Formation of a Preinitiation Complex by S-phase Cyclin CDK–Dependent Loading of Cdc45p onto Chromatin

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Cdc45p, a protein essential for initiation of DNA replication, associates with chromatin after "start" in late G_1 and during the S phase of the cell cycle. Binding of Cdc45p to chromatin depends on Clb-Cdc28 kinase activity as well as functional Cdc6p and Mcm2p, which suggests that Cdc45p associates with the prereplication complex after activation of S-phase cyclin-dependent kinases (CDKs). As indicated by the timing and the CDK dependence, binding of Cdc45p to chromatin is crucial for commitment to initiation of DNA replication. During S phase, Cdc45p physically interacts with minichromosome maintenance (MCM) proteins on chromatin; however, dissociation of Cdc45p from chromatin is slower than that of MCMs, which indicates that the proteins are released by different mechanisms.

In the yeast *Saccharomyces cerevisiae*, the chromatin structure at origins of DNA replication alters between the prereplication complex (pre-RC) in G_1 and the post-RC in S, G_2, and M phases of the cell division cycle (*1*–*8*). The origin recognition complex (ORC), an initiator protein for DNA replication, is present in both pre- and post-RCs (*2*, *3*), whereas Cdc6p and MCM proteins are associated with the pre-RC and then are released during S phase (*3*–*7*). Genomic footprints of pre-RCs were detected at both active and inactive chromosomal origins (*3*), which suggests that additional proteins are required to form an RC that is competent for initiation. To initiate DNA replication, the pre-RC needs to be activated in late G_1 and at the G_1-S transition by a second set of factors, including the S-phase cyclin CDKs (Clb-Cdc28) and Cdc7-Dbf4 protein kinase (*8*). Unlike cytokinesis and spindle-pole duplication, S phase depends on activation of Clb-Cdc28 kinase activity by the SCF (Skp1/Cdc53/F-box protein) complex that degrades the CDK inhibitor Sic1p after "start" (*8*, *9*). But how origins are committed to initiation by the S-phase cyclin CDKs is not known.

Like ORC, Cdc6p, and the MCMs, Cdc45p is essential for initiation of DNA replication (*10*, *11*). A mutation in *CDC45* interacts genetically with mutations in genes encoding ORC and MCM proteins as well as with replication origins (*11*). To test whether Cdc45p bound to chromatin like the known pre-RC components, we prepared yeast extracts by detergent lysis and isolated a chromatin-enriched fraction by low-speed centrifugation (*7*, *12*). Less than half the Cdc45p was sedimented (Fig. 1A), but like Orc3p, a subunit of ORC, some of the Cdc45p was released with polynucleosomes by limited micrococcal nuclease (MNase) digestion. The released Cdc45p was sedimented by high-speed centrifugation, showing that it remained tightly bound to polynucleosomes. Moreover, extended MNase digestion resulted in complete release of Cdc45p from the low-speed sediment (*13*), indicating that all the Cdc45p in this fraction was associated with chromatin.

To examine the chromatin association of Cdc45p during the cell cycle, we synchronized cells in G_1 with α factor or in mitosis by a temperature-sensitive allele of *DBF2* (*1*), and then we released the cells into the cell cycle (*14*). Cdc45p was present in the supernatant fraction throughout the cell cycle (Figs. 1B and 2A). The amount of Cdc45p in the supernatant was low after prolonged incubation in α factor, but this probably was not due to normal cell cycle regulation because no decline of Cdc45p was observed in the G_1 phase of the second cell cycle or in G_1 after release from a *dbf2*

