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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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 L. Zou and B. Stillman, unpublished results.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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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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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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that derive from the tumor suppressor gene product APC (Fig. 1, B and C). The fragments (APC #1 to #4) encode three nonoverlapping sequences within the 20–amino acid repeat region; APC #2 and APC #3 do not contain 20–amino acid repeats. Each fragment contains a previously unidentified repeated element, a SAMP (Ser-Ala-Met-Pro) sequence, in its core (Fig. 1C). The interaction between APC and conductin occurs through these SAMP elements because mutation of SAMP to AALP (Ala-Ala-Leu-Pro) abolished binding to conductin (*16*). Yeast two-hybrid analysis indicated that the GSK3β binding domain of conductin is close to the β-catenin binding site (Fig. 1B).

Interaction of conductin with β-catenin, APC, and GSK3β in mammalian cells was also demonstrated by coimmunoprecipitation (*17*). Conductin–β-catenin complexes could be precipitated from transiently transfected Neuro2A cells with antibodies to either protein (Fig. 2A, upper panels). Endogenous conductin–β-catenin complexes were observed in SW48 colon carcinoma (Fig. 2A, lower panels). Interaction of conductin with the APC fragments was also observed by coimmunoprecipitation from transiently transfected Neuro2A cells (Fig. 2B); β-catenin was coprecipitated through its association with conductin. Endogenous GSK3β also coimmunoprecipitated with conductin in Neuro2A cells (Fig. 2C); this interaction required the GSK3β binding domain of conductin. These results suggest that conductin in mammalian cells forms a multiprotein complex that includes β-catenin, APC, and GSK3β.

The functional importance of β-catenin–conductin interactions was analyzed in SW480 colon carcinoma and Neuro2A cells that contain mutant and wild-type APC genes, respectively (*9*, *11*, *18*). In SW480 cells only a COOH-terminally truncated APC protein is produced that lacks all SAMP and six 20–amino acid repeats. As a consequence, β-catenin accumulates in the cytoplasm and nucleus; introduction of wild-type APC causes β-catenin degradation (*9*, *11*). When we transiently expressed conductin in SW480 cells, β-catenin staining was lost in the transfected cells (Fig. 3, A and B). Neighboring cells that did not express conductin retained strong nuclear staining of β-catenin. Coexpression of LEF-1 prevented β-catenin degradation (Fig. 3, C and D). The β-catenin binding domain is required for reducing β-catenin amounts (Fig. 3, E and F). In Neuro2A cells the stability of endogenous β-catenin is low (*1*, *5*). To interfere with β-catenin degradation in these cells, we used mutants of conductin that might act antagonistically with endogenous conductin. Indeed, expression of a conductin deletion mutant that lacks the binding sites for β-catenin, APC, and GSK3β stabilized endogenous β-catenin effectively (Fig. 3, G and H). We also used conductin-binding

