reporter activity (Fig. 1A). Equally strong inhibition of ER-α signaling by *wtBRCA1* was observed in breast cancer cell line T47D, in two other human breast cancer cell lines (MCF-7 and MDA-MB-231), and in two other human prostate cancer cell lines (LNCaP and TsuPr-1) (Fig. 1, A and B). However, three human cervical cancer cell lines showed relatively weak inhibition of ER-α signaling by *wtBRCA1*: E2-stimulated reporter activity in the presence of *wtBRCA1* was about 40% to 50% that in the presence of empty vector (Fig. 1B).

We used the gal4-ER/gal4-Luc assay system (*11*) to determine whether BRCA1 directly inhibits the highly conserved COOH-terminal activation function (AF-2) of ER-α, independently of any effect it might have on the DNA binding activity of the receptor. When linked to the yeast gal4 DNA binding domain (DBD), the AF-2 domain of ER-α stimulated activity of a reporter containing a multimeric gal4 binding site in an E2-dependent fashion (Fig. 1C). In both DU-145 and T47D cells, *wtBRCA1* reduced gal4-ER/gal4-Luc activity to background levels, whereas pcDNA3 vector had little or no effect on reporter activity. These findings suggest that BRCA1 inhibits the AF-2 function of ER-α, but they do not rule out the possibility that BRCA1 targets additional steps in ER-α activation.

In both the ER-α and gal4-ER assays, basal reporter activity was very low, so it was not possible to determine whether BRCA1 acted as a nonspecific transcriptional repressor. We tested the ability of *wtBRCA1* to regulate the activity of other transcription factors. Expression vectors for cell cycle–regulated transcription factors E2F1 and Sp1 caused activation of luciferase reporters controlled by the corresponding binding elements, but the *wtBRCA1* gene did not inhibit activation of either reporter in DU-145 or T47D cells (Fig. 1D). *wtBRCA1* also failed to repress activation of the gal4-Luc reporter by vectors encoding the gal4 DBD linked to the transcriptional activation domains of c-Jun and E2F1 (Fig. 1E). In fact, in some of these assays, reporter activity was slightly higher in the presence of *wtBRCA1*. These results suggest that BRCA1 is not an indiscriminate repressor of transcription.

Previous studies have linked expression of the *BRCA1* gene with differentiation of specific cell types, including mammary epithelium (*12*), but the molecular targets of BRCA1 for mammary differentiation have not been identified. Our findings suggest that the wild-type BRCA1 protein may function, in part, to suppress estrogen-dependent mammary epithelial proliferation by inhibiting ER-α-mediated transcriptional pathways related to cell proliferation. Conversely, inactivation of the ability to repress ER-α transcriptional activity by mutation or by loss of *BRCA1* expression may contribute to mammary carcinogenesis. Interestingly, the relatively weak inhibition of ER-α signaling in human cervical cancer cell lines by the *wtBRCA1* gene correlates with the absence of an association of cervical cancer, another estrogen-dependent tumor type, with germ line mutations of *BRCA1*. To establish the validity of these hypotheses, it is necessary to confirm the ability of BRCA1 to regulate the ER-α response in in vivo models.

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13. The wild-type *BRCA1* expression plasmid (*wtBRCA1*) was created by cloning the *BRCA1* cDNA into the pcDNA3 vector (Invitrogen) with artificially engineered 5′ Hin dIII and 3′ Not I sites. For transcription assays, we incubated asynchronously proliferating cells overnight at 50% to 70% of confluency in 24-well dishes with 0.5 μg of each vector (except where otherwise indicated) in serum-free Dulbecco's modified Eagle's medium (DMEM) containing Lipofectamine (Life Technologies). Cells were washed, incubated in serum-free phenolphthalein-free DMEM (0.2 ml per well) with or without 17β-estradiol (1 μM) for another 24 hours, and harvested for luciferase assays. Values are means ± SEM of four replicate wells and are representative of at least three experiments. Results were similar whether ER-α assays were performed in serum-free DMEM or in the presence of 2% charcoal-stripped serum.
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Self-Organization of Microtubule Asters Induced in *Xenopus* Egg Extracts by GTP-Bound Ran

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The nucleotide exchange activity of RCC1, the only known nucleotide exchange factor for Ran, a Ras-like small guanosine triphosphatase, was required for microtubule aster formation with or without demembranated sperm in *Xenopus* egg extracts arrested in meiosis II. Consistently, in the RCC1-depleted egg extracts, Ran guanosine triphosphate (RanGTP), but not Ran guanosine diphosphate (RanGDP), induced self-organization of microtubule asters, and the process required the activity of dynein. Thus, Ran was shown to regulate formation of the microtubule network.

