Critical role for the EB1 and APC interaction in the regulation of microtubule polymerization

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Human EB1 was originally cloned as a protein that interacts with the COOH terminus of adenomatous polyposis coli (APC) [1]. Interestingly, this interaction is often disrupted in colon cancer, due to mutations in *APC*. EB1 also interacts with the plus-ends of microtubules and targets APC to microtubule tips [2–6]. Since APC is detected on the kinetochores of chromosomes, it has been hypothesized that the EB1-APC interaction connects microtubule spindles to the kinetochores and regulates microtubule stability [7–9]. In yeast, EB1 regulates microtubule dynamics [6, 10, 11], and its binding domain in APC may be conserved in Kar9, an EB1 binding protein involved in the microtubule-capturing mechanism [12–14]. These results suggest that the interaction of EB1 and APC is important and may be conserved. However, it is largely unknown whether the EB1-APC interaction affects microtubule dynamics. Here, we show that EB1 potently promotes microtubule polymerization in vitro and in permeabilized cells, but, surprisingly, only in the presence of the COOH-terminal EB1 binding domain of APC (C-APC). Significantly, this C-APC activity is abolished by phosphorylation, which also disrupts its ability to bind to EB1. Furthermore, yeast EB1 protein effectively substitutes for the human protein but also requires C-APC in promoting microtubule polymerization. Finally, C-APC is able to promote microtubule polymerization when stably expressed in APC mutant cells, demonstrating the ability of C-APC to promote microtubule assembly in vivo. Thus, the interaction between EB1 and APC plays an essential role in the regulation of microtubule polymerization, and a similar mechanism may be conserved in yeast.

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Results and discussion
### C-APC binding confers the ability of EB1 to promote microtubule polymerization

To examine whether EB1 directly affects microtubule polymerization, we produced EB1 as a GST fusion protein (Figure 1a) and incubated it with purified tubulins in a microtubule-stabilizing buffer, followed by the monitoring of microtubule polymerization using a time-lapsed light-scattering assay (Figure 1b) and immunostaining with an anti-tubulin monoclonal antibody (mAb) (Figure 1c) [15]. In this assay, EB1 protein did not promote microtubule polymerization, suggesting that it might require another factor for its microtubule assembly activity.

To examine this possibility, we generated the C terminus of APC (C-APC, 2560–2843 aa) as a His-tagged fusion protein (Figure 1a), which is known to bind EB1, but not microtubules (Figure 2a) [1, 5]. Like EB1, C-APC protein also had no detectable activity when added to purified tubulins in the assembly assay (Figure 1b,c). However, when EB1 and C-APC were incubated together with tubulins, we observed a surprising rise in turbidity in a time-dependent manner, with the potency being close to that of Taxol, a strong inducer of microtubule polymerization (Figure 1b). These results were microscopically confirmed by the formation of organized microtubule fibers, which were only generated in the presence of both EB1 and C-APC (Figure 1c). In contrast, neither the N- (ΔC, 1–106) nor C-terminal (ΔN, 107–268) fragment of EB1 promoted microtubule assembly in the presence of C-APC (data not shown), indicating that full-length EB1 is required for microtubule polymerization. These results demonstrate that EB1 has microtubule assembly activity, but this activity is absolutely dependent on the presence of C-APC.

This synergistic effect of C-APC and EB1 on microtubule polymerization was further examined using permeabilized cells. Permeabilized NIH3T3 cells were incubated with EB1 and tubulins in the presence or absence of C-APC, then fixed at various times and immunostained with the anti-tubulin mAb. After 1 min, short nucleated microtubule asters were observed, despite the presence or absence of C-APC (Figure 1d). However, in cells incubated with C-APC, nucleated microtubules were elongated more rapidly than those in cells lacking C-APC at progressive time points up to 8 min (Figure 1d). These results suggest that C-APC and EB1 act synergistically to increase the elongation speed of microtubules from centrosomes in permeabilized cells.