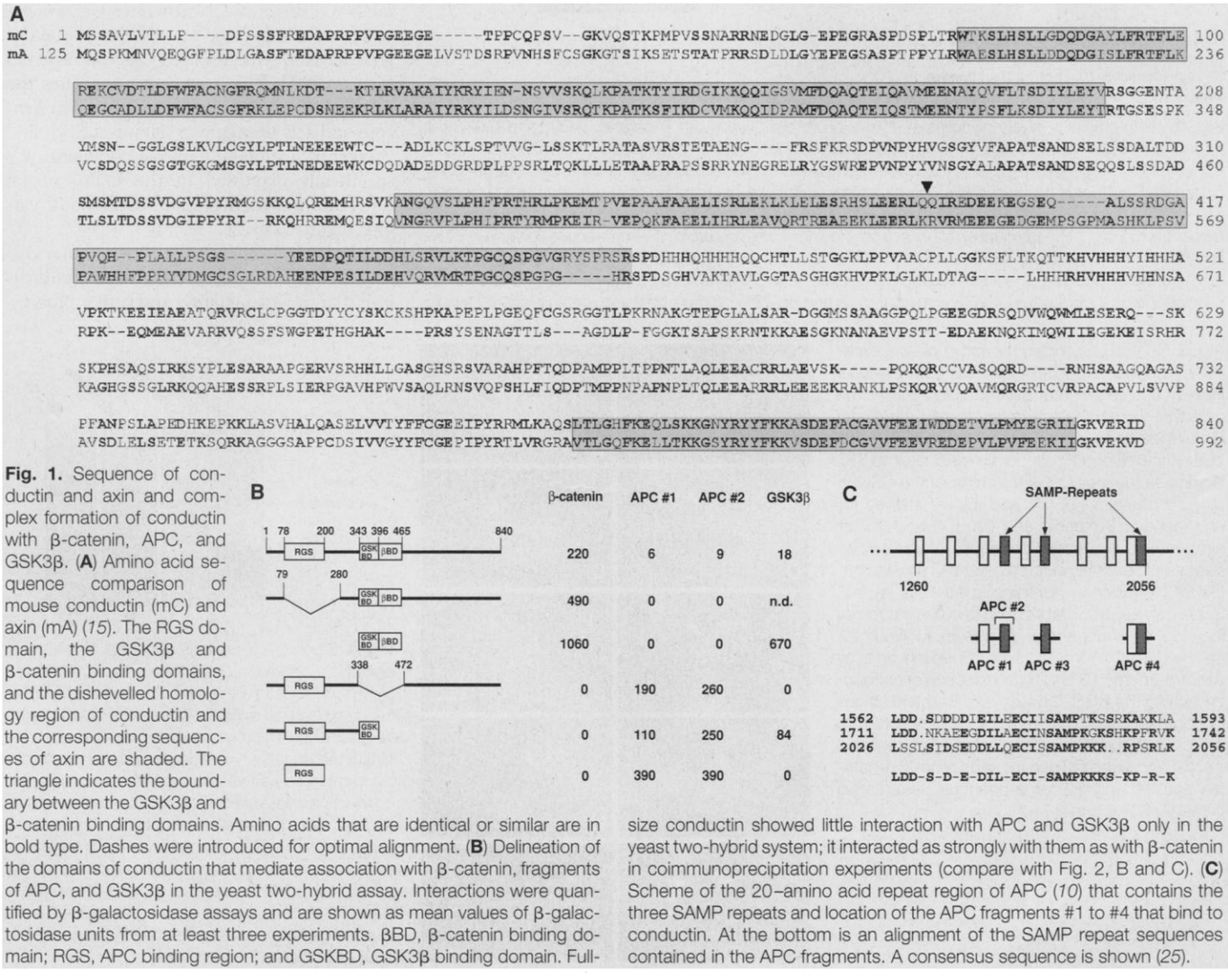


Fig. 1. Sequence of conductin and axin and complex formation of conductin with β-catenin, APC, and GSK3β. (**A**) Amino acid sequence comparison of mouse conductin (mC) and axin (mA) (*15*). The RGS domain, the GSK3β and β-catenin binding domains, and the dishevelled homology region of conductin and the corresponding sequences of axin are shaded. The triangle indicates the boundary between the GSK3β and β-catenin binding domains. Amino acids that are identical or similar are in bold type. Dashes were introduced for optimal alignment. (**B**) Delineation of the domains of conductin that mediate association with β-catenin, fragments of APC, and GSK3β in the yeast two-hybrid assay. Interactions were quantified by β-galactosidase assays and are shown as mean values of β-galactosidase units from at least three experiments. βBD, β-catenin binding domain; RGS, APC binding region; and GSKBD, GSK3β binding domain. Full-size conductin showed little interaction with APC and GSK3β only in the yeast two-hybrid system; it interacted as strongly with them as with β-catenin in coimmunoprecipitation experiments (compare with Fig. 2, B and C). (**C**) Scheme of the 20–amino acid repeat region of APC (*10*) that contains the three SAMP repeats and location of the APC fragments #1 to #4 that bind to conductin. At the bottom is an alignment of the SAMP repeat sequences contained in the APC fragments. A consensus sequence is shown (*25*).

APC fragments to interfere with β-catenin degradation. For instance, the APC fragment #4, but not a mutant of its SAMP repeat, effectively stabilized β-catenin [Fig. 3, I and K (*16*, *19*)]. This fragment also prevented APC-induced degradation of β-catenin in SW480 cells.

Conductin-induced degradation of β-catenin in SW480 cells was also observed by biochemical analysis; this required amino acids 338 to 472 encoding the β-catenin and GSK3β binding sites but not the RGS domain of conductin (Fig. 4A). The NH_2-terminal phosphorylation sites in β-catenin were previously reported to be essential for its degradation (*11*, *20*). The stability of a β-catenin mutant that lacks these sites was not affected by conductin (Fig. 4B). Specific degradation of β-catenin by conductin and the stability of the β-catenin mutant was observed in three independent experiments. In Neuro2A cells, the effective stabilization of β-catenin by APC fragment #4 was also seen in biochemical experiments (Fig. 4C). Together, the results on β-catenin stability suggest that conductin is a key component of the β-catenin degradation machinery. Our conductin mutant may interfere with a downstream step by antagonizing endogenous conductin, whereas the APC fragment #4 may cause a block of the endogenous APC-conductin interaction. We suggest that the assembly of a multiprotein complex by conductin controls the stability of β-catenin.

