

Wnt1 and MEK1 Cooperate to Promote Cyclin D1 Accumulation and Cellular Transformation*

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Members of the Wnt family of signal transducers regulate cellular differentiation/reorganization and cellular proliferation. However, few pro-proliferative targets of Wnt have been identified. We now show that cyclin D1, a critical mediator of cell cycle progression, is a downstream target of Wnt-dependent signaling. NIH-3T3 cell lines engineered to overexpress Wnt1 displayed reduced glycogen synthase kinase-3 β activity. Wnt1-dependent glycogen synthase kinase-3 β inhibition corresponded with decreased cyclin D1 proteolysis and, thus, hyperaccumulation of active cyclin D1-CDK4 (cyclin-dependent kinase 4) kinase. However, in the absence of serum-derived growth factors, Wnt-1 was not sufficient to drive cyclin D1 accumulation or S-phase entry. In contrast, cells engineered to co-express Wnt1 and activated MEK1 accumulated high levels of cyclin D1 and entered the DNA synthetic phase in the absence of serum-derived growth factors, a characteristic of neoplastic transformation. The ability of a dominant-negative cyclin D1 mutant, D1-T156A, to inhibit Wnt1/MEK1-dependent S-phase entry suggests that cyclin D1 is a critical downstream target for Wnt1- and MEK1-dependent cellular proliferation.

Wnts comprise a family of secreted glycoproteins that function as ligands that, upon receptor binding, initiate signaling pathways that regulate cell growth and differentiation (1). The Wnt ligand binds to Frizzled, its membrane bound receptor, transmitting its signals through Disheveled (Dvl), culminating in the inhibition of glycogen synthase kinase-3 β (GSK-3 β)¹ and the stabilization of β -catenin (2–6). Active GSK-3 β triggers β -catenin proteolysis through site-specific phosphorylation (2). Upon accumulation, β -catenin is free to translocate to the nucleus as a component of a transcription factor complex composed of TCF/LEF-1 family members and activate transcription of genes whose promoters contain binding sites for TCF/LEF-1 (7).

In addition to providing pivotal signals in the determination of embryonic development (1), certain Wnt family members are also implicated in tumorigenesis. For example, the proto-typical Wnt family member Wnt1 can transform cultured murine epithelial cells (8) and reduce growth factor requirements and increase saturation densities when expressed in rodent fibroblasts (9). A direct demonstration of the oncogenic capacity of Wnt1 was the observation that mice expressing a Wnt1 transgene develop mammary carcinoma (8).

GSK-3 β may be one of the key effectors of Wnt-dependent signaling, as it directly regulates the accumulation and, thus, activity of several pro-proliferative molecules, such as cyclin D1 (10). In general, the mitogen-responsive D-type cyclins (D1, D2, D3) regulate G₁-phase progression in concert with their catalytic subunit, cyclin-dependent kinase 4 (CDK4) or 6 (CDK6) (11). G₁ progression also requires the activity of CDK2 in combination with cyclin E (11). Mitogenic stimuli trigger the accumulation of active cyclin D1-CDK4 complexes through both increased expression and decreased proteolysis of cyclin D1 and through the promotion of cyclin D1-CDK4 association (11). Mitogen-dependent expression of cyclin D1 depends upon growth factor-mediated activation of a signal transduction cascade consisting of Ras, Raf-1, and ERK1 and ERK2 (12–18). Accumulation of cyclin D1 during G₁ also relies upon mitogen-dependent inhibition of GSK-3 β through activation of PI3K and AKT (protein kinase B) (10). GSK-3 β -dependent phosphorylation of cyclin D1 on a single threonine residue, threonine 286, triggers the cytoplasmic proteolysis of cyclin D1 via the 26 S proteasome (10). The significance of the precise regulation of cyclin D1 accumulation is emphasized by the observed overexpression of cyclin D1 in a high percentage of human malignancies (19).

Given the capacity of GSK-3 β to regulate the accumulation of cyclin D1, we reasoned that Wnt1-induced growth alterations might be attributed, in part, to its capacity to promote increased accumulation of cyclin D1. In this paper we demonstrate that Wnt1-dependent down-regulation of GSK-3 β promotes the deregulation of cyclin D1 accumulation through reduced cyclin D1 proteolysis, increased accumulation of cyclin D1 message, and increased nuclear accumulation of cyclin D1. We also demonstrate that cells overexpressing Wnt1 and activated MEK1 promote growth factor independent S-phase entry, which is dependent upon the accumulation of cyclin D1-dependent kinase.

EXPERIMENTAL PROCEDURES

Tissue Culture Conditions, Cell Lines, and Transfections—NIH-3T3 cells and their derivatives were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (FCS), and antibiotics (Fisher). NIH-3T3 cells expressing zinc-inducible MEK1* (provided by Dr. Charles Sherr, St. Jude Children's Research Hospital) were established previously, and their characteristics are described in

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¹ The abbreviations used are: GSK-3 β , glycogen synthase kinase-3 β ; BrdUrd, bromodeoxyuridine; ERK, extracellular signal-regulated kinase; MEK, mitogen-activated protein kinase kinase; MEK1*, constitutively active MEK1; PI3K, phosphatidylinositol 3-kinase; HFc, HFc position of the mouse immunoglobulin IgG₁; LEF-1, lymphoid enhancer factor-1; TCF, T-cell factor; CDK, cyclin-dependent kinase; FCS, fetal calf serum; GST, glutathione S-transferase.

detail elsewhere (15). NIH-3T3 cells that express zinc-inducible MEK1* and constitutively express Wnt1 were established by transfection of a plasmid encoding Hfc-tagged Wnt1 (provided by Dr. Stuart Aaronson, Mount Sinai School of Medicine) and a second plasmid encoding the puromycin resistance gene into MEK1*-3T3 cells using the calcium phosphate co-precipitation protocol (20). Cell lines were selected in complete medium containing 800 $\mu\text{g/ml}$ G418 and 7.5 $\mu\text{g/ml}$ puromycin. NIH-3T3 cells expressing HFc-Wnt1 were established using the calcium phosphate co-precipitation protocol (20) and selected in complete medium containing 800 $\mu\text{g/ml}$ G418.

