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Supplemental Figure Legends

Supplemental Figures

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Supplemental Experimental Procedures

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SUPPLEMENTAL FIGURE LEGENDS

Figure S1. A high concentration of RanBP1 buffers RCC1 activity in CSF-XEE. Related to Figure 1.

(**A**) Total CSF- XEE and different amount of recombinant xRCC1 or xRanBP1 were analyzed by immunoblotting with antibodies against RCC1 or RanBP1. The band intensity was quantified and the endogenous protein concentration of RCC1 and RanBP1 was calculated. The estimated endogenous concentrations of both proteins is listed in the lower table.

(**B**) Recombinant xRCC1 was added at different concentrations (3x, 10x, or 30x relative to endogenous RCC1 level) to CSF-XEE containing rhodamine-labeled α-tubulin (20 μg/ml) and demembraned chromatin (1,000 units/μl). After 30 minutes at RT, aliquots of each reaction were fixed, stained with Hoechst 33342 and processed for fluorescent microscopy. Images were taken for chromatin (left, blue) and tubulin (middle, red). Scale bar, 10 μm. Spindles with chromatin in vicinity were counted as one structure. Percentage of bipolar spindles with chromatin correctly localizing at the mid-plate was plotted as mean ± SEM (N = 3 XEEs, 50 structures counted in each XEE).

(**C**) Total reactions as in (**B**) were subjected to immunoblotting with antibodies against RCC1 and Histone H3.

Figure S2. RanBP1 does not associate to chromatin and $RCC1^{Ran}$ characterization. Related to Figure 2.

(**A**) Buffer or recombinant xRanBP1 (10x endogenous RanBP1 level) were added to CSF-XEE containing demembraned sperm chromatin (10,000 units/μl). After a 30 minute incubation at RT, the chromatin was re-purified. Samples from each reaction (lane 1,2) and corresponding isolated chromatin fractions (lane 3,4) were subjected to Western blotting with antibodies against RanBP1 and Histone H3.

(**B**) Recombinant His_6-S-Ran-GTP was incubated with recombinant wild type ($RCC1^{WT}$) or mutant ($RCC1^{Ran}$) RCC1. S protein beads were added to each sample and incubated for 60 minutes at RT. $RCC1^{WT}$ alone without His_6-S-Ran-GTP was incubated with S protein beads in parallel. Both total protein input (upper panel) and proteins bound to the S protein beads (lower panel) were separated by SDS-PAGE and visualized by CBB staining. The identity of each band was indicated to the right of each panel.

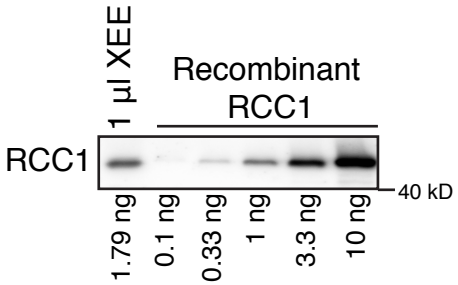
Figure S3. RanBP1 Ser 60 is phosphorylated in early anaphase cycling XEE. Related to Figure 4.

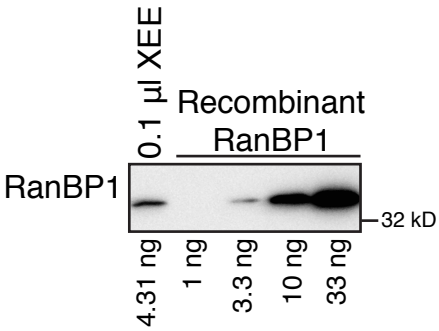
(A) MS/MS spectrum confirming the presence of phosphorylated Ser 60 in RanBP1 purified from early anaphase cycling XEE. Ionized peptide fragments b_6 and b_8 contain the phosphorylated Ser 60. "$-H_3PO_4$" or "$-H_2O$" indicate loss of phosphate or water during ionization. "+H" indicates protonation during ionization. m/z, mass to charge ratio.