Figure 1

C-APC binding confers the ability of EB1 to promote microtubule polymerization both in vitro and in permeabilized cells. **(a)** Purified His-C-APC and GST-EB1 were analyzed on SDS-containing gels. **(b,c)** Microtubule assembly induced by EB1 and C-APC in vitro. EB1 and C-APC were incubated separately or together with purified tubulin in microtubule-assembly buffer at 30°C. (b) Microtubule polymerization was measured by changes in turbidity. The turbidity in the presence of either Taxol alone or C-APC and EB1 at 10 min of incubation is defined as 100%. (c) The reaction mixtures at 10 min were transferred onto coverslips, followed by immunostaining with anti-tubulin mAb to examine microtubule polymerization. **(d)** Microtubule polymerization induced by EB1 and C-APC in permeabilized cells. Permeabilized NIH3T3 cells were incubated with EB1 and tubulins in the presence (+) or absence (−) of C-APC and fixed at indicated times, followed by tubulin immunostaining. Centrosomes are indicated by arrows.
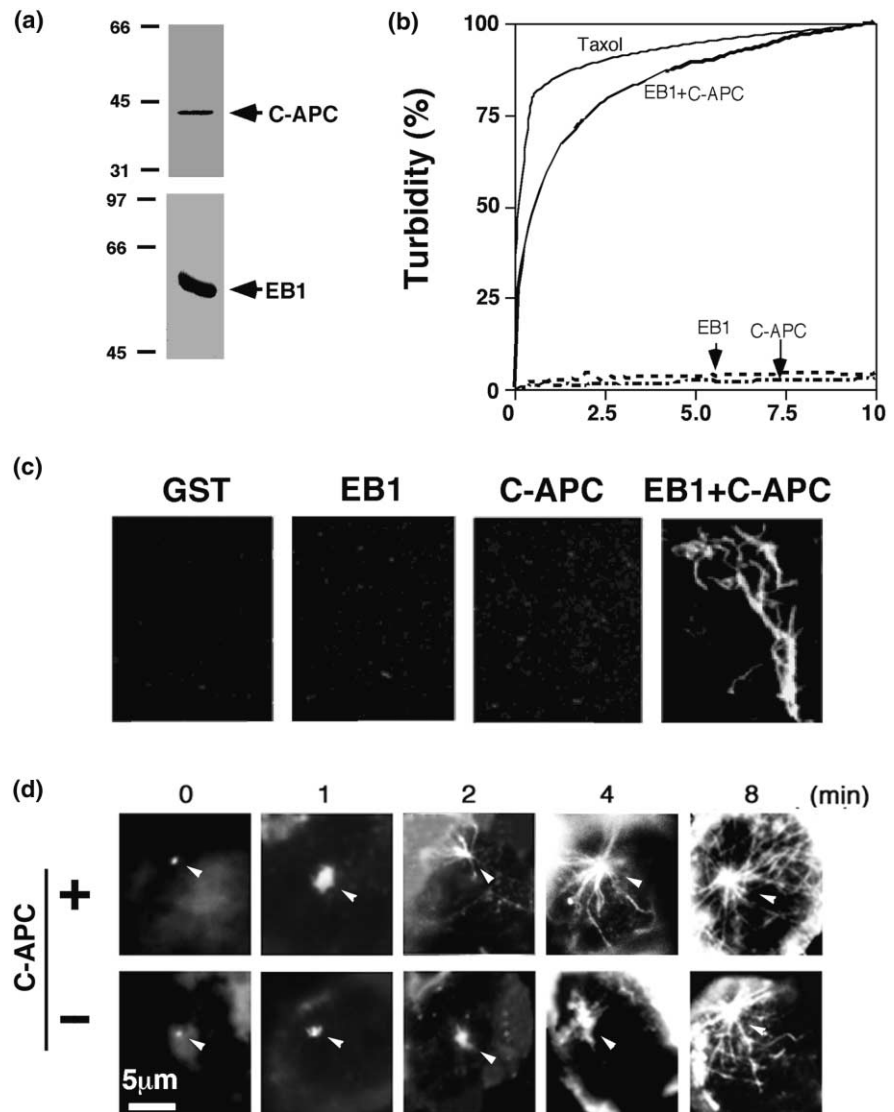