For focus formation assays, the indicated cell lines were plated at a density of $5 \times 10^5/60\text{-mm}$ dish in Dulbecco's modified Eagle's medium supplemented with antibiotics, 5% FCS, and, where indicated, 80 μM zinc sulfate. The medium was changed every 4 days, and cells were stained with Giemsa (Sigma) after 3 weeks.

Immunoblotting, Immunoprecipitations, and Protein Kinase Assays—For direct Western analysis, cells were lysed in EBC buffer (50 mM Tris-HCl, pH 7.5, 120 mM NaCl, 0.5% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 20 units/ml aprotinin, 5 $\mu\text{g/ml}$ leupeptin, 0.4 mM Na_3VO_4 , and 0.4 mM NaF). 150 μg of total cellular proteins were resolved on denaturing polyacrylamide gels, electrophoretically transferred to nitrocellulose membranes (MSI, Westborough MA), and blotted with the indicated primary antibodies. Immunoblot analysis of Myc-tagged MEK1*, cyclin D1, CDK4, p21^{cip1}, and p27^{kip1} was otherwise performed as described previously (15, 21). For detection of Hfc-tagged Wnt1, cell lysates were prepared as above, and Wnt1-HFc was detected with a mouse-specific Hfc antiserum (DAKO; Carpinteria, CA). Sites of antibody binding were visualized using anti-mouse-conjugated horseradish peroxidase (Amersham Pharmacia Biotech) followed by enhanced chemiluminescence detection (NEN Life Science Products).

For detection of total CDK2-associated catalytic activity, cells were lysed in Tween 20 immunoprecipitation buffer (50 mM HEPES, pH 7.5, 10 mM MgCl_2 , 2.5 mM EGTA, 1 mM EDTA, 1 mM dithiothreitol, and the protease and phosphatase inhibitors indicated above). CDK2 complexes were isolated from clarified lysates (150 $\mu\text{g}/\text{sample}$) with antiserum directed to CDK2 (R_{DD}) and protein A-Sepharose for 1 h at 4 °C. Immune complexes were washed 4 times with immunoprecipitation buffer and 2 times with kinase buffer (50 mM HEPES, pH 7.5, 10 mM MgCl_2 , 1 mM dithiothreitol, 20 μM unlabeled ATP, and the protease and phosphatase inhibitors indicated above). Reactions were initiated by the addition 10 μCi of [$\gamma\text{-}^{32}\text{P}$]ATP (6000 Ci/mmol; NEN Life Science Products) and incubated at 30 °C for 25 min (with linear incorporation kinetics). Labeled proteins were denatured in sample buffer and separated on denaturing polyacrylamide gels before autoradiography. Rb kinase and GSK-3 β kinase assays were performed as described previously (10, 22).

Immunofluorescence—For detection of BrdUrd and FLAG-tagged cyclin D1 in a single cell, transfected cells seeded on coverglass were fixed and permeabilized with ice-cold methanol-acetone (1:1) for 10 min at -20 °C. The coverglass was then incubated with FLAG-specific Octaprobe antiserum (1 $\mu\text{g/ml}$; Santa Cruz Biotechnology; Santa Cruz, CA) for 1 h at room temperature. The coverslips were washed with phosphate-buffered saline and incubated with rabbit anti-goat IgG (1 $\mu\text{g/ml}$; Fisher) for 30 min. To detect the antibody-antigen complexes, a third incubation was performed with anti-rabbit-conjugated fluorescein isothiocyanate (1:400; Amersham Pharmacia Biotech) for 30 min. Following treatment of cells with 1.5 N HCl, cells were stained with a BrdUrd-specific antibody (Amersham Pharmacia Biotech) followed by biotinylated anti-mouse (1:400; Vector Laboratories, Burlingame, CA) and streptavidin Texas Red (1:400; Vector Laboratories). All incubations were performed in phosphate-buffered saline containing 1% non-fat dry milk. Finally, after a final wash in phosphate-buffered saline, DNA was stained with Hoechst dye 33258 (Sigma). Detection of endogenous cyclin D1 was performed as described elsewhere (21). Cells were visualized using a Nikon microscope fitted with the appropriate filters.

Northern Blotting—Total cellular RNA (15 $\mu\text{g}/\text{lane}$) was separated by formaldehyde-agarose gel electrophoresis and transferred to a nylon membrane (Sigma). After cross-linking, membranes were hybridized with a ³²P-labeled cyclin D1 probe or $\gamma\text{-actin}$ (provided by Dr. Joseph Brewer, Loyola University) probes prepared by random priming, washed, and subjected to autoradiography.