(B) Cycling XEE was frozen at indicated time points and total XEE from each aliquot was immunoblotted with anti-Cyclin B antibody. RanBP1 was immunoprecipitated from each aliquot and immunoblotted with either anti-pSer60-xRanBP1 phospho-specific antibody or anti-xRanBP1 antibody.

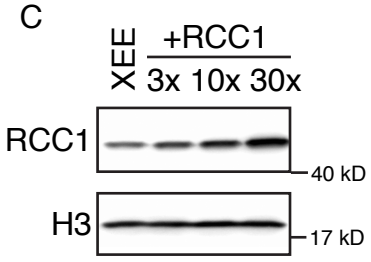
Figure S4. Protein sequence alignment of RanBP1, RanBP2, and RanBP3 from H.*sapiens*. Serine 60 is highlighted in red. Related to Figure 5.

A



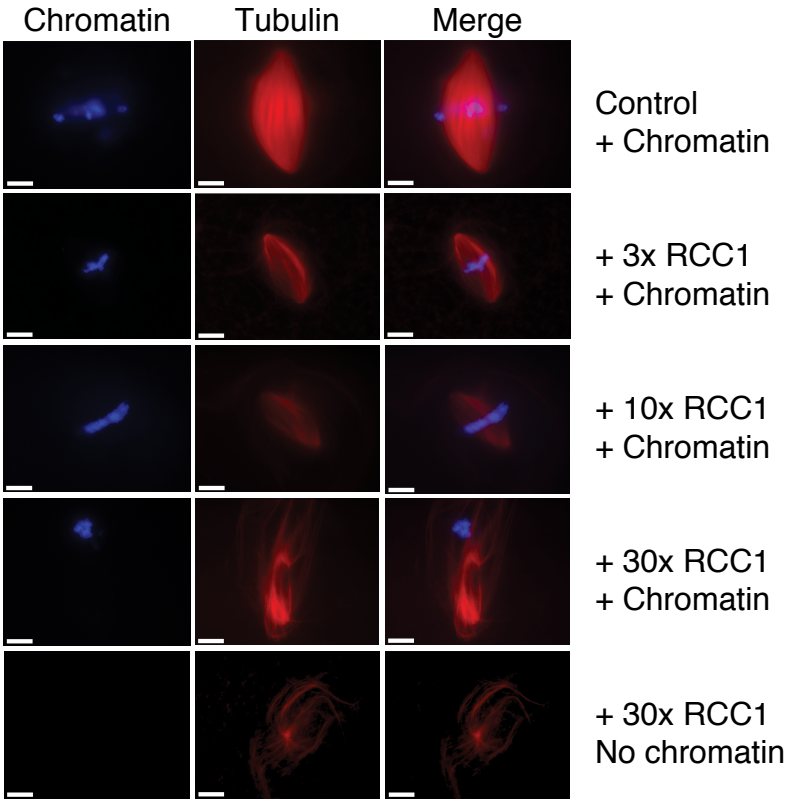


C



	Concentration (µg/ml)	Molarity (µM)
RCC1	1.79	0.04
RanBP1	43.11	1.77

B



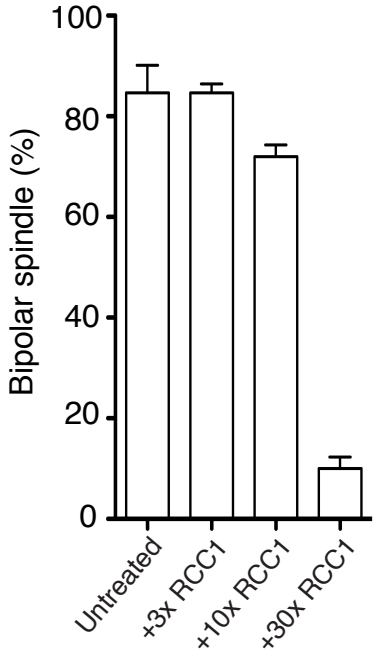
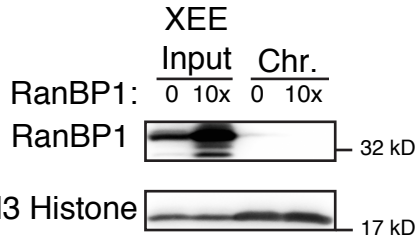