To examine whether the EB1 and C-APC interaction is essential for EB1 to promote microtubule assembly, we used Cdc2, which can phosphorylate APC at two in vivo phosphorylation sites (Figure 2a) and prevent it from binding EB1 [4, 16–18]. When C-APC and EB1 were incubated with Cdc2 in the presence of [^{32}P]-ATP, C-APC, but not EB1, was efficiently phosphorylated (Figure 2b, left panel), as shown previously [18]. To confirm that phosphorylation of C-APC by Cdc2 disrupts its ability to bind to EB1, C-APC was preincubated with either Cdc2 or control buffer, then incubated with EB1 and Taxol-stabilized microtubules, followed by centrifugation to sediment both microtubules and binding proteins. Nonphosphorylated C-APC was cosedimented with microtubules, but only in the presence of EB1 (Figure 2b, right panels),

consistent with C-APC binding of microtubules via EB1. Cdc2- phosphorylated C-APC failed to cosediment with microtubules, even in the presence of EB1 (Figure 2b, right panels), confirming that Cdc2 phosphorylation disrupts the ability of C-APC to bind EB1 [18]. To examine the microtubule assembly activity of phosphorylated C-APC, C-APC protein was first phosphorylated by Cdc2 and then incubated with EB1 and tubulins, followed by the assembly assay and immunostaining with the anti-tubulin mAb. No microtubule assembly activity was detected by either the turbidity assay or immunostaining (Figure 2c,d). These results together indicate that C-APC binding confers the ability of EB1 to promote microtubule assembly and that this APC activity is regulated by Cdc2 phosphorylation.

Figure 2

Cdc2 phosphorylation disrupts the ability of C-APC to bind EB1 and to promote microtubule assembly. **(a)** The APC domain structure. The COOH terminus of APC (2560–2843, C-APC) contains two Cdc2 consensus phosphorylation sites indicated by asterisks. **(b)** The failure of Cdc2-phosphorylated C-APC to bind EB1. EB1 and C-APC were phosphorylated by Cdc2 in the presence of [^{32}P]-ATP and separated on SDS gels, followed by autoradiography (left panel). C-APC protein was incubated with Cdc2 or the control buffer in the presence of cold ATP (middle), and then incubated with GST or GST-EB1 and Taxol-stabilized microtubules, followed by centrifugation to sediment microtubules and its binding proteins. Bound C-APC was detected by immunoblotting analysis (right). **(c,d)** The failure of Cdc2-phosphorylated C-APC to promote microtubule assembly. C-APC was phosphorylated by Cdc2 (C-APC~P) or incubated in control buffer (C-APC) and then subjected to a microtubule polymerization assay in the presence or absence of EB1, as determined by (c) turbidity changes or (d) tubulin immunostaining.
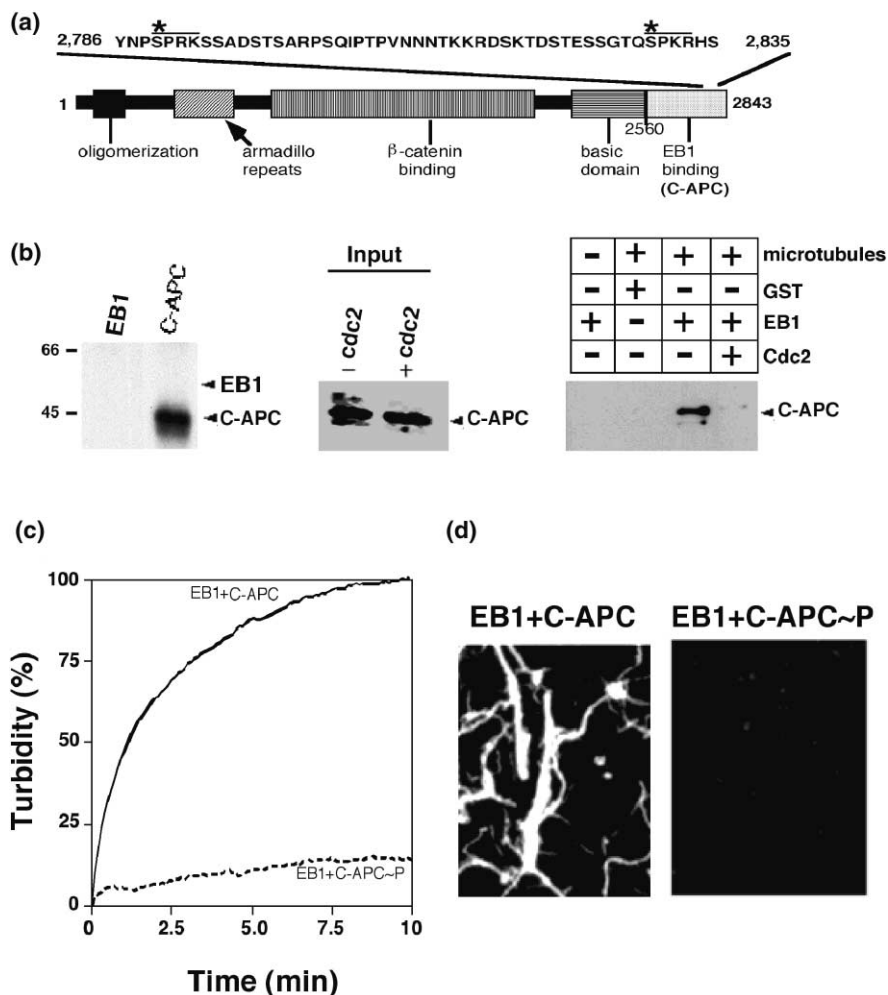
Synergy of EB1 and C-APC on microtubule polymerization is conserved