RESULTS

Wnt1 Modulates GSK-3 β Activity in NIH-3T3 Cells—We established NIH-3T3 cells lines that constitutively overexpress HFc-tagged Wnt1 (Wnt1-3T3). To confirm expression of Wnt1-HFc, whole cell lysates prepared from proliferating NIH-3T3 or

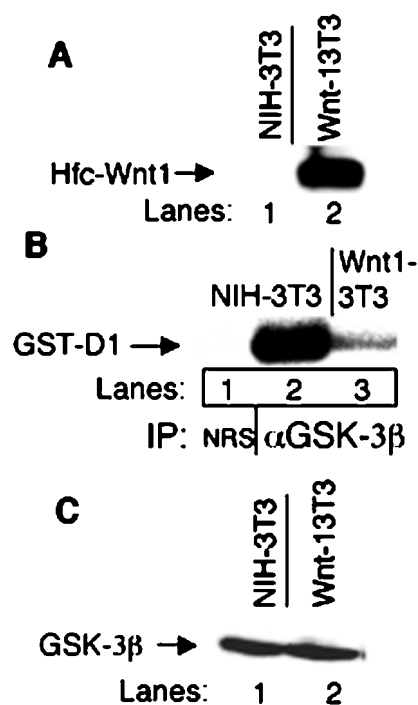


FIG. 1. Decreased GSK-3 β activity in Wnt1-overexpressing NIH-3T3 cells. A and C, whole cell lysates prepared from either asynchronously proliferating parental NIH-3T3 or Wnt1-3T3 cells were separated on a denaturing polyacrylamide gel, transferred to nitrocellulose, and blotted with an antibody specific for murine Hfc (A) or GSK-3 β (C). Antibody complexes were visualized by enhanced chemiluminescence. B, lysates prepared from the indicated asynchronously proliferating cell lines (200 μg) were precipitated with non-immune rabbit serum (NRS) or with an antibody specific for GSK-3 β . The resulting complexes were assayed for kinase activity using GST-D1 as the substrate. IP, immunoprecipitation.

Wnt1-3T3 cells were separated on denaturing polyacrylamide gels and subsequently transferred to nitrocellulose membrane. Direct Western blot analysis with an antibody that recognizes the mouse-Hfc tag confirmed that Wnt1 was expressed in Wnt1-3T3 but not parental NIH-3T3 cells (Fig. 1A).

Although Wnt1 is normally not expressed in NIH-3T3 cells (23), others have demonstrated that components of the Wnt signaling pathway are functional in these cells (9, 24, 25). Activation of Wnt1-dependent signaling pathways should inhibit the catalytic activity of GSK-3 β (26). Although this inhibition is potentially due to the incorporation of GSK-3 β into catalytically inactive complexes (24, 27) or through inhibitory phosphorylation of GSK-3 β at serine 9 (26), the end result of either mechanism should be a net reduction in GSK-3 β catalytic activity. To confirm that ectopically expressed Wnt1 regulates GSK-3 β , we assessed GSK-3 β kinase activity in asynchronously proliferating parental NIH-3T3 and Wnt1-3T3 cells. GSK-3 β precipitated from whole cell lysates was assayed for its ability to phosphorylate glutathione S-transferase fused with the carboxyl-terminal 41 amino acids of cyclin D1 (GST-D1). We have previously demonstrated that GSK-3 β specifically phosphorylates a single residue, threonine-286, located within the cyclin D1 moiety of this recombinant protein (10). GSK-3 β precipitated from NIH-3T3 cell lysates incorporated significantly more phosphate into the cyclin D1 substrate than GSK-3 β precipitated from Wnt1-3T3 cellular lysates (Fig. 1B), demonstrating that overexpression of Wnt1 results in inhibition of GSK-3 β activity. Equivalent levels of GSK-3 β were expressed in both cell lines (Fig. 1C), demonstrating that the observed decrease in GSK-3 β activity did not reflect its decreased expression.

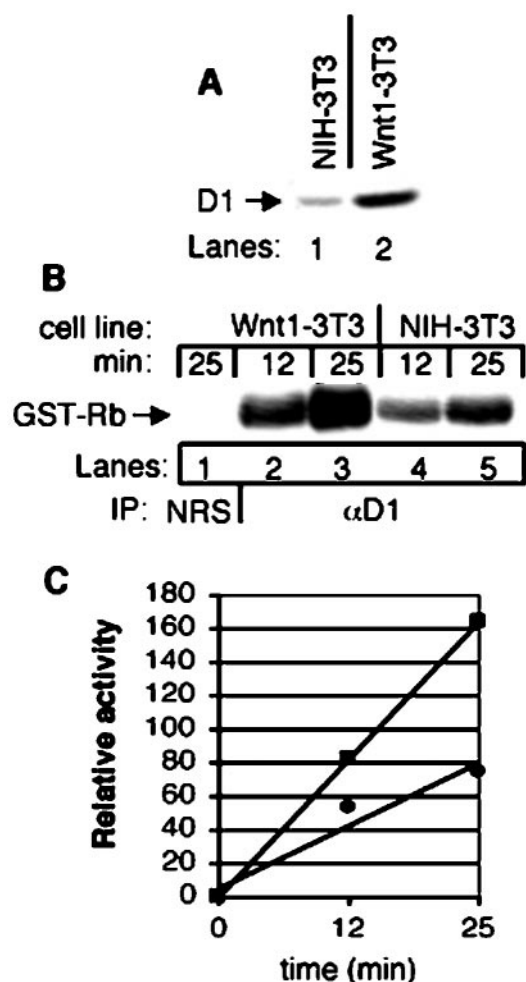


FIG. 2. Wnt1-3T3 cells accumulate high levels of cyclin D1. *A*, cell lysates prepared from asynchronously proliferating parental 3T3 (lane 1) or Wnt1-3T3 (lane 2) were blotted with a cyclin D1-specific monoclonal antibody, and antibody complexes were visualized by enhanced chemiluminescence. *B*, lysates prepared from the indicated, proliferating cell lines were precipitated with nonimmune rabbit serum (NRS) or with an antibody specific for cyclin D1. The resulting complexes were assayed for kinase activity for the indicated times using GST-Rb as the substrate and quantitated (*C*) by spot densitometry. The experiment shown is representative of several such experiments. *IP*, immunoprecipitation.