Ran is an abundant Ras-like nuclear small guanosine triphosphatase (GTPase) essential for nucleus-cytosol exchange of macromolecules (*1–4*). Its GTPase activity is activated by RanGAP1 or Rna1p, and its nucleotide is exchanged by RCC1 (*1*, *2*). Although all of the yeast *ran* mutants thus far isolated show nuclear protein import defects (*3*), hamster and yeast *rcc1* mutants show diverse phenotypes, such as suppression of receptor-less mating process, chromosome instability, premature chromatin condensation, lack of chromosome decondensation, abnormal mRNA metabolism, and mRNA export defects (*1*). How Ran regulates these processes is controversial. In addition to the RanBP1 and importin β families, which are required for nucleus-cytosol exchange of macromolecules (*4*), Dis3p (*5*) and RanBPM (*6*) are reported to bind to RanGTP. Although Dis3p is required for ribosomal RNA processing (*7*), RanBPM is localized in the centrosomes and the antibodies to RanBPM inhibit microtubule aster formation in vitro (*6*).

The centrosome consists of a pair of centrioles surrounded by pericentriolar material (PCM) (*8*). *Xenopus* γ-tubulin ring complex-

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es, which are made up of γ-tubulin and eight other polypeptides including Xgrip109 (*9*), are localized in the PCM and act as microtubule-nucleating units that cap the minus ends of microtubules (*10*). Although it is unknown where in the centrosome RanBPM is localized or how RanBPM participates in microtubule nucleation, the existence of RanBPM at the centrosome suggests the possibility that the Ran GTPase cycle may control microtubule organization. To address this issue, we used the in vitro microtubule assembly system of *Xenopus* egg extracts (*11*) and studied the role of Ran for microtubule organization.

The extracts were prepared from *Xenopus* eggs arrested in meiosis II by cytostatic factor, a cytoplasmic endogenous meiotic inhibitor (*12*), as described (*11*, *13*). After incubation of the egg extracts with demembranated sperm nuclei at 24°C for 10 min, microtubule asters were formed (Fig. 1). To address the question of whether Ran participates in control of microtubule organization, we immunodepleted RCC1, which is the only known nucleotide exchange factor for Ran (*1*), from egg extracts (*14*) (Fig. 1A). More than 91% of RCC1 was removed from the egg extracts. In these RCC1-depleted egg extracts, aster formation was severely inhibited, whereas asters were formed in mock-depleted egg extracts (Fig. 1B). Consistent with this finding, RanT24N, a Ran mutant that is locked in the guanosine diphosphate (GDP)–bound form and inhibits RanGEF activity of RCC1 (*15*), inhibited the aster-forming action in egg extracts (Fig. 1B).

It is possible that RCC1-depleted egg extracts failed to form asters because of loss of factors immunodepleted in association with RCC1. To exclude this possibility, we produced recombinant human RCC1 (hRCC1) in *Escherichia coli* and purified it (*16*). Purified hRCC1 was added to the RCC1-depleted egg extracts, and then mixtures were incubated with demembranated sperm nuclei. After incubation at 24°C for 10 min, microtubule asters were formed, but not in the absence of hRCC1 (Fig. 2A). Hence, RCC1 was required for microtubule aster formation in the egg extracts. Asters were also observed in the extracts where no sperm DNA was present (Fig. 2A, panel a, arrow). Indeed, when hRCC1 was added at the concentration of hRCC1, 0.22 μM, corresponding to the endogenous RCC1 concentration of egg extracts, sperm-free asters were formed (Table 1). Thus, the self-organization of microtubule asters reported previously (*17*) occurred in the RCC1-depleted egg extracts by addition of recombinant hRCC1.