When we microinjected conductin mRNA into dorsal blastomeres of *Xenopus* embryos, we observed ventralization and defects in axial structures (*21*), as was reported for axin (*15*). Thus, conductin represses the activity of the wnt signaling pathway in *Xenopus* and acts upstream of siamois, LEF-1, and β-catenin. In mouse embryos conductin was specifically expressed in the brain, neural tube, and mesenchyme and epithelia of various organs (*22*).

Control of the concentration of cytoplasmic and nuclear β-catenin/armadillo is essential in wnt signaling and tumor progres-

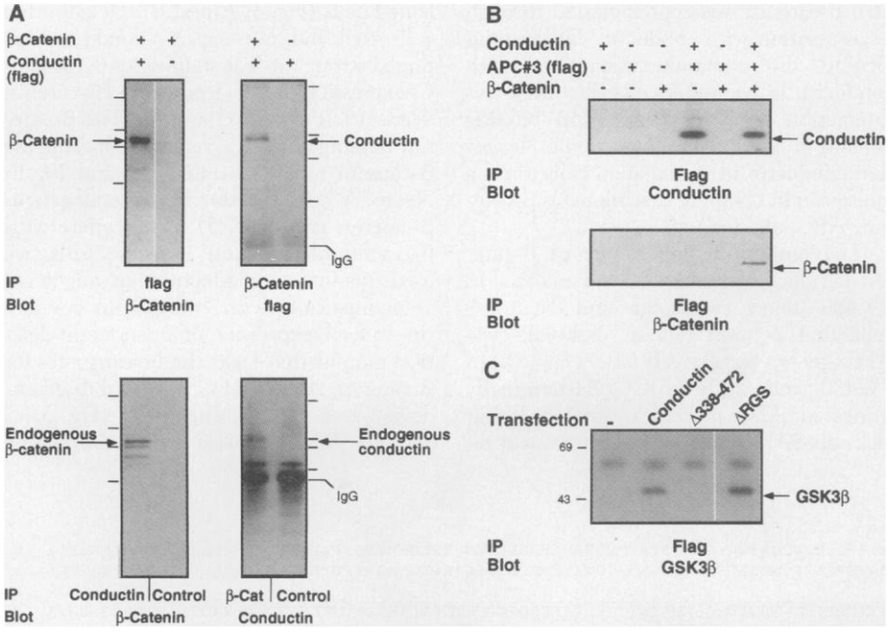
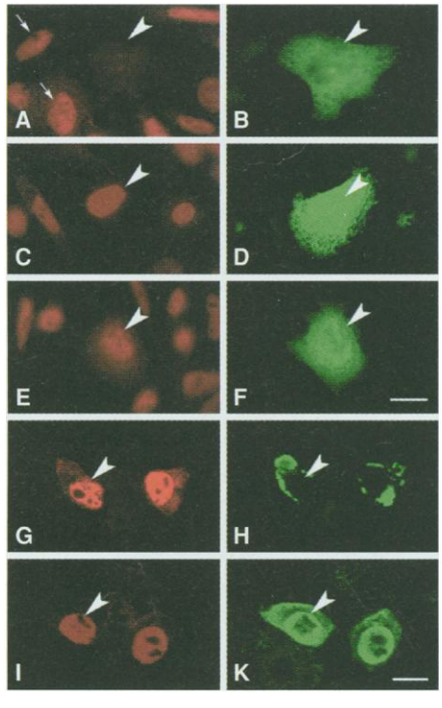


Fig. 2. Biochemical analysis of complex formation of conductin with β-catenin, APC, and GSK3β in mammalian cells. (**A**) Detection of exogenous β-catenin–conductin complexes in Neuro2A cells (upper panels) and endogenous β-catenin–conductin complexes in SW48 cells (lower panels) by coimmunoprecipitation. SW48 cells contained stabilized β-catenin with a mutation in the NH_2-terminus (*11*). Transfection expression constructs and specific antibodies used are indicated above and below the panels, respectively. Flag, NH_2-terminal epitope tag; control, nonimmune serum; IP, immunoprecipitated protein; Blot, immunoblot antibody. (**B**) Detection of conductin-APC complexes in transfected Neuro2A cells by coimmunoprecipitation. The APC fragment #3 contains a SAMP but not a 20–amino acid repeat. (**C**) Association of endogenous GSK3β with conductin and deletion (Δ) mutants in transfected Neuro2A cells.