Wnt1-3T3 Cells Accumulate High Levels of Cyclin D1—Cyclin D1 accumulation is exquisitely sensitive to a variety of signaling pathways (28). Mitogens regulate cyclin D1 accumulation via Ras-dependent activation of at least two distinct pathways. The first, which proceeds via Raf-1-MEK1-ERK, regulates cyclin D1 gene expression and assembly with CDK4 (12–18). The second Ras-dependent pathway, involving PI3K and AKT, triggers the inhibition of GSK-3 β , thereby stabilizing cyclin D1 protein (10). Signal-dependent cyclin D1 accumulation may also be subject to regulation via Ras-independent mechanisms. For example, Wnt-dependent signal transduction pathways also regulate GSK-3 β , apparently in a Ras-independent fashion (2). Thus, we considered the possibility that Wnt1-3T3 cells might overexpress cyclin D1. Consistent with this hypothesis, lysates prepared from asynchronously proliferating Wnt1-3T3 cells contained 4-fold more cyclin D1 than parental NIH-3T3 cells (Fig. 2A), as determined by immunoblot analysis using a cyclin D1-specific monoclonal antibody.

In mouse fibroblasts, CDK4 is the primary catalytic partner of cyclin D1 (29). As steady-state levels of CDK4 exceed that of cyclin D1 (30), the increase in cyclin D1 abundance in Wnt1-

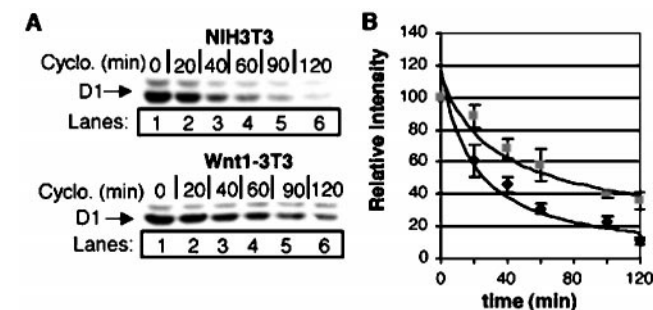


FIG. 3. Decreased cyclin D1 turnover in Wnt1-3T3 cells. *A*, cycloheximide was added to the media of the indicated asynchronously proliferating cell lines at a final concentration of 50 μ g/ml. Whole cell lysates were prepared at the indicated time points following the addition of cycloheximide and blotted with the cyclin D1-specific antibody. Antibody complexes were visualized by enhanced chemiluminescence, and the relative intensity was determined by spot densitometry and normalized to the maximum signal. *B*, the results shown are the mean and S.D. of four independent experiments. The curves were estimated using a best fit. Cyclin D1 decay in normal NIH-3T3 cells is denoted by filled diamonds, and in Wnt1-3T3, by filled squares.

3T3 cells should result in a corresponding increase in cyclin D1-CDK4 kinase activity (22). Cyclin D1-CDK4 complexes were precipitated from whole cell lysates prepared from asynchronously proliferating NIH-3T3 or Wnt1-3T3 cells using a cyclin D1-specific monoclonal antibody that supports cyclin D1-CDK4 activity (22). Cyclin D1-CDK4 activity was then determined by immune complex kinase assays using recombinant GST-Rb as the substrate (22). Cyclin D1-CDK4 complexes isolated from Wnt1-3T3 cells displayed an increased propensity to phosphorylate GST-Rb (Fig. 2B, lanes 2–3) relative to those precipitated from normal, proliferating NIH-3T3 cells (lanes 4–5). Densitometric scanning revealed that the absolute catalytic activity of the cyclin D1-dependent kinase was enhanced at least 2-fold in the Wnt1-overexpressing cells (Fig. 2C). These results demonstrate that Wnt1 promotes the increased accumulation of catalytically active cyclin D1-dependent kinase.

It has previously been demonstrated that cells overexpressing cyclin D1 exhibit a contracted G₁-phase interval (30). Given the capacity of Wnt1 to promote increased accumulation of cyclin D1, we determined the percentage of asynchronously proliferating Wnt1-3T3 or parental NIH-3T3 cells in each stage of the cell cycle by flow cytometric analysis. Surprisingly, we failed to detect significant alterations in the percentage of G₁- or S-phase Wnt1-3T3 versus NIH-3T3 cells (negative data not shown). Our inability to detect alterations in the cell cycle profile of Wnt1-3T3 versus NIH-3T3 is likely a reflection on the modest level of cyclin D1 overexpression in these cells in the absence of a collaborating oncogene (see below).