Figure S1

A


XEE
Input
Chr.
RanBP1: 0 10x 0 10x
RanBP1
32 kD
H3 Histone
17 kD

B

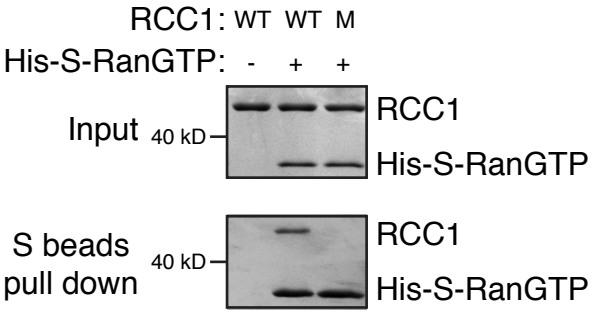
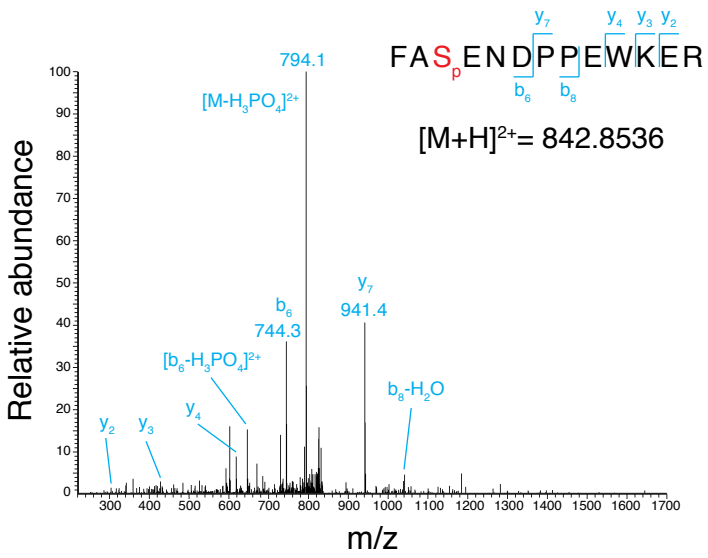

RCC1: WT WT M
His-S-RanGTP: - + +
Input
40 kD
RCC1
His-S-RanGTP
S beads
pull down
40 kD
RCC1
His-S-RanGTP

Figure S2

A


FASpENDPPEWKER
y7 y4 y3 y2
b6 b8
[M+H]2+= 842.8536
794.1
[M-H3PO4]2+
y7
941.4
b6
744.3
[b6-H3PO4]2+
b8-H2O
y4
y2
y3
Relative abundance
m/z

B

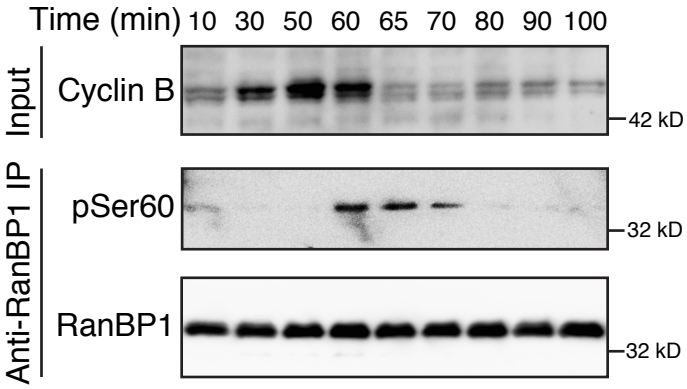

Time (min) 10 30 50 60 65 70 80 90 100
Input
Cyclin B
42 kD
Anti-RanBP1 IP
pSer60
32 kD
RanBP1
32 kD