Since human *EB1* can rescue the *EB1* deletion in yeast [10], we examined whether Mal3 substitutes for human EB1 in the microtubule assembly assay. When purified GST-Mal3 and its truncation mutant (Figure 3a) were used in the microtubule assembly assay in the presence or absence of C-APC protein, like EB1, Mal3 alone failed to promote microtubule polymerization, but potently induced microtubule assembly in the presence of C-APC (Figure 3b,d). In contrast, the C-terminal deleted mutant of Mal3, Mal3mt (1–130 aa), had no activity in the presence of C-APC (Figure 3c), as is the case for the EB1 mutants (data not shown). These results demonstrate that C-APC can also confer the ability of Mal3 to promote microtubule polymerization. Since yeast does not have an *APC* homolog, it remains unclear which yeast protein interacts with Mal3 to promote microtubule assembly. Interestingly, Kar9p, a yeast EB1 binding protein involved in the microtubule-capturing mechanism, shows a significant sequence similarity to the EB1 binding sequences

in APC [12]. It would be interesting to examine whether Kar9 or other EB1 binding proteins can confer the ability of the yeast EB1 protein to promote microtubule assembly.

C-APC promotes microtubule assembly in vivo

To investigate whether the interaction of EB1 and C-APC affects microtubule assembly in vivo, we expressed C-APC in the SW480 colon cancer cell line, which contains a mutation in the *APC* gene, resulting in a C-terminal deletion, which includes the EB1 binding domain [2]. SW480 cells were transfected with the vector GFP or GFP-C-APC, and stable cell lines were generated by G418 selection. To examine the localization of both EB1 and C-APC, cells grown on coverslips were incubated on ice for 1 hr to depolymerize microtubules and shifted to warm medium for 1 min, followed by immunostaining with anti-EB1 or anti-tubulin antibodies. GFP-C-APC colocalized with EB1 on elongating microtubule plus-ends (Figure 4a,b), which is consistent with the previous findings that EB1 targets APC to the microtubule distal tips [2–6].