Wnt1 Overexpression Promotes Decreased Cyclin D1 Proteolysis—We next considered the possibility that the increased cyclin D1 accumulation in Wnt1-3T3 cells might result from decreased cyclin D1 proteolysis. To test this hypothesis, we utilized the protein synthesis inhibitor cycloheximide to block new protein synthesis (50 μ g/ml blocks >95% of total protein synthesis within 2 min; data not shown) and followed the rate of cyclin D1 decay by direct Western blot analysis with a cyclin D1 monoclonal antibody. Cycloheximide was added to asynchronously proliferating NIH-3T3 or Wnt1-3T3 cells, and lysates were prepared at the indicated intervals. The cyclin D1 half-life in parental NIH-3T3 cells was 25 min (Fig. 3A, upper panel), in agreement with the previously determined half-life of cyclin D1 (10). In contrast, the cyclin D1 half-life in Wnt1-3T3 was approximately 60 min (Fig. 3A, lower panel). The results of a series of independent pulse-chase experiments are represented graphically in Fig. 3B and demonstrate a cyclin D1

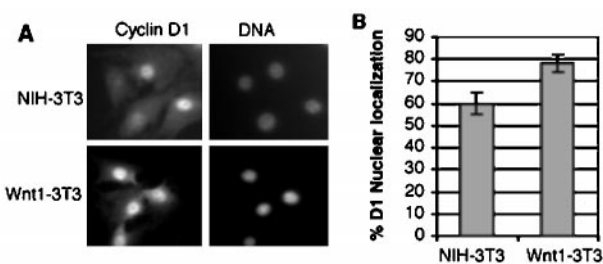


FIG. 4. Increased nuclear accumulation of cyclin D1 in Wnt1-overexpressing cells. A, immunofluorescent detection of cyclin D1 in either NIH-3T3 (top) or Wnt1-3T3 (bottom) cells. The indicated cells were fixed and stained with either a cyclin D1-specific monoclonal antibody (left panels) or Hoechst stain (right panels). B, the average percentage of cells, prepared as above, in which cyclin D1 exhibited primarily nuclear localization was determined and is expressed relative to the total population of cells.

half-life of 25 min in parental NIH-3T3 cells versus 65 min in Wnt1-3T3 cells. These results demonstrate that cyclin D1 turnover is compromised in Wnt1-overexpressing NIH-3T3 cells.

Increased Nuclear Accumulation of Cyclin D1 in Wnt1-3T3 Cells—Although cyclin D1 is a nuclear protein during G₁ phase, it relocalizes to the cytoplasm during S phase (10, 31), a process that is also regulated by GSK-3 β (10). We thus considered the possibility that overexpression of Wnt1 might perturb the normal subcellular distribution of cyclin D1. To address this issue, Wnt1-3T3 and NIH-3T3 cells seeded on glass coverslips were fixed, and the subcellular localization of cyclin D1 was determined by immunofluorescent staining with a monoclonal antibody specific for cyclin D1. Representative immunofluorescent data are shown in Fig. 4A. As previously seen, cyclin D1 localized primarily to the nucleus in approximately 60% of asynchronously proliferating fibroblasts (Fig. 4, A (top panels) and B (left column)). In contrast, 78% of Wnt1-3T3 cells contained nuclear cyclin D1 (Fig. 4, A (bottom panel) and B (right column)). These findings demonstrate that Wnt1 overexpression perturbs normal cyclin D1 subcellular distribution, resulting in the increased nuclear accumulation of cyclin D1.

Wnt1-3T3 Cells Accumulate Increased Levels of Cyclin D1 mRNA—Given the propensity of β -catenin to positively regulate cyclin D1 expression (32, 33) and the capacity of Wnt1 overexpressing cells to accumulate high levels of free β -catenin (7), we considered the possibility that Wnt1 might also promote increased accumulation of cyclin D1 message. Total RNA was isolated from asynchronously proliferating or serum-starved parental NIH-3T3 or Wnt1-3T3 cells. Equal quantities of total RNA were transferred to membranes and probed with either a cyclin D1-specific probe or a γ actin-specific probe. Northern blotting revealed a modest but significant increase of approximately 2-fold steady-state cyclin D1 mRNA in the asynchronously proliferating Wnt1-3T3 cells relative to that observed in proliferating NIH-3T3 cells (Fig. 5; compare lanes 2 and 4). As has previously been observed for many cell types, no cyclin D1 message was detectable in either serum-starved NIH-3T3 or Wnt1-3T3 cells (Fig. 5; compare lanes 1 and 3). The inability of Wnt1 to drive cyclin D1 gene expression in the absence of serum likely reflects the inability of Wnt1 to activate ERK1 or 2 in these cells, both of which are required for cyclin D1 gene expression (9).

Wnt1 and activated MEK1* Cooperatively Promote Growth Factor-independent S-phase Entry—Overexpression of Wnt1 is not sufficient to support either cyclin D1 synthesis or S-phase entry in the absence of serum-derived growth factors (Figs. 5 and 6). This is potentially due to the failure of Wnt1-dependent signals to activate the growth factor inducible extracellular signal-regulated kinases ERK1 and ERK2 (9). In contrast, con-

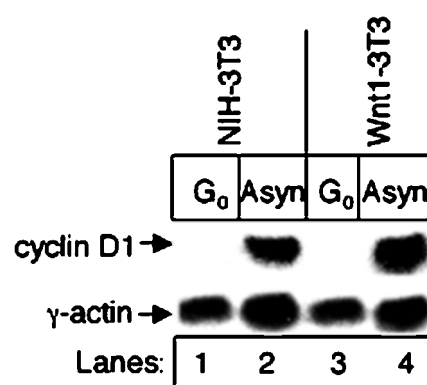


FIG. 5. Overexpression of Wnt1 increases steady-state accumulation of cyclin D1 message. Shown is total RNA isolated from either serum-starved (G₀) or asynchronously proliferating (Asyn) cells and blotted with either a cyclin D1-specific probe (top panel) or a γ -actin probe (bottom panels).