Fig. 3. Control of cytoplasmic and nuclear β-catenin by conductin in cultured cells. Double-label immunofluorescence of SW480 colon carcinoma cells that were transiently transfected with (**A** and **B**) full-size conductin, (**C** and **D**) conductin plus LEF-1, and (**E** and **F**) conductin residues 1 to 396 lacking sequences COOH-terminal of the GSK3β binding domain. (A), (C), and (E) are stained for endogenous β-catenin and (B), (D), and (F) for exogenously expressed conductin. Large arrowheads, nuclei of transfected cells; thin arrows, nuclei of neighboring nontransfected cells [in (A)]. Scale bar, 20 μm. Stabilization and nuclear translocation of endogenous β-catenin in Neuro2A cells induced by mutants of conductin and by APC fragments: (**G** and **H**) transfection of conductin lacking the RGS, GSK3β, and β-catenin binding domains (amino acids 79 to 280 and 338 to 472) and (**I** and **K**) transfection of APC fragment #4. (G) and (I) are stained for endogenous β-catenin and (H) and (K) for exogenously expressed proteins. Scale bar, 30 μm.



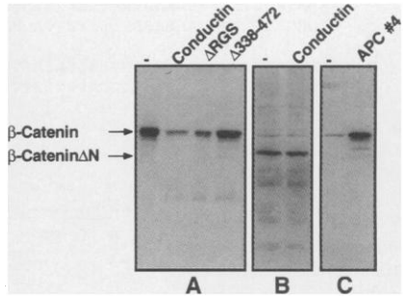


Fig. 4. Biochemical analysis of control of β-catenin stability by conductin. SW480 cells were transfected by using Lipofectin with expression constructs for (**A**) full-size β-catenin or (**B**) a deletion mutant lacking the NH_2-terminus (*1*) from amino acids 1 to 131 (β-cateninΔN) together with conductin or conductin deletion mutants as indicated above the lanes; -, no conduction transfected. Cell extracts were prepared 2 days after transfection and immunoblotted for β-catenin with antibody to the myc tag. Transfection efficiency was adjusted by using β-galactosidase expression (*26*). (**C**) Biochemical analysis of the stabilization of endogenous β-catenin in Neuro2A cells after exogenous expression of the APC fragment #4; -, no APC fragment transfected.

sion (*3*, *4*, *9*, *11*, *23*, *24*). APC and GSK3β have been implicated in the control of β-catenin stability. We demonstrate here that conductin interacts with β-catenin, APC, and GSK3β. Conductin overexpression directs β-catenin to the degradation pathway without functional APC. Mutants of conductin act antagonistically in this pathway. Moreover, APC fragments that interfere with conductin-APC interaction inhibit degradation of β-catenin. Conductin, therefore, is positioned downstream of APC in this regulatory pathway. The related protein axin, which suppresses axis formation in mice (*15*), might function similarly.