Figure S3

*hs*RanBP1 -MRAKLFRFASENDLPEWKER-
*hs*RanBP2 -NRAKLFRFDVES - - KEWKER-
*hs*RanBP3 -MQCKLFVFDKTS - - QSWVER-

Figure S4

SUPPLEMENTAL EXPERIMENT PROCEDURES

DNA Constructs

Wild type *Xenopus laevis* RCC1 (xRCC1) was cloned into a pET8a vector as described previously (Dasso et al., 1992). Wild type human TC4/Ran (hRan) was cloned into a pET3a vector as described (Dasso et al., 1994). His_6-S-hRan was cloned into a pET28a vector. Wild type *Xenopus laevis* RanBP1 (xRanBP1) and xRCC1-HA were cloned into a pET28a vector between the *Nco*1 and *Xho*1 sites. xRCC1 Ran binding mutant $xRCC1^{Ran}$ (D50A, R153A, N155A, and N273D), xRanBP1 S60A, and xRanBP1 S60D mutants were generated by PCR using Herculase II (Agilent Technologies #600679).

Recombinant Protein Expression

Recombinant wild type xRCC1, wild type xRCC1-HA, and $xRCC1^{Ran}$ were expressed by transforming constructs into E.*coli* BL21-CodonPlus-RIL (Stratagene). Cells were grown at 37°C and induced with 0.1 mM IPTG, followed by overnight incubation at 18°C. Cells were harvested and lysed in lysis buffer (20mM Na_2PO_4, pH 7.2, 50 mM NaCl, 2 mM $MgCl_2$, 1 mM DTT) by sonication. Lysed cells were centrifuged at 28,000 g for 30 min, and the supernatant was applied to HiTrap S ion exchange column (GE Healthcare). Proteins were eluted using a linear salt gradient from 50 mM NaCl to 800 mM NaCl in lysis buffer. The most pure fractions containing RCC1 were pooled and concentrated by Amicon (Millipore).

Recombinant wild type xRanBP1, $xRanBP1^{S60A}$, and $xRanBP1^{S60D}$ were expressed by transforming construct into E.*coli* BL21 (DE3) (Invitrogen). The cells were grown at 37 °C and induced by 0.5 mM IPTG followed by 3 hrs incubation at 37 °C. The cells were harvested and lysed in lysis buffer (20mM HEPEs pH=7.8, 50 mM NaCl, 2 mM $MgCl_2$, 1 mM DTT) by sonication. Lysed cells were centrifuged at 28,000 g for 30 min and the supernatant was applied to HiTrap Q ion exchange column (GE Healthcare). The proteins were eluted using a linear salt gradient from 50 mM NaCl to 800 mM NaCl in lysis buffer. The most pure fractions containing RanBP1 were pooled and concentrated by Amicon (Millipore).

Recombinant His_6-S-Ran was expressed by transforming the construct into E.*coli* BL21 (DE3) (Invitrogen). The cells were grown at 37 °C and induced with 0.5 mM IPTG followed overnight incubation at RT. The protein was

purified using a Ni-NTA resin (Qiagen) according to manufacturer's instructions. Purified His_6-S-Ran was charged with GDP or GTP as described (Dasso et al., 1994).

Expression and purification of untagged Human RanGAP1 was described previously (Haberland and Gerke, 1999).

Estimation of endogenous protein concentrations

To estimate the endogenous RCC1 concentration in CSF-XEE, a series of standard solutions were made by diluting recombinant xRCC1 to concentrations of 0.1, 0.33, 1, 3.3, 10 ng/µl. 1 µl CSF-XEE and the standard xRCC1 solutions were separated by SDS-PAGE and subjected to Western blotting with anti-xRCC1 antibodies. The band intensity was quantified and a linear standard curve was constructed. The amount of RCC1 in 1 µl CSF-XEE was calculated by fitting to the standard curve. Endogenous RanBP1 concentration was estimated similarly with the exception that only 0.1 µl CSF-XEE was loaded, so that the band intensity of endogenous RanBP1 fell within the linear range of standard curve.