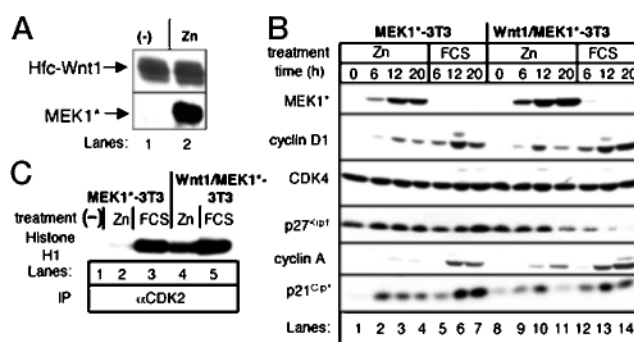


FIG. 6. Wnt1 and constitutively active MEK1 cooperatively promote growth factor independent S-phase entry. A, whole cell lysates were prepared from either untreated (-) or ZnSO₄-stimulated Wnt1/MEK1* cells. Lysates were blotted with either a mouse Hfc-specific antibody, to detect ectopic Wnt1, or the 9E10 monoclonal antibody, to detect Myc-tagged MEK1*. B, in parallel with the experiment in A, serum-starved cells were stimulated with ZnSO₄ or FCS for the indicated intervals, after which whole cell lysates were prepared, and the levels of MEK1*, cyclin D1, CDK4, p27^{kip1}, p21^{cip1}, and cyclin A were determined by immunoblot. C, CDK2 precipitates recovered from serum-starved cells or cells stimulated with either ZnSO₄ or FCS for 20 h were assayed for their ability to phosphorylate Histone H1.

stitutively active MEK1 (MEK1*), the upstream activator of ERK1 and -2, can promote both the expression of cyclin D1 and the assembly of cyclin D1-CDK4 complexes in the absence of serum-derived growth factors (15, 17). However, MEK1*, like Wnt1, cannot support S-phase entry in the absence of serum-derived growth factors (see below) (15, 17). The inability of cells expressing MEK1* to enter S phase apparently resulted from the failure of these cells to accumulate sufficient levels of cyclin D1-CDK4 complexes necessary to titrate the CDK inhibitor, p27^{kip1} (15). The sub-optimal levels of cyclin D1 result from the inability of MEK1-dependent pathways to inhibit GSK-3 β (10). Ultimately, the failure of cyclin D1-CDK4 complexes to titrate p27^{kip1} results in “free” p27^{kip1} that can associate with and inhibit the CDK2 kinases (both cyclins A/CDK2 and E/CDK2), both of which are required for S-phase entry (34).

We reasoned that ectopic expression of Wnt1, which reduces cyclin D1 proteolysis along with MEK1*, which strongly induces cyclin D1 expression, might provide sufficient levels of cyclin D1-CDK4 complexes to promote S-phase entry. NIH-3T3 cells, which already expressed a Myc epitope-tagged MEK1* under the control of the zinc-inducible sheep metallothionein promoter (MEK1*-3T3) (15), were engineered to constitutively overexpress Wnt1-HFc (Wnt1/MEK1*-3T3). High levels of Wnt1-HFc were detectable in the presence or absence of zinc

(Fig. 6A, top panel), whereas MEK1* expression was only detected following addition of zinc sulfate (Fig. 6A, bottom panel).

Before we assessed the ability of Wnt1 and MEK1* to support growth factor-independent S-phase entry, we first determined their ability to induce expression of G₁- and S-phase-specific markers. Parental MEK1*-3T3 and Wnt1/MEK1*-3T3 cells arrested by serum removal for 30 h were stimulated to reenter the cell cycle by addition of either 20 μ M zinc sulfate or 10% FCS. Stimulation of arrested MEK1*/Wnt1-3T3 cells with zinc resulted in the accumulation of cyclin D1, decreased levels of p27^{kip1}, and increased expression of S-phase-specific cyclin A (Fig. 6B). The addition of zinc to Wnt1/MEK1*-3T3 cells also facilitated accumulation of active CDK2 kinase (Fig. 6C, compare lanes 1 and 4), which is representative of both cyclin E and cyclin A/CDK2 kinases, as both productively associate with CDK2 (21). In contrast, although the addition of zinc to arrested MEK1*-3T3 did facilitate cyclin D1 and p21^{cip1} accumulation as previously reported (15), it failed to induce any decrease in p27^{kip1} levels or induce significant expression of cyclin A (Fig. 6B). Consistent with this observation, we failed to detect activation of CDK2 kinases in zinc-stimulated MEK1*-3T3 cells, whereas significant levels were present in serum-stimulated cells (Fig. 6C, compare lanes 1–3).

To directly assess the ability of Wnt1 and MEK1* to coordinately drive S-phase entry in the absence of serum-derived growth factors, G₀-arrested MEK1*-3T3 and Wnt1/MEK1*-3T3 cells were stimulated to reenter the cell cycle with either zinc sulfate or 10% FCS (Fig. 7A). S-phase entry was monitored by labeling of DNA with BrdUrd. As expected, medium containing 0.1% serum did not support S-phase entry of either parental MEK1*-3T3 or Wnt1/MEK1*-3T3, as determined by BrdUrd incorporation (Fig. 7A, first and fourth bars). In addition, MEK1*-3T3 failed to enter S-phase following the induction of MEK1* with zinc sulfate (second bar) but efficiently entered S phase following the addition of 10% FCS (third bar). In contrast, the addition of zinc sulfate to Wnt1/MEK1*-3T3 cells resulted in a 4-fold increase in the number of cells entering S phase relative to untreated cells (compare fourth and fifth bars). The addition of serum resulted in an additional 2-fold increase in the number of Wnt1/MEK1*-3T3 cells entering S phase (sixth bar).