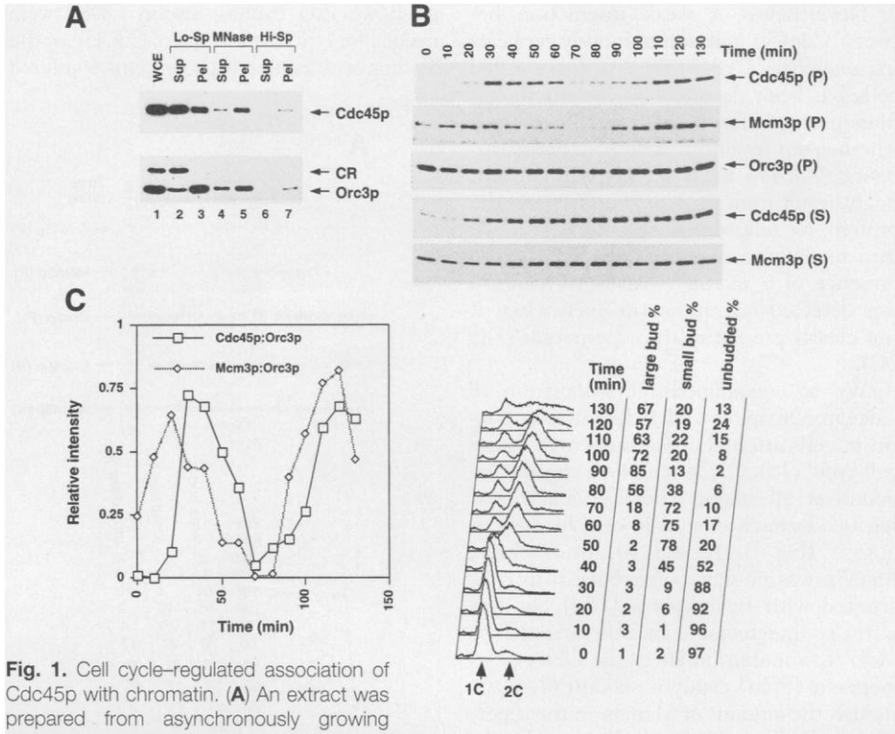

Fig. 1. Cell cycle–regulated association of Cdc45p with chromatin. (**A**) An extract was prepared from asynchronously growing wild-type cells that expressed Cdc45-HA3p and was fractionated as described in (*7*). Cdc45-HA3p and Orc3p were detected by immunoblotting (*12*). WCE, whole-cell extract; Lo-Sp, low speed; MNase, micrococcal nuclease; Hi-Sp, high speed; Sup, supernatant; Pel, sedimented material; CR, a soluble, cross-reacting protein detected by the antibody to Orc3p. (**B**) Cell cycle regulation of Cdc45 association with chromatin. Wild-type cells were arrested in G_1 with α factor (10 μg/ml) for 3 hours at 30°C and then released at 25°C. Cells withdrawn at the indicated time points were analyzed for DNA content and budding index (lower), and extracts were fractionated (*14*). Cdc45-HA3p, Mcm3p, and Orc3p present in the supernatant (S) or sediment (P) were detected by immunoblotting (upper) (*12*). (**C**) Distinct patterns of association of Cdc45p and Mcm3p with chromatin. The intensity of individual Cdc45-HA3p, Mcm3p, and Orc3p bands (P fractions) on the immunoblot shown in (B) was quantified with the IS-1000 digital imaging system. Relative intensity refers to the signal ratios of either Cdc45-HA3p to Orc3p or Mcm3p to Orc3p at the indicated times.

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arrest. Orc3p was present in the chromatin fraction throughout the cell cycle, whereas Mcm3p bound to chromatin during exit from mitosis and then gradually came off during S phase (Figs. 1B and 2A). Cdc45p was not associated with chromatin in α factor–arrested cells but bound to chromatin in late G_1, remained bound during S phase, and gradually dissociated from chromatin in late S or G_2 (Fig. 1B). After release from a *dbf2* block, Cdc45p bound to chromatin after Mcm3p at the time when small buds emerged (Fig. 2A). Although dissociation of Cdc45p from chromatin was slower than that of Mcm3p, most if not all of it was released by mitosis (Figs. 1C and 2B).

In α factor–arrested cells, both Cdc6p and MCMs are bound to chromatin at origins of replication, and the pre-RC footprint can be detected (*1*). As Cdc45p is not associated with chromatin in α factor–arrested cells, our data suggest that Cdc45p is not a component of the pre-RC. Indeed, the pre-RC footprint was detected in *cdc45* mutant cells arrested at the nonpermissive temperature (*15*).