Antibodies and Immunoblots

SDS-PAGE and membrane transfer were done in a standard procedure. The primary antibodies were diluted in blocking buffer at 1:1,000 (v/v) except for anti-xCyclin B2 that is diluted at 1:500 (v/v) and anti-pSer60-RanBP1 antibody diluted at 1:100 (v/v). The secondary HRP-linked antibodies against rabbit IgG or mouse IgG (GE Healthcare) were diluted in blocking buffer at 1:10,000 (v/v). SuperSignal Chemiluminescent Substrate (Thermo Scientific) was used for detection. The images were captured with Gel Logic 6000 Pro system (Carestream) and analysis (Figures 2A-C, 4B and S1A) was done using Carestream Imaging software (Carestream).

Antibody crosslinking and Immunodepletion

To immunodeplete RanBP1 from 1 ml CSF-XEE, 2 mg anti-xRanBP1 antibodies were coupled to 100 µl settled Protein A Sepharose beads (GE Healthcare). Antibodies were crosslinked by incubating in buffer (0.2 M triethanolamine, pH 8.3) containing 5 mg/ml Dimethyl pimelimidate (DMP) (Thermo Scientific) for 40 min at RT. 1 ml

CSF-XEE was incubated with 50 µl coupled beads at RT for 30 min, with rotation. The beads were removed by centrifugation at 1,000 g for 1 min, followed by a second round of depletion with another 50 µl of antibody-coupled beads.

To immunodeplete RCC1 from 1 ml CSF-XEE, 250 µg anti-xRCC1 antibody was coupled to 1 ml protein A magnetic beads (Invitrogen). Antibodies were crosslinked similarly. 1 ml CSF-XEE was incubated with 0.5 ml coupled beads at RT for 30 min on an end-by-end rotator. Beads were removed by standing briefly on magnetic tube holder and the depletion was repeated once with another 0.5 ml coupled beads.

2D Gel Electrophoresis

Samples were prepared in DeStreak Rehydration Solution (GE Healthcare) and loaded into Immobiline DryStrip pH 4-7, 7 cm (GE Healthcare) polyacrylamide gel strips with an immobilized pH gradient. The loaded strips were subject to isoelectric focusing (IEF) in IPGphor (Pharmacia Biotech) according to manufacture's instructions. After IEF, the strips were further separated on a second dimension by SDS-PAGE. Immunoblotting was performed, and the image was captured and analyzed (Figure 4C and 4D) using an Odyssey Infrared Imaging System (Li-cor), following instructions from the manufacturer.

Detection of endogenous RanBP1 phoshorylation

Cycling XEE was prepared as described (Murray, 1991). 10 µl ATP, [γ-^{32}P]ATP (3,000 Ci/mmol, 10 mCi/ml) (PerkinElmer) was added to 1 ml cycling XEE. The XEE started cycling after being warmed up to RT. 50 µl aliquots were snap frozen in liquid nitrogen at different times. Frozen aliquots were thawed and diluted 1:10 (v/v) in denatured buffer (20 mM HEPEs, pH 7.7, 150 mM NaCl, 0.2% Triton-X100, 1 M urea). RanBP1 was precipitated from individual samples by incubating with 50 µl protein A magnetic beads (Invitrogen) coupled with 15 µg anti-xRanBP1 antibodies for 90 min at 4°C. The beads were washed three times with denatured buffer and eluted by 30 µl 0.1 M glycine (pH 2.4). The eluent was neutralized with 20% (v/v) 1 M Tris-HCl (pH 8.0) and boiled in SDS sample buffer. After separation by SDS-PAGE, the gel was dried and exposing to a storage phosphor screen (Amersham Biosciences)

for 24 hrs. The image was scanned and analyzed (Figure 4A and 4B) using a Storm Imaging System (Amersham Biosciences).