The above experiments demonstrate that ectopic expression of both Wnt1 and MEK1* promote growth factor-independent S-phase entry. We next wished to determine if Wnt1/MEK1*-induced S-phase entry is dependent on the accumulation of active cyclin D1-dependent kinase. To assess the role of cyclin D1 in Wnt1/MEK1*-dependent S-phase entry, we made use of a dominant-negative mutant of cyclin D1 (D1-T156A), which upon expression recruits CDK4 into inhibitory, cytoplasmic complexes, thereby inducing G₁-phase arrest (35). Wnt1/MEK1*-3T3 cells seeded on glass coverslips were transfected with plasmids encoding either wild-type cyclin D1 or D1-T156A. Both wild-type cyclin D1 and D1-T156A express an amino-terminal FLAG epitope tag to facilitate their detection. Transfected cells were rendered quiescent by serum removal for 24 h. Cells were stimulated to re-enter the cell cycle by the addition of zinc sulfate and were labeled with BrdUrd for 20 h to monitor S-phase entry. Cells were fixed and stained with antibodies specific for the amino-terminal FLAG epitope tag and BrdUrd, followed by staining with Hoechst dye. Representative immunofluorescent data are shown in Fig. 7B, and the numbers of cells staining positively for both cyclin D1 and BrdUrd are shown graphically in Fig. 7C.

Because ectopically expressed cyclin D1 can be incorporated into catalytically active cyclin D1-CDK4 complexes (22), overexpression of cyclin D1 should not prevent S-phase entry. In-

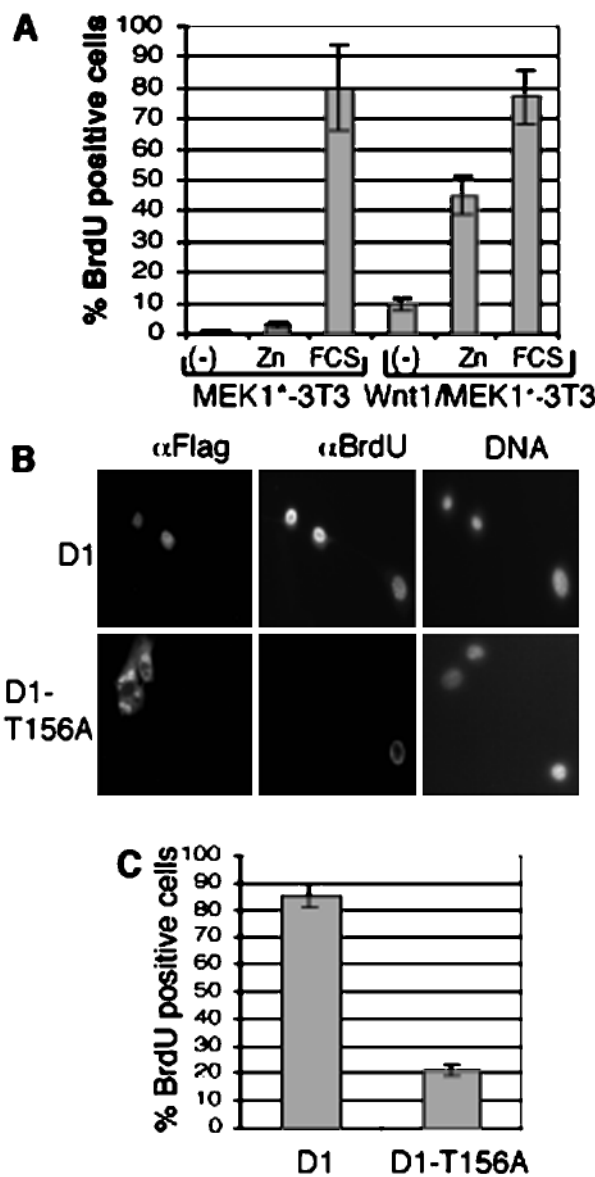


FIG. 7. Dominant-negative cyclin D1 inhibits Wnt1/MEK1*-dependent S-phase entry. A, cells expressing inducible MEK1* or inducible MEK1* and Wnt1 were serum-starved and re-stimulated with either ZnSO₄ or FCS in the presence of BrdUrd. After 20 h of stimulation, cells were harvested and processed for immunofluorescence using a BrdUrd-specific monoclonal antibody. The number of cells that stained positively for BrdUrd incorporation is expressed relative to the total population of cells. B, Wnt1/MEK1*-3T3 cells were transfected with a vector encoding either FLAG-tagged wild-type cyclin D1 (top panels) or cyclin D1-T156A (bottom panels). 24 h after transfection, the cells were arrested by serum withdrawal for 24 h and subsequently stimulated with ZnSO₄ and labeled with BrdUrd for 20 h. Cells were fixed and stained with either the FLAG-specific octa-probe antibody, a BrdUrd specific antibody, and Hoechst dye. C, graphic representation of the average percentage of cells that stained positively for both ectopic cyclin D1 and BrdUrd. The average shown was determined from four transfections.