Nevertheless, a weak interaction between Cdc45p and autonomously replicating sequences (ARSs) in α factor–arrested cells has been detected by chromatin immunoprecipitation (CHIP) (6). To address whether our inability to detect chromatin-bound Cdc45p in α factor–arrested cells might result from the low abundance of the protein, we released cells from a *dbf2* block into medium containing α factor. In the presence of α factor, virtually no Cdc45p was detected on chromatin even when it was clearly present in the supernatant (Fig. 2C).

We also examined the association of Cdc45p, Orc3p, and Mcm3p with chromatin in cells arrested at various stages in the cell cycle (*16*). Orc3p remained chromatin-bound at all stages, whereas Mcm3p was released from chromatin in cells blocked in mitosis (Fig. 3, A and B). In S phase, Mcm3p was present on chromatin in cells arrested with hydroxyurea (HU) but was virtually undetectable in cells arrested by *cdc17-1*, a mutant allele of the DNA polymerase α (Polα) catalytic subunit (*17*). Although the amount of Mcm3p in the supernatant also declined in *cdc17*-arrested cells, the decrease in the chromatin fraction was more pronounced (Fig. 3B). Thus, the association of Mcm3p with chromatin in S phase may depend on its interaction with Polα.

Cdc45p was absent on chromatin from G_1 cells arrested by *cdc28-4* or α factor but was clearly bound to chromatin in cells arrested by *cdc7-1* at the G_1-S boundary (Fig. 3A). Thus, chromatin binding of Cdc45p is temporally distinct from formation of the pre-RC. Furthermore, Cdc45p loading is independent of Cdc7 protein kinase activity but, in the absence of functional Cdc7p, binding of Cdc45p to chromatin is insufficient to convert the pre-RC into the post-RC. In contrast to Mcm3p, Cdc45p remained bound to chromatin in cells arrested by *cdc17-1* (Fig. 3A), which suggests that Mcm3p was not required to maintain association of Cdc45p with chromatin in S phase. In cells arrested in G_2 by *cdc13*, or at G_2-M by nocodazole, a small amount of chromatin-bound Cdc45p remained detectable. No Cdc45p was detected, however, on the chromatin from cells arrested in late mitosis by *dbf2* or *cdc15*.

The absence of chromatin-bound Cdc45p in *cdc28*-arrested cells might mean that kinase activity of Cdc28 is required for loading Cdc45p onto chromatin or, alternatively, that the cells were arrested before Cdc45p could be loaded. The amount of chromatin-bound Cdc45p was also very low in *cdc34*-arrested cells (Fig. 3, A and B), which failed to degrade Sic1p and therefore lacked Clb-Cdc28 activity (*18*). To distinguish whether S-phase cyclin CDKs were needed for efficient loading of Cdc45p or the loading was inhibited by proteins stabilized in the *cdc34* mutant, we analyzed Cdc45p loading in a *clb5,6* mutant strain. In this mutant, G_1 cyclin CDKs (Cln-Cdc28) become active on schedule and induce bud formation, but Clb-Cdc28 activation is postponed, causing a delay in S phase (*19*). After the *clb5,6* cells were released from an α factor block, chromatin binding of Cdc45p was delayed relative to budding (Fig. 3C), which was independent of Clb-Cdc28 activation. Together, these results suggest that Cdc45p loading requires activation of Clb-Cdc28 in late G_1. Moreover, the G_1 cyclin CDKs cannot induce Cdc45p loading.

Establishment of the pre-RC requires a period of low CDK activity (*20*). The opposite requirement for Clb-Cdc28 activity and the difference in timing clearly distinguish the binding of Cdc45p to chromatin from that of the pre-RC components.

To test whether the binding of Cdc45p to chromatin depends on the pre-RC, we arrested wild-type, *cdc6*, and *mcm2* cells (*16*) with nocodazole at G_2-M and then released them into HU-containing medium at either 25° or 37°C. After nocodazole arrest, a small amount of Cdc45p was detected on chromatin in all three strains (Fig. 4A). In wild-type cells, Cdc45p was efficiently loaded onto chromatin after pas-