Measuring guanine nucleotide release of endogenous RCC1

Recombinant Ran was loaded with [α-^{32}P]GTP (PerkinElmer) as described (Richards et al., 1995). Ran-[α-^{32}P]GTP was incubated with 0.3% (molar ratio) recombinant human RanGAP1 for 30 min at RT to allow GTP hydrolysis to form Ran-[α-^{32}P]GDP. To measure endogenous guanine nucleotide release activity, 10 μM recombinant Ran-GDP, containing loaded Ran-[α-^{32}P]GDP, was added to either CSF-XB buffer or XEE. Aliquots of each reaction were taken at 0.5 min, 1.5 min, 5 min and 15 min, and diluted in stop buffer (20 mM Tris-HCl pH 7.5, 25 mM $MgCl_2$, 100 mM NaCl, and 1mM DTT) on ice. Diluted samples were filtered through 0.45 μm filter paper (Whatman). The filter paper was washed twice with stop buffer and was transferred to 5 ml CytoScint (MP biomedicals). The radioactivity retained on the filter paper was measured using a scintillation counter.

***In vitro* protein binding assay**

To check the Ran-GTP binding affinity of WT/mutated xRanBP1 (Figure 5B), recombinant human His_6-S-RanGTP was diluted in 150 μl protein binding buffer (20 mM HEPES, 150 mM NaCl, 50 μg/ml Digitonin (Calbiochem), and 2 mM $MgCl_2$) to a final concentration of 0.2 mg/ml. Either WT or mutated recombinant xRanBP1 was added to each sample at a final concentration of 0.2 mg/ml. A control incubation was made in parallel with WT xRanBP1 but no His_6-S-RanGTP. Ni-NTA resin (Qiagen) was equilibrated in binding buffer. 20 μl resin was added to each sample and incubated at RT for 60 min with end-by-end rotation, followed by pelleting at 2,000 g for 1 min. Five washes with binding buffer were performed, using similar pelleting conditions in each case. Bound proteins were eluted by incubating with binding buffer supplemented with 250 mM imidazole for 3 min.

To test RCC1/Ran/RanBP1 complex formation with WT/mutated RanBP1 (Figure 5C), recombinant xRCC1-HA and human His_6-S-RanGDP are diluted in 150 μl protein binding buffer, to a final concentration of 0.2 mg/ml. After 10 min incubation at RT, WT or mutated xRanBP1 were added to each sample to a final concentration of 0.4 mg/ml. A control was incubated in parallel, including xRCC1-HA and WT/mutated xRanBP1 but omitting His_6-S-

RanGDP. 20 µl anti-HA affinity gel (Sigma-Aldrich, E6779) equilibrated in binding buffer was added to each sample and incubated at RT for 60 min with rotation. The beads were pelleted by centrifugation at 2,000 g for 1 min, and washed five times with binding buffer with similar pelleting after each wash. Bound proteins were eluted by incubating with 50 µl elution buffer (0.1 M glycine, pH=2.4) for 1 min. After centrifugation, supernatants were transferred into new tubes, and neutralized with 10 µl buffer (1 M Tris-HCl, pH 8.0).

To characterize the Ran binding xRCC1 mutant (xRCC1^{Ran}), recombinant xRCC1^{WT} or xRCC1^{Ran} were diluted in binding buffer to a final concentration of 0.2 mg/ml. Recombinant His_6-S-RanGTP was added to each sample at a final concentration of 0.2 mg/ml. A control sample was incubated in parallel with xRCC1^{WT} but no His_6-S-RanGTP. 20 µl S protein agarose beads (Novagen) equilibrated in binding buffer were added to each sample and incubated at RT for 60 min with rotation. The remainder of the procedure was identical to anti-HA immunoprecipitation, as above.

SUPPLEMENTAL REFERENCES:

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Dasso, M., Seki, T., Azuma, Y., Ohba, T., and Nishimoto, T. (1994). A mutant form of the Ran/TC4 protein disrupts nuclear function in Xenopus laevis egg extracts by inhibiting the RCC1 protein, a regulator of chromosome condensation. EMBO J *13*, 5732-5744.

Haberland, J., and Gerke, V. (1999). Conserved charged residues in the leucine-rich repeat domain of the Ran GTPase activating protein are required for Ran binding and GTPase activation. Biochem J *343 Pt 3*, 653-662.

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