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12. Yeast two-hybrid screens were performed with the region of β-catenin encoding the 13 arm repeats (*1*) or with the RGS domain of conductin (amino acids 78 to 200) in a DNA binding domain vector and a cDNA library from 10.5-day mouse embryos in a VP16 activation domain vector (*6*). Full-length conductin was isolated from λgt10 mouse embryo cDNA libraries (Stratagene). Deletion mutants of conductin and β-catenin were made by restriction digests or polymerase chain reaction and tested for interaction in a quantitative β-galactosidase assay. Sequence comparisons were made with BLAST and ClustalW (1.7) programs [S. F. Altschul, W. Gish, W. Miller, E. M. Myers, D. J. Lipman, *J. Mol. Biol.* **215**, 403 (1990)].
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16. Mutations were introduced [K. M. Weidner *et al.*, *Nature* **384**, 173 (1996)] into the APC fragment #4, and interactions with the RGS domain of conductin were measured in yeast two-hybrid assays. The SAMP repeat was mutated from SAMP to AALP.
17. Transfection of mammalian cells, immunoprecipitations, and protein immunoblots were performed as described (*1*) with antibody to β-catenin (anti–β-catenin), anti-GSK3β, anti–myc tag (9E10), and anti-flag (Kodak). Antibodies to conductin were prepared in rabbits by immunization with a recombinant fragment of conductin containing amino acids 7 to 396. The antibody was purified by affinity chromatography and shown to be specific for conductin by immunoprecipitation and protein immunoblot analysis. Anti-GSK3β was purchased from StressGen (Victoria, Canada). Immunofluorescence staining was performed on cells fixed with 2% formaldehyde and permeabilized with Triton X-100 (*1*).
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19. The other fragments of APC were also effective in stabilizing β-catenin in Neuro2A cells, although to a lesser degree.
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21. The degree of ventralization of *Xenopus* embryos was dependent on the concentration of the injected conductin mRNA. We noticed a decrease in β-catenin concentrations in the injected embryos. Axis formation was rescued by co-injection of mRNA that encodes LEF-1 but not of a LEF-1 deletion mutant that lacks the NH_2-terminal β-catenin binding domain. Axis formation was also rescued by co-injection of mRNAs encoding β-catenin or siamois but not Xwnt-8. The mRNA for *Xenopus* injection was produced by in vitro transcription with the pSP64T3 vector and SP6 RNA polymerase [M. Kühl, S. Finnemann, O. Binder, D. Wedlich, *Mech. Dev.* **54**, 71 (1996)] and was injected into dorsal blastomeres of 4-cell-stage embryos. Control injections of prepro-lactin mRNA resulted in no morphological changes.
22. A 4.7-kb conductin mRNA was found in mouse embryos by Northern (RNA) blot analysis at all developmental stages tested (E7 through E17) and in many adult tissues; highest expression levels in the adult are observed in brain, lung, and liver. In situ hybridization of E11 and E12 mouse embryos revealed distinct patterns of conductin expression, for example, in the dorsal neural tube, distinct regions of the brain, the mesenchyme below the epidermis, lung mesenchyme, and kidney epithelia. Multiple-tissue Northern blots (Clontech) and in situ hybridization of mouse embryos were carried out as described [F. D. Bladt, D. Riethmacher, D. S. Isenmann, A. Aguzzi, C. Birchmeier, *Nature* **376**, 768 (1996)].
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25. Sequences related to the SAMP repeat are found in APC of *Caenorhabditis elegans* [C. E. Rocheleau *et al.*, *Cell* **90**, 707 (1997)]. The published APC sequence of *Drosophila* does not contain SAMP repeats [S. Hayashi *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **94**, 242 (1997)].
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Activation of the Protein Kinase p38 in the Spindle Assembly Checkpoint and Mitotic Arrest

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The mitogen-activated protein kinase (MAPK) superfamily comprises classical MAPK (also called ERK), c-Jun amino-terminal or stress-activated protein kinase (JNK or SAPK), and p38. Although MAPK is essential for meiotic processes in *Xenopus* oocytes and the spindle assembly checkpoint in *Xenopus* egg extracts, the role of members of the MAPK superfamily in M phase or the spindle assembly checkpoint during somatic cell cycles has not been elucidated. The kinase p38, but not MAPK or JNK, was activated in mammalian cultured cells when the cells were arrested in M phase by disruption of the spindle with nocodazole. Addition of activated recombinant p38 to *Xenopus* cell-free extracts caused arrest of the extracts in M phase, and injection of activated p38 into cleaving embryos induced mitotic arrest. Treatment of NIH 3T3 cells with a specific inhibitor of p38 suppressed activation of the checkpoint by nocodazole. Thus, p38 functions as a component of the spindle assembly checkpoint in somatic cell cycles.

MAPKs are serine-threonine kinases that are activated by various mitogens that induce transition from the quiescent state into the cell cycle division (*1*). MAPK is also activated during meiotic maturation in *Xenopus* oocytes and has an essential role in both the transition from G_2 to M phase of meiosis and metaphase arrest of mature oocytes (*2*–*4*). Although these results suggested the possible role of MAPK/ERK in the mitotic M phase also, little or no activation of MAPK is detected during M phase of somatic cell cycles. In an in vitro cell cycle system derived from *Xenopus* egg extracts, MAPK is required for the spindle assembly checkpoint mechanism (*5*, *6*), a mechanism conserved evolutionarily and essential for accurate transmission of genetic information to the daughter cells (*7*). Although a requirement for MAPK in the spindle assembly checkpoint in somatic cells has also been suggested because injection of MAPK phosphatase (XCL100) overcomes the checkpoint (*8*), activation of MAPK in cells arrested in M phase as a result of spindle assembly defects has not been observed. However, one or more MAPK-related molecules appears to be activated in the nocodazole-treated cells arrested in M phase (*9*). We therefore tested whether JNK or p38 (also known as MPK2, CSBP, or HOG1) might participate in the spindle assembly checkpoint of somatic cell cycles.

Lysates of nocodazole-arrested mitotic NIH 3T3 cells released from flasks by shak-

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