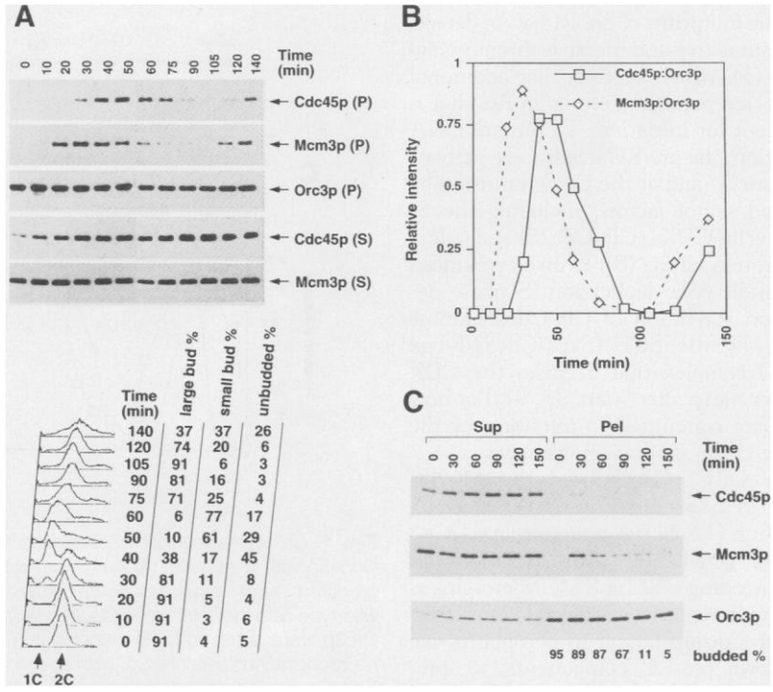

Fig. 2. Cell cycle timing of association of Cdc45p with chromatin. (**A**) Binding of Cdc45p to chromatin after that of Mcm3p. Cdc45-HA3p–expressing *dbf2* cells (*16*) were arrested for 2 hours at 37°C and then released at 25°C. Cells withdrawn at the indicated times were subjected to chromatin fractionation and immunoblotting (upper) (*14*) and were analyzed for DNA content and budding index (lower). (**B**) Relative intensity of Cdc45-HA3p and Mcm3p to that of Orc3p (P fractions) on the immunoblot shown in (A). (**C**) Cdc45p is not on chromatin at the α factor block. *dbf2* cells were arrested and then released into medium containing α factor at 25°C. Immunoblots of the supernatant and sedimented fractions are shown with the budding index.

sage through G_1 at both temperatures. In contrast, Cdc45p loading was reduced in *cdc6* and *mcm2* cells at 37°C, showing the requirement of functional Cdc6p and Mcm2p. Given that Cdc6p and the MCMs are present in the pre-RC (*4–7*), formation of pre-RC appears likely to be a prerequisite for Cdc45p loading. We therefore examined whether Cdc45p physically interacted with the pre-RC components. Cdc45p coimmunoprecipitated with Mcm2p, Mcm3p, and Mcm5p from extracts derived from asynchronously growing cells (*13*). Consistent with these findings, Cdc45p interacts with Mcm5p and Mcm7p and comigrates with large MCM-containing complexes over a gel-filtration column (*21*).

Only a fraction of MCM proteins are present in the pre-RC during G_1; the bulk of them remain free from chromatin throughout the cell cycle (Figs. 1 and 2) (*7, 22*). Therefore, binding to MCMs does not necessarily indicate association with the pre-RC. To test whether Cdc45p interacted with MCMs on chromatin, we immunoprecipitated both the supernatant and the sedimented fractions derived from cells synchronized with α factor or HU (*23*). Deoxyribonuclease I (DNase I) was used to release chromatin-bound proteins in the sediments. Cdc45p coprecipitated with Mcm2p from both the supernatant and the sedimented fractions derived from HU-blocked cells but not α factor–arrested cells (Fig. 4B). Thus, Cdc45p interacts with Mcm2p on and off the chromatin in a cell cycle–regulated manner.

In a *dbf2* block-and-release time course, Cdc45p coprecipitated with Mcm2p and Mcm5p specifically in late G_1 and S phase (Fig. 4C) (*13*). Furthermore, Mcm2p coprecipitated with Cdc45p during the same portion of the cell cycle. In late G_1, when the pre-RC is present, Cdc45p interacts with MCMs on chromatin. However, the interaction also occurs in the supernatant and persists throughout S phase, indicating that the stability of the Cdc45p-MCM complex does not rely on the presence of the pre-RC. Indeed, in the whole cell extract, Cdc45p remained associated with MCMs in late S phase after most of the MCMs had dissociated from the chromatin. It could be that Cdc45p or certain MCMs are specifically modified during this period of the cell cycle so that they can form a complex on and off the chromatin.