Figure 3

C-APC confers the ability of the yeast EB1 protein Mal3 to promote microtubule polymerization in vitro. **(a)** Mal3 and its C-terminal truncated mutant were purified and analyzed on SDS gels. **(b–d)** Microtubules induced by Mal3 and C-APC. (b,d) C-APC and Mal3 or (c,d) its mutant were incubated either separately or together with tubulins and subjected to the microtubule polymerization assay, as determined by (b,c) turbidity changes or (d) tubulin immunostaining.
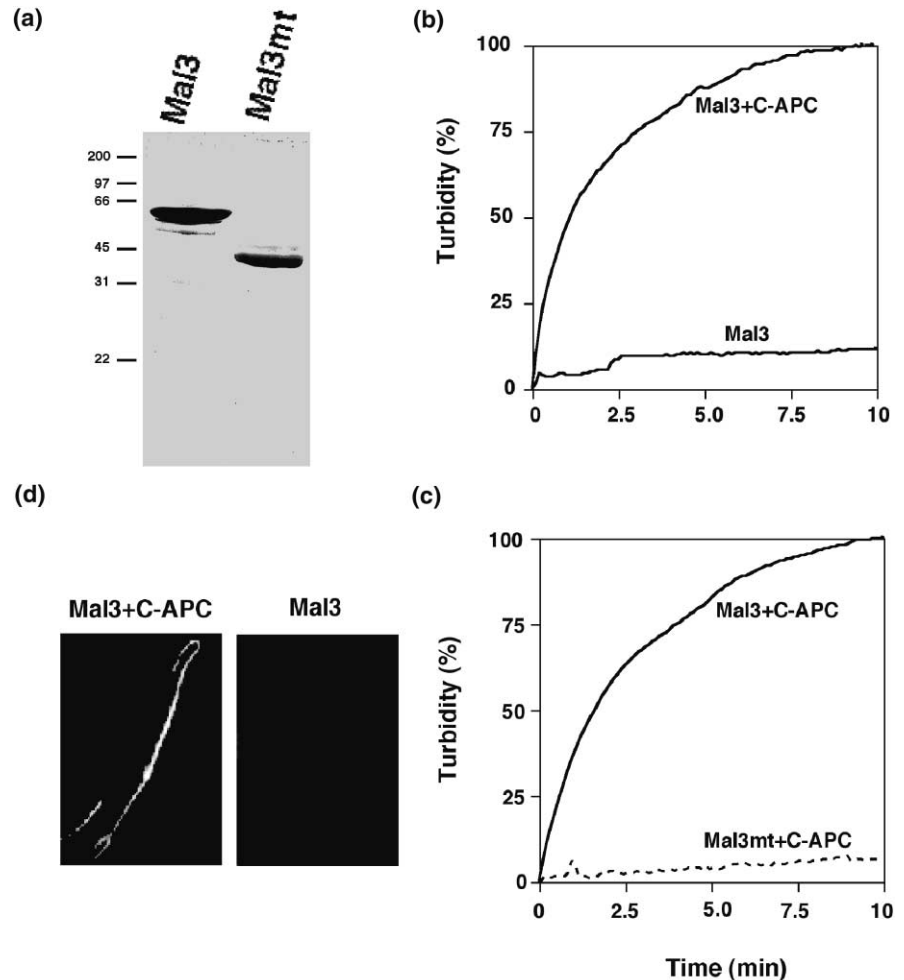
To examine microtubule assembly activity in these SW480 cells stably expressing GFP-C-APC, they were incubated on ice for 1 hr and subjected to anti-tubulin immunostaining either directly or following incubation in warm media (30°C) for different times. As shown previously [19], microtubules were depolymerized after 1 hr on ice (Figure 4c). At the 1 min time point following incubation in the temperature shift, the GFP control cells contained rather short microtubules (Figure 4c). In contrast, most SW480 cells stably expressing C-APC had much longer microtubules (Figure 4c). We quantified these differences by counting the number of interphase cells with microtubule-asters that had a diameter longer than 5 μm. More than 80% of C-APC-expressing cells, but less than 15% of GFP-expressing cells, had asters longer than 5 μm after 1 min at warm media (Figure 4d). However, at the 5 min time point following the temperature shift, no obvious difference in the density or the length of microtubules was observed between the control GFP and GFP-C-APC-expressing cell lines; both had extended asters long enough to reach the periphery of the cells. This results indicates that the differences in C-APC- and vector control-expressing cells at 1 min are not due to the defects in the nucleation from centrosomes or the quantity of tubulin heterodimers, but rather due to the differences in the microtubule-polymerizing activity in these cells. These results demonstrate that C-APC promotes microtubule assembly in vivo and indicate that APC mutant cells have a defect in microtubule assembly.

In mammalian cells, the interaction of EB1 and APC targets APC to the microtubule distal tips, and this targeting mechanism is thought to allow APC to modulate microtubule dynamics [4, 20–22]. EB1 regulates microtubule dynamics in vivo in yeast, and human *EB1* can functionally replace the *Mal3* gene in fission yeast, indicating the functional conservation of EB1 proteins [10]. Our surprising finding that EB1 promotes microtubule polymerization only in the presence of its binding protein, APC, uncovers a unique feature of EB1. It suggests that the ability of EB1 to regulate microtubule polymerization is likely to depend on its binding proteins such as APC,