Microtubule asters induced by addition of RCC1 without sperm nuclei were stained with antibodies to Xgrip109, a *Xenopus* γ ring protein (*9*), and NuMA, a nuclear protein associated with mitotic apparatus (*18*), both of which are associated with microtubule minus ends. Xgrip109 and NuMA were enriched and highly focused in the poles (Fig. 2B). NuMA binds to dynein and dynactin and functions in spindle pole formation by cross-linking and sliding microtubule minus ends

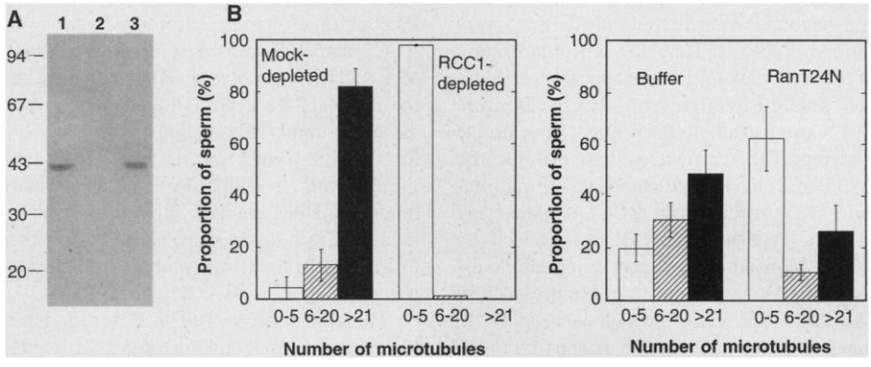

Fig. 1. Requirement of RCC1 for sperm-aster formation. (**A**) Depletion of RCC1 from *Xenopus* egg extracts. Two microliters of egg extracts, untreated (lane 1), RCC1-depleted (lane 2), and mock-depleted (lane 3), was run on an SDS-polyacrylamide (10%) slab gel, transferred to a polyvinylidene difluoride membrane, and probed with antibodies to *Xenopus* RCC1. (**B**) Distribution of the number of microtubules nucleated per sperm DNA. Demembranated sperm was incubated at 24°C for 10 min with egg extracts that had been immunodepleted with antibodies to *Xenopus* RCC1 (RCC1-depleted) or control IgG (mock-depleted) (left) or had been supplemented with RanT24N (42 μM) or buffer alone (right). More than 100 centrosomes were observed in each case. The data and error bars represent averages and standard deviations of values obtained from three different experiments.

Table 1. Aster formation by recombinant hRCC1 and hRan. Recombinant hRCC1 or hRan bound to the indicated nucleotide was added at the indicated final concentration into the RCC1-depleted *Xenopus* egg extracts. After incubation at 24°C for 40 min without sperm, total asters on the cover slips were counted.

	Final concentration (μM)	Numbers of asters per cover glass
RCC1	0.22	16
	2.2	54
RCC1 D182	0.22	0
	2.2	0
RanGTP	24	0
	76	0
	240	67, 75
RanGTPγS	24	358
	76	1046
	240	2734
RanGDP	24	0
	76	0
	240	0

