining one-proton quartet at 4.17 ppm is from H-5.

Mammalian Cell Culture, Transfection, and Synchronization

HeLa Tet-On cells were grown in DMEM (Invitrogen) supplemented with 10% fetal bovine serum (FBS) and 2 mM L-glutamine. When cells reached a confluency of 50%, plasmid transfection was performed using the Effectene reagent (Qiagen) according to the manufacturer's protocols. All mammalian expression plasmids used in this study were derived

from modified pCS2 vectors. The human Wapl and Pds5B cDNAs (which contained silent mutations in the siRNA-targeted region) were inserted into these vectors. The final vectors encoded RNAi-resistant Wapl and Pds5B proteins fused to GFP or Myc at their N-termini. Wapl and Pds5B mutants were constructed with site-directed mutagenesis. All constructs were verified by DNA sequencing. For making stable cell lines, HeLa Tet-On cells were transfected with a pTRE2 plasmid encoding human Smc1 with its C-terminus fused to GFP. Clones were selected with 200 μg/ml hygromycin B. Inducible expression of Smc1-GFP was screened in the absence or presence of 1 μg/ml doxycycline (Invitrogen). For siRNA transfection, cells were transfected with Lipofectamine RNAiMAX (Invitrogen) at 20%-40% confluency according to the manufacturer's protocols, and analyzed at 24–48 h after transfection. The siRNA oligonucleotides targeting human Pds5A (siPds5A; 5'-UGUAAAAGCUCUCAACGAA-3'), Pds5B (siPds5B; GAACUUCUACCUUAAGAUU-3'), Wapl (siWapl; 5'-CGGACTACCCTTAGCACAA-3'), Sgo1 (siSgo1; 5'-GAGGGGACCCUUUUACAGATT-3'), Sororin (siSororin; 5'-CAGAAAGCCCAUCGUCUUA-3'), Esco1 and Esco2 (ON-TARGETplus Set of 4) were synthesized by GE Healthcare. The siRNAs were transfected at a final concentration of 5 nM. For experiments in Figure 1F and 3C, HeLa Tet-On cells were transfected first siWapl or siPds5A/B for 24 h and then with GFP-Wapl or Myc-Pds5B WT or mutant plasmids for another 24 h. The expression levels of GFP-Wapl proteins were controlled by using different doses of GFP-Wapl plasmids. For mitotic synchronization, cells were treated with 2 mM thymidine for 16–18 h, released into fresh medium for 9 h, and blocked at mitosis with the addition of 5 μM nocodazole (Sigma) for 2 h.

Antibodies, Immunoblotting, and Immunoprecipitation

The anti-Wapl antibody was generated against a C-terminal fragment of human Wapl (residues 601–1190) as described previously (Wu et al., 2012). Rabbit polyclonal antibodies against eGFP, human Sororin$_{91–252}$, and human Pds5B$_{1140–1310}$ were raised at Yenzym Antibodies with purified recombinant proteins as antigens. The following antibodies were purchased from the indicated commercial sources: anti-Myc (Roche, 11667203001), anti-HA (Roche, 11583816001), anti-mCherry (BioVision Inc, 5993-100), anti-Smc1 (Bethyl Laboratories, A300-055A), anti-Smc3 (Bethyl Laboratories, A300-060A), anti-SA2 (Santa Cruz, Biotechnology, sc-81852), anti-Pds5A (Bethyl Laboratories, A300-089A), anti-β-tubulin (Sigma, T4026), anti-Esco2 (Bethyl Laboratories, A301-689A), MPM2 (Millipore, 05-368), and CREST serum (ImmunoVision). Anti-Esco1 and anti-Smc3 K105Ac antibodies were gifts from Susannah Rankin (Oklahoma Medical Research Foundation) and Prasad Jallepalli (Memorial Sloan Kettering Cancer Center), respectively. The antibodies to Myc and GFP have been validated for immunoblotting and immunoprecipitation applications. The antibodies to HA, mCherry, Smc1, Smc3, SA2, Pds5A and Tubulin have been validated for immunoblotting. The antibody to MPM2 has been validated for flow cytometry. The relevant validation information and references can be found at the manufacturers' websites. The antibody to Sororin is validated for immunoblotting, and the anti-Pds5B antibody is validated for both immunoblotting and immunoprecipitation in this study.

For immunoblotting, cells were lysed in the SDS sample buffer (pH 6.8), sonicated, and boiled. The lysates were separated by SDS-PAGE and blotted with the desired primary antibodies. The primary antibodies were used at a final concentration of 1 μg/ml. Anti-mouse IgG (H+L) (Dylight 680 conjugates), anti-rabbit IgG (H+L) (Dylight 800 conjugates) (Cell Signaling), or horseradish peroxidase-linked donkey anti-rabbit or sheep anti-mouse IgGs (GE healthcare) were used as secondary antibodies. The blots were either scanned with an Odyssey Infrared Imaging System (LI-COR) or developed with the SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific) according to the manufacturer's protocols.