Figure 4
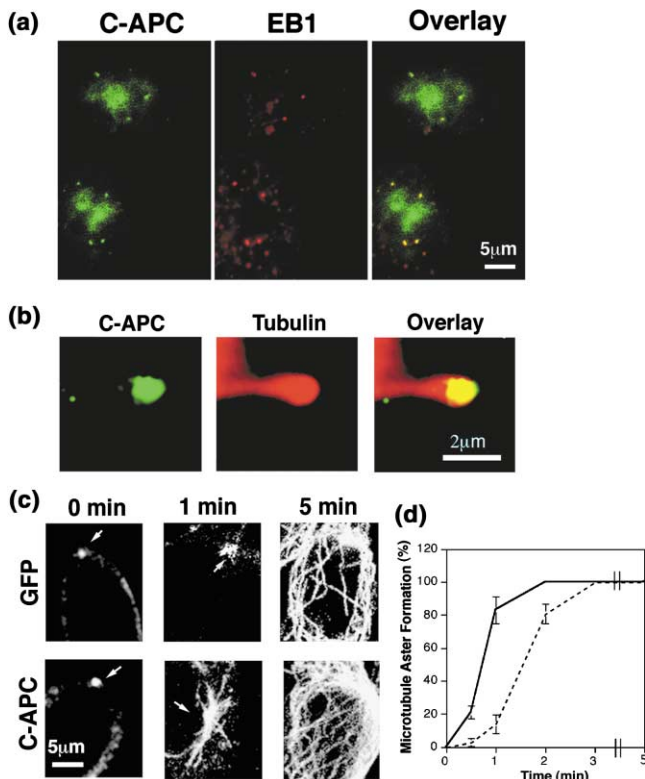
C-APC promotes microtubule assembly in APC mutant cells. **(a,b)** The colocalization of EB1 and C-APC on microtubule distal tips. SW480 cell lines stably expressing GFP or GFP-C-APC (green) were incubated on ice for 60 min to depolymerize microtubules and then at 30°C for 1 min, followed by immunostaining with (a) anti-EB1 or (b) anti-tubulin antibodies. Note, control GFP was not localized at microtubules (data not shown). **(c,d)** Microtubule assembly promoted by C-APC in vivo. Stable cells were treated as described in Figure 4 a,b and incubated at 30°C for various times indicated. (c) Cells were fixed and stained by the anti-tubulin mAb, followed by confocal microscopy. Cells containing microtubule asters with a diameter longer than 5 μm were scored. (d) The results from a representative experiment in triplicate are shown. Centrosomes are indicated by arrows.

which may provide a new mechanism to control microtubule dynamics.

Interestingly, EB1 and APC are reported to play an important role in a microtubule-capture mechanism, in which the EB1-APC protein complex may connect the ends of spindle microtubules to the kinetochores of chromosomes [8]. It has been shown that EB1 is required for the APC-mediated attachment of microtubules to kinetochores and that the localization of APC on kinetochores is lost when the microtubules become depolymerized [7–9]. If the EB1-APC complex regulates microtubule polymerization, it may stabilize the localization of APC on kinetochores and stabilize the connection of microtubules and kinetochores with other microtubule binding domains of APC.

Yeast EB1 and its binding protein Kar9 also play a crucial role in a similar microtubule-capture mechanism during nuclear migration [13, 14]. Furthermore, it has been reported recently that the EB1 binding sequence in APC is conserved in the EB1 binding site in Kar9p [12]. Consistently, we here show that C-APC can also confer the ability of Mal3 to promote microtubule polymerization. These results suggest that, in the microtubule-capture mechanism, EB1 may capture kinetochores and cell cortex and also change the dynamics of microtubules on captured chromosomes or cell cortex through its binding partners, such as APC or Kar9p. This may link the microtubule-capture mechanism to microtubule dynamics.

In summary, we demonstrate that human and yeast EB1 proteins potently promote microtubule assembly only when bound to the C terminus of APC. Furthermore, this activity of the EB1-APC complex is regulated by phosphorylation. Finally, stable expression of the C-terminal APC fragment in APC mutant cells promotes microtubule polymerization, demonstrating its ability to promote microtubule assembly in vivo. These results indicate that the interaction between EB1 and APC plays an essential role in the regulation of microtubule polymerization in vitro and in vivo, and a similar mechanism may also be conserved in yeast.

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