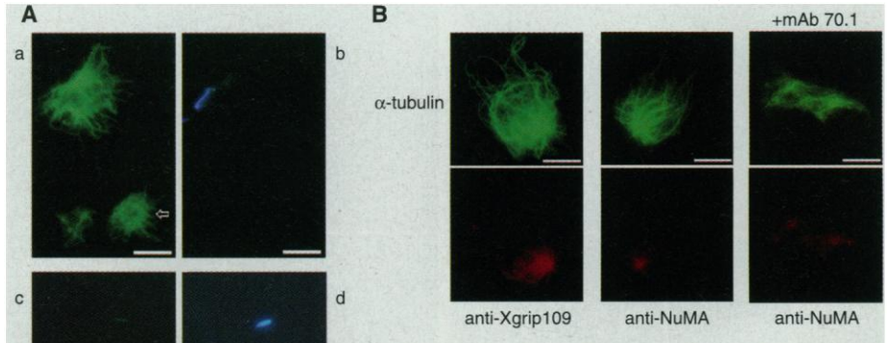

Fig. 2. Aster formation in RCC1-depleted egg extracts induced by RCC1. (**A**) RCC1-depleted egg extracts were preincubated for 30 min with recombinant hRCC1 (2.2 μM final concentration) (**a** and **b**) or buffer alone (**c** and **d**) and then for 10 min at 24°C with demembranated sperm nuclei and stained with mAb to α-tubulin (a and c) or with Hoechst 33342 (b and d). The arrow indicates an aster formed without sperm DNA. Scale bar, 20 μm. (**B**) Recombinant hRCC1 (0.22 μM final concentration) was added to RCC1-depleted egg extracts, and mixtures were incubated without sperm for 40 min at 24°C. Asters on the cover slips were stained with antibodies to Xgrip109 and NuMA as indicated. In the two right-hand panels (+mAb 70.1), the RCC1-depleted extracts were incubated with recombinant hRCC1 (0.22 μM final concentration) for 30 min and with mAb 70.1 to the dynein intermediate chain (Sigma D-5167) (final concentration of 4 μg/ml) for another 10 min at 24°C. Scale bar, 20 μm.

together (*18*). Hence, the presence of NuMA may indicate that the observed self-organization of microtubule asters occurred in a dynein-dependent manner. When a monoclonal antibody (mAb) to the dynein intermediate chain (mAb 70.1) (*17*) was added after the formation of asters, the integrity of asters was disrupted within 10 min (Fig. 2B). Thus, the dynein-dependent translocation of microtubules is apparently required for self-organization of asters induced by addition of RCC1.

To test whether the observed effect of RCC1 was dependent on its RanGEF activity, we prepared recombinant proteins of the RCC1 mutant D182, which has very little RanGEF activity (*16*). When recombinant hRCC1 D182 was added to RCC1-depleted egg extracts, no microtubule asters were formed (Table 1). Thus, the self-organization of microtubules into asters appears to require the RanGEF activity of RCC1. Because RCC1 catalyzes both directions of nucleotide exchange (*2*), we next examined a specific effect of RanGTP and RanGDP on microtubule aster formation in RCC1-depleted egg extracts. When hRanGTP was added to RCC1-depleted egg extracts, microtubule aster formation was induced in the presence of 240 μM of RanGTP, which is roughly 10 times the endogenous Ran concentration of egg extracts (Table 1). At the same concentration, RanGDP induced no aster. Thus, RanGTP, but not RanGDP, is required for microtubule aster formation.

Compared with RanGTP, hRanGTPγS induced aster formation, even in the presence of 24 μM (Table 1). Asters formed after addition of RanGTPγS, however, were very small (Fig. 3), although the number of asters formed was more than 10 times greater than that formed by the addition of RanGTP. NuMA was localized at the centers of asters induced by RanGTPγS (Fig. 3, bottom panels), revealing that microtubules were assembled into poles at the minus ends. The size of asters induced by RanGTPγS did not increase after prolonged incubation. Thus, the hydrolysis of GTP bound to Ran is essential for microtubule elongation. Neither GTP nor GTPγS alone induced microtubule organization at 240 μM. The finding that the inhibition of microtubule assembly caused by RCC1 depletion can be rescued by addition of RanGTP, but not RanGDP, indicates that the Ran GTPase cycle is involved in microtubule assembly. By analogy with Ras (*19*), RanGTP may carry out its function through the effectors to which RanGTP specifically binds. RanBPM is a likely effector, because it is known to bind to RanGTP and antibodies to RanBPM inhibit microtubule aster formation (*6*).

The egg extracts used in this experiment were prepared from unfertilized eggs arrested in metaphase (*11*), and therefore no nuclear membrane was formed during the experiments. Thus, Ran affects microtubule organization independently of its role in the nucleus-cytosol exchange of macromolecules. Consistent with the notion that Ran is involved in mitotic spindle formation, RanGAP is reported to be localized in mitotic spindles (*20*).