Given that several MCMs associate specifically with ARSs in late G_1 (*5, 6*), our results suggest that Cdc45p physically associates with the pre-RC before it is transformed into the post-RC. However, our data do not rule out the presence of Cdc45p elsewhere on chromatin. Cdc45p was shown to associate with ARSs in a CHIP assay (6), but we suggest that this occurs when Cdc45p is loaded onto chromatin in late G_1, after activation of the S-phase–specific cyclin CDKs. Binding of Cdc45p to the pre-RC might activate the proteins in the complex or recruit additional factors to the origins. The sequential loading of Cdc6p, MCMs, and Cdc45p to replication origins throughout late mitosis and G_1 reveals a cascade of protein assembly and acquisition of origin competence.

After initiation of replication and throughout the remaining cell cycle, Clb-Cdc28 kinases block reinitiation of DNA replication during a single cell cycle by interfering with Cdc6p function (*7, 8, 24*). Paradoxically, Clb-Cdc28 activity is also essential for activating initiation of DNA replication, although how this occurs is not clear. One positive role for the S-phase cyclin CDKs in initiation of DNA replica-

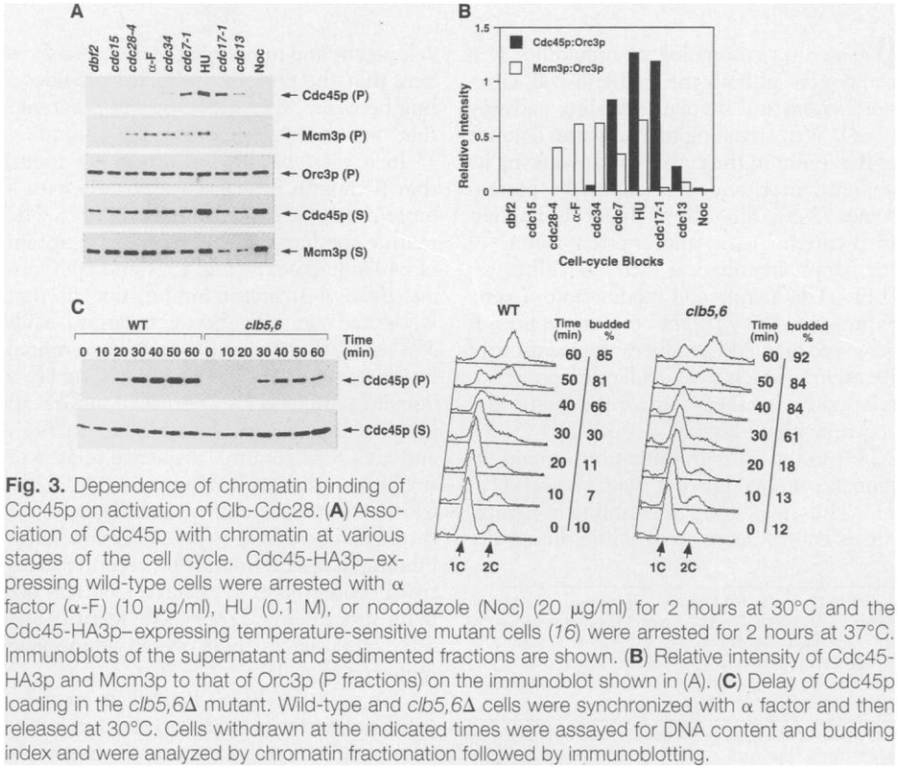

Fig. 3. Dependence of chromatin binding of Cdc45p on activation of Clb-Cdc28. (**A**) Association of Cdc45p with chromatin at various stages of the cell cycle. Cdc45-HA3p–expressing wild-type cells were arrested with α factor (α-F) (10 μg/ml), HU (0.1 M), or nocodazole (Noc) (20 μg/ml) for 2 hours at 30°C and the Cdc45-HA3p–expressing temperature-sensitive mutant cells (*16*) were arrested for 2 hours at 37°C. Immunoblots of the supernatant and sedimented fractions are shown. (**B**) Relative intensity of Cdc45-HA3p and Mcm3p to that of Orc3p (P fractions) on the immunoblot shown in (A). (**C**) Delay of Cdc45p loading in the *clb5,6*Δ mutant. Wild-type and *clb5,6*Δ cells were synchronized with α factor and then released at 30°C. Cells withdrawn at the indicated times were assayed for DNA content and budding index and were analyzed by chromatin fractionation followed by immunoblotting.