For immunoprecipitation, the anti-Myc, anti-GFP, anti-Pds5B, anti-Sororin, or anti-Wapl antibodies were coupled to Affi-Prep Protein A beads (Bio-Rad) at a concentration of 1 mg/ml. Cells were lysed with the lysis buffer containing 25 mM Tris-HCl (pH 7.7), 50 mM NaCl, 0.1% (v/v) Nonidet P-40, 2 mM $MgCl_2$, 10% (v/v) glycerol, 5 mM NaF, 0.3 mM Na_3VO_4, 10 mM β-glycerophosphate, 1 mM DTT, protease inhibitor mixture (Roche), and 50 units/ml Turbo Nuclease (Accelagen). After a 1-h incubation on ice and a 10-min incubation at 37˚C, all lysates were centrifuged at 4˚C at 20,817 g for 20 min. The supernatants were incubated with the desired antibody beads for 3 h at 4˚C. The beads were then washed three times with the lysis buffer containing 200 mM NaCl. Proteins bound to beads were dissolved in SDS sample buffer, separated by SDS-PAGE, and blotted with the appropriate antibodies.

Flow Cytometry

Cells were harvested with trypsinization and fixed in 70% ice-cold ethanol overnight. After being washed with PBS, cells were permeabilized with PBS containing 0.25% Triton X-100 on ice for 5 min. Then, cells were incubated with the antibody to MPM2 in PBS containing 1% BSA for 3 h at room temperature, followed by an incubation with a fluorescent secondary antibody (Invitrogen) for 30 min. After being washed with PBS, cells were resuspended in PBS containing 0.1% Triton X-100, RNase A, and propidium iodide, and analyzed with a flow cytometer. Data were processed with FlowJo.

Metaphase Spreads and Immunofluorescence

After synchronization, mitotic HeLa Tet-On cells were collected by shake-off. Cells were washed once with PBS, treated with 55 mM KCl hypotonic solution at 37˚C for 15 min, and spun onto microscope slides with a Shandon Cytospin centrifuge. Cells on the slides were first permeabilized with the PHEM buffer (25 mM HEPES pH 7.5, 10 mM EGTA pH 8.0, 60 mM PIPES pH 7.0, 2 mM $MgCl_2$) containing 0.3% Triton X-100 for 5 min and then fixed in 4% paraformaldehyde for 10

min. Fixed cells were washed three times with PBS containing 0.1% Triton X-100 for 2 min each time, and incubated with CREST in PBS containing 3% BSA and 0.1% Triton X-100 at 4˚C overnight. Cells were then washed three times with PBS containing 0.1% Triton X-100 for 2 min each time, and incubated with fluorescent secondary antibodies (Molecular Probes) in PBS containing 3% BSA and 0.1% Triton X-100 for 1 h at room temperature. Cells were again washed three times with PBS containing 0.1% Triton X-100 and stained with 1 μg/ml DAPI for 2 min. After the final washes, the slides were sealed with nail polish and viewed with a 100X objective on a DeltaVision fluorescence microscope (GE Healthcare). Image processing and quantification were performed with Image J.

Fluorescence In Situ Hybridization (FISH)

The BAC clone RP11-466L19 was purchased from Empire Genomics. FISH probes were labeled with 5-Fluorescein dUTP (Enzo Life Sciences) using the Nick Translation Kit (Abbott Molecular). Human cot-1 DNA (Invitrogen) and salmon sperm DNA (Invitrogen) were added, and probes were precipitated and resuspended in the hybridization buffer (Cytocell). HeLa Tet-on cells were transfected with Pds5A/B or Sororin siRNAs before synchronization with 2 mM thymidine for 16-18 h. Cells were then released intro fresh medium for 4 h and fixed with methanol and acetic acid (ratio 3:1). Fixed cells were dropped onto slides and in situ hybridized at 80˚C with DNA probes. Slides were sequentially washed with 0.1% SDS in 0.5 X SSC at 70 ˚C for 5 min, 1 X PBS at room temperature for 10 min and 0.1% Tween 20 in 1 X PBS at room temperature for 10 min. Slides were then mounted with ProLong Gold (Life Technologies) and viewed with a 100X objective on a DeltaVision fluorescence microscope (GE Healthcare). Image processing and quantification were performed with ImageJ.

Fluorescence Recovery after Photobleaching (FRAP)

HeLa cells stably expressing Smc1-GFP were plated into chambered coverglass, transfected with empty vector or RNAi-resistant mCherry-Pds5B WT or mutant plasmids, and then transfected with siPds5A and siPds5B. A final concentration of 1 μg/ml doxycycline was always maintained in the culture medium. FRAP was performed using a custom built spinning disk confocal microscope (BioVision) equipped for live-cell imaging and operated with the MetaMorph and iLas2 software. Single stack images were captured with a 100X objective. A small circular region in each cell was bleached with a 405 nm laser at 100% intensity. Images were acquired with both 488-nm and 561-nm lasers before bleaching, and with a 488-nm laser at 30 sec intervals for 60 min after bleaching. For data analysis, a region of interest (ROI) that equals to the bleached area was defined in ImageJ. The integrated density of GFP in this region was measured before bleaching as I_{pre}, immediately after bleaching as I_0, and at each time point after bleaching as I_t. $(I_t-I_0)/I_{pre}$ was plotted against time. Individual data sets were fitted to a single exponential function using the GraphPad Prism software to determine the half-life and plateau of recovery.

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