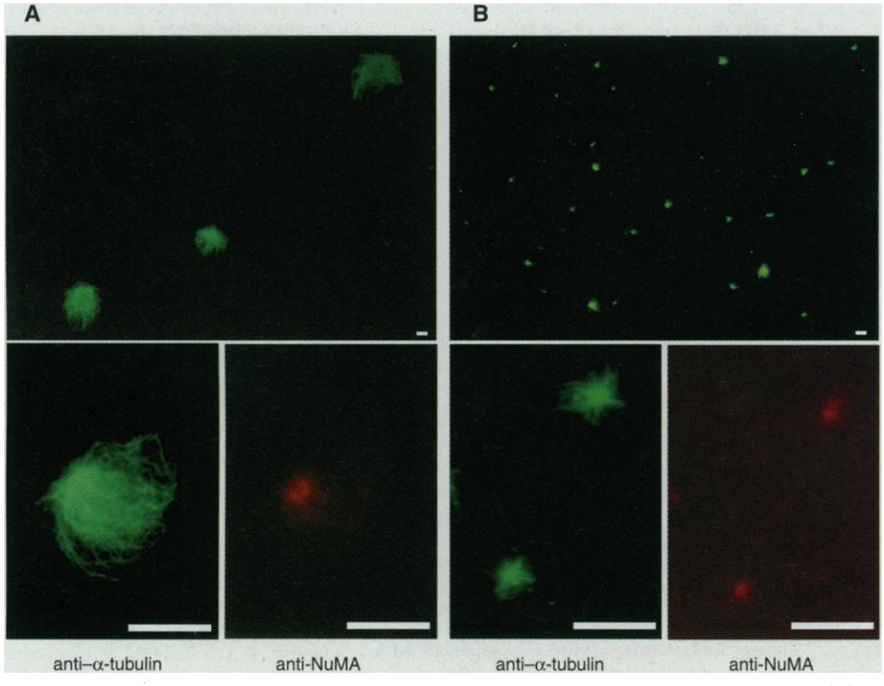

Fig. 3. RanGTP-induced aster formation. Asters formed by addition of recombinant RanGTP (**A**) or RanGTPγS (**B**) (240 μM) to RCC1-depleted egg extracts without sperm were stained with antibodies to α-tubulin (anti–α-tubulin) (green) and NuMA (anti-NuMA) (red) as described (*13*). Note that asters induced by RanGTPγS were small. Scale bar, 20 μm.

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13. Ten microliters of egg extract mixed with 0.3 μl of permeabilized sperm (1000 sperm) was incubated at 24°C for 10 min. The reaction was stopped by addition of 500 μl of permeabilization buffer [80 mM PIPES (pH 6.8), 5 mM EGTA, 1 mM $MgCl_2$, and 0.5% Triton X-100] and centrifuged onto poly-L-lysine–coated cover slips as described (*11*) at 14,000 rpm in a HITACHI RPS40T-816 rotor for 15 min. Samples were fixed by cold MeOH (−20°C) for 5 min, rehydrated with buffer [80 mM PIPES (pH 6.8), 5 mM EGTA, 1 mM $MgCl_2$, 0.1% Triton X-100, and 3% bovine serum albumin] for 15 min, and stained with the indicated primary antibodies and then with the secondary antibodies, fluorescein isothiocyanate–conjugated goat antibody to mouse immunoglobulin G (IgG) (Biosource, Camarillo, CA) or Rhodamine-conjugated goat antibody to rabbit IgG (CAPPEL, Aurora, OH). Sperm chromatin was stained with Hoechst 33342.
14. According to M. Dasso, H. Nishitani, S. Kornbluth, T. Nishimoto, and J. W. Newport [*Mol. Cell. Biol.* **12**, 3337 (1992)], 5 μl of affinity-purified antibodies to *Xenopus* RCC1 (1 mg/ml) [H. Nishitani, H. Kobayashi, M. Ohtsubo, T. Nishimoto, *J. Biochem.* **107**, 228 (1990)] was mixed with 3 μl of protein A–sepharose beads, and the mixture was incubated at 4°C for 2 hours with rotation. After incubation with antibodies, the beads were washed and mixed with 10 μl of *Xenopus* egg extract, and the mixture was incubated for 30 min on ice. The beads were pelleted by centrifugation, and the cytosol was removed and reincubated with fresh beads as described above.
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