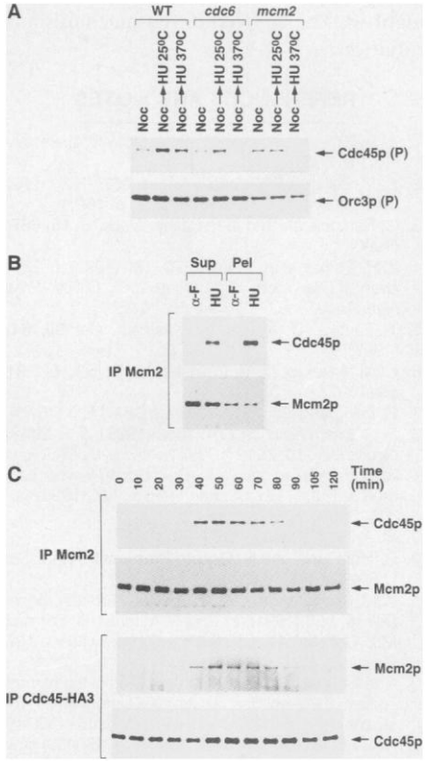

Fig. 4. Cell cycle–regulated binding of Cdc45p to Mcm2p. (**A**) Binding of Cdc45p with chromatin depends on functional Cdc6p and Mcm2p. Cdc45-HA3p–expressing wild-type, *cdc6,* and *mcm2* cells were arrested with nocodazole for 2 hours at 25°C and then released into medium containing HU at either 25° or 37°C. Cells were harvested 2 hours after release and then analyzed by chromatin fractionation and immunoblotting. (**B**) Binding of Cdc45p to Mcm2p on chromatin. Wild-type cells were arrested with either α factor or HU for 2 hours at 30°C. The low-speed supernatant and sedimented fractions were immunoprecipitated with the Mcm2p monoclonal antibody in the presence of DNase I (*23*). (**C**) Interaction of Cdc45p with MCMs in late G_1 and S phase. Cdc45-HA3p–expressing *dbf2* cells were arrested and released as described in Fig. 2A. Whole-cell extracts were immunoprecipitated in the presence of DNase I (*23*).

tion is loading of Cdc45p onto the pre-RC at active replication origins. This may create a new protein complex (the preinitiation complex; pre-IC) that then needs only Cdc7-Dbf4 protein kinase activity for firing of the origin and actual DNA synthesis. The association of Cdc45p with chromatin occurs at the G_1-S transition as CDKs phosphorylate Orc6p (*7*, *25*), and as Cdc6p loses its ability to promote MCM loading onto DNA (*26*). Because Cdc6p and the S-phase cyclin CDKs interact (*25–27*), it is possible that Cdc45p loading might be mediated by the control of Cdc6p function by cyclin CDKs. If this were to occur at each origin coincident with initiation of DNA replication at that site, then the mechanism that restricts replication to once per cell cycle might well be coupled to the mechanism of initiation.

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12. Cdc45p was tagged with three copies of the hemagglutinin (HA) epitope at its COOH-terminus. The strains generated (YB0469, *MAT***a**, *cdc45:: CDC45-HA3-LEU2*; YB0470, *MAT*α, same as YB0469) grew identically to the parental strain (W303-1) at 25° and 37°C. Extract preparation, centrifugal fractionation, and MNase digestion were done essentially as described in (*7*), except that the extraction buffer contained 1% Triton X-100. Samples were resolved on 10% SDS-polyacrylamide gels. Cdc45-HA3p, Orc3p, Mcm2p, and Mcm3p were detected with monoclonal antibodies 12CA5 (HA antibody), SB3 (Orc3p antibody), Mcm239, and Mcm34, respectively.
13. L. Zou and B. Stillman, unpublished results.
14. Cells (~4 × 10^7) were collected at each time point. Low-speed supernatant and sedimented fractions were prepared as described in (*7*).
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16. The following Cdc45-HA3p–expressing mutants are in the W303 strain background. YB0471, *cdc6-1*; YB0472, *cdc7-1*; YB0473, *cdc15-2*; YB0474, *cdc28-4*; YB0475, *cdc34*; YB0476, *mcm2*; YB0477, *Clb5::URA3, Clb6::LEU2*. The following Cdc45-HA3p–expressing strains are not in the W303 strain background: YB0478, *cdc13*; YB0479, *cdc17-1*; YB0480, *dbf2-2*. All these strains exhibit temperature sensitivity similar to the parental mutant strains.
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23. Whole cell extract for each immunoprecipitation was prepared from ~5 × 10^8 cells. Immunoprecipitation was done essentially as described in (*10*) except that 150 Kunitz units of DNase I (Sigma) was added to each reaction mixture. Antibodies used were 12CA5 (for Cdc45-HA3p) and Mcm229. For the immunoprecipitations from supernatant and pellet fractions, spheroplasts were prepared as described in (*7*), washed four times with wash buffer, and then lysed in lysis buffer (*10*) with 1% Triton X-100. The low-speed supernatant and the suspension of the sedimented material were subjected to immunoprecipitation as described above.
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28. We thank S. Bell and M. Akiyama for antibodies, C. Liang for advice on chromatin fractionation, and J. Chong, A. Verreault, and H. Yang for critical reading of the manuscript. Supported by a grant from NIH (GM45436 to B.S.).

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Functional Interaction of an Axin Homolog, Conductin, with β-Catenin, APC, and GSK3β

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Control of stability of β-catenin is central in the wnt signaling pathway. Here, the protein conductin was found to form a complex with both β-catenin and the tumor suppressor gene product adenomatous polyposis coli (APC). Conductin induced β-catenin degradation, whereas mutants of conductin that were deficient in complex formation stabilized β-catenin. Fragments of APC that contained a conductin-binding domain also blocked β-catenin degradation. Thus, conductin is a component of the multiprotein complex that directs β-catenin to degradation and is located downstream of APC. In *Xenopus* embryos, conductin interfered with wnt-induced axis formation.

β-Catenin, a homolog of armadillo, is a component of both the cadherin cell adhesion system and the wnt signaling pathway (*1–4*). Wnt signaling increases the amount of β-catenin in the cytosol by preventing its ubiquitination and degradation by proteasomes (*3–5*). This allows direct interaction of β-catenin with transcription factors of the lymphoid enhancer factor–T cell factor (LEF-TCF) family and modulation of gene expression (*6*, *7*). The tumor suppressor gene product APC induces degradation of β-catenin, which is dependent on phosphorylation by the serine-threonine kinase glycogen synthase kinase 3β (GSK3β) (*8*, *9*). APC and β-catenin mutations found in human tumors prevent degradation (*10*, *11*). Thus, regulating the stability of β-catenin is central for wnt signaling during development and tumor progression. We show here that the protein conductin provides a link between APC, GSK3β, and β-catenin that modulates degradation of β-catenin.

In a yeast two-hybrid screen we found that β-catenin interacts specifically with a protein that we named conductin (*12*). The murine conductin cDNA encodes a protein of 840 amino acids (Fig. 1, A and B). Conductin has a β-catenin binding domain that is located centrally between amino acids 396 and 465, it contains an NH_2-terminal RGS [regulator of G protein signaling (*13*)] domain (amino acids 78 to 200), a GSK3β binding domain (amino acids 343 to 396), and a COOH-terminal sequence related to the protein dishevelled [amino acids 783 to 833 (*14*)]. In β-catenin, armadillo repeats 3 through 7 are responsible for conductin binding. Conductin and the recently identified protein axin (*15*) have identical domain structures and show 45% identity and 58% similarity in their overall amino acid sequence (Fig. 1A).

We used the RGS domain in an independent yeast two-hybrid screen and isolated several interacting protein fragments

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