deed, greater than 80% of the Wnt1/MEK1* cells expressing wild-type cyclin D1 entered S phase (Fig. 7B (top panels) and C (left bar)). In contrast, only 20% of the cells expressing D1-T156A entered the DNA synthetic phase (Fig. 7, B (bottom panels) and C (right bar)). 45% of the total population of transfected Wnt1/MEK1*-3T3 cells that did not take up DNA (as determined by immunofluorescence) entered S phase after the addition of zinc sulfate, demonstrating little or no toxicity due to the transfection protocol (data not shown). Thus, Wnt1/

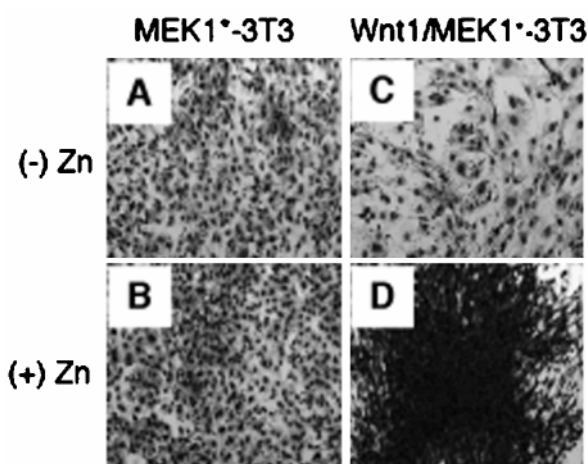


FIG. 8. Co-expression of Wnt1/MEK1* promotes foci formation. MEK1*-3T3 (A and B) or Wnt1/MEK1*-3T3 (C and D) seeded on dishes in 5% FCS in the absence (A and C) or presence (B and D) of zinc sulfate. After 3 weeks, cells were stained with Giemsa and foci were counted, and representative phase/contrast pictures were taken.

MEK1*-dependent S-phase entry requires functionally active cyclin D1-dependent protein kinase.

Based on the ability of Wnt1 and MEK1* to promote growth factor-independent S-phase entry, we reasoned that Wnt1/MEK1*-3T3 cells might exhibit additional properties of transformation such as loss of contact inhibition. To test this possibility, we determined the ability of MEK1*/Wnt1-3T3 cells to form foci. MEK1*-3T3 and MEK1*/Wnt1-3T3 were grown in medium containing 5% serum (to reduce toxicity due to zinc sulfate) with or without zinc sulfate (to induce MEK1* expression). The MEK1*-3T3 cells did not form detectable foci in the absence or presence of zinc (Fig. 8, A and B). Multiple foci were detected in MEK1*/Wnt1-3T3 cells stimulated with zinc sulfate but not in the absence of zinc (8, C and D). These data argue that MEK1*- and Wnt-signaling cooperate to transform NIH-3T3 cells.

DISCUSSION

Wnt1 Regulates the Accumulation of Active Cyclin D1-CDK4 Complexes—Wnt1 was initially identified as a proto-oncogene that becomes activated upon proviral insertion by the mouse mammary tumor virus (36), implicating Wnt gene products in the control of cellular proliferation. It is now clear that the primary function of Wnt family members is the regulation of various aspects of cellular differentiation and reorganization (1). Wnts elicit their effects in part through the activation of a signaling pathway, which inhibits GSK-3 β (2). Inhibition of GSK-3 β promotes the accumulation of β -catenin, which functions as a co-transcriptional activator of TCF/LEF-1-dependent transcription (7). The ability of β -catenin in conjunction with TCF/LEF-1 to promote expression of both *c-myc* (37) and *cyclin D1* (32, 33), both of which are known regulators of cellular proliferation, suggests that both may contribute to Wnt-induced proliferation.

We now demonstrate that cyclin D1 is a downstream target of Wnt-dependent signals. Wnt1 overexpression resulted in decreased GSK-3 β activity and increased cyclin D1 accumulation. We detected a dramatic decrease in cyclin D1 proteolysis (3-fold) in Wnt1-overexpressing cells. Although decreased cyclin D1 proteolysis likely made the largest contribution to cyclin D1 overexpression in Wnt1-3T3 cells, we also detected a modest increase in steady-state levels of cyclin D1 message, suggesting that increased cyclin D1 expression might also contribute to its overexpression in these cells. Given the ability of GSK-3 β to directly regulate cyclin D1 proteolysis and indirectly

regulate cyclin D1 expression through the modulation of β -catenin accumulation, we propose that the increased accumulation of cyclin D1 in Wnt1-overexpressing cells is the direct result of Wnt1-dependent inhibition of GSK-3 β . The modest increase in cyclin D1 expression in response to Wnt1 may reflect the transcriptional strength of the β -catenin-TCF-LEF-1 complex relative to the strength of mitogen-inducible transcriptional activators that regulate cyclin D1 expression. We also detected increased nuclear accumulation of cyclin D1 in cells overexpressing Wnt1. Again, this is likely a feature of Wnt1-dependent inhibition of GSK-3 β , as we have shown that GSK-3 β -dependent phosphorylation of cyclin D1 results in its nuclear to cytoplasmic redistribution (10).

Regulation of cyclin D1 may be a general property of Wnt family members. We also found that NIH-3T3 cells overexpressing Wnt2 exhibited a phenotype similar to that observed with the Wnt1-3T3 cells (data not shown). As cyclin D1 expression has profound effects on cellular differentiation (38) as well as proliferation (39–43), the ability of Wnt to regulate cyclin D1 may be critical not only for Wnt-induced cellular proliferation but for certain aspects of Wnt-induced cellular differentiation.

Wnt1/MEK1*-induced S-phase Entry—Although Wnt1 has oncogenic properties, expression of Wnt1 alone cannot transform most cell types. NIH-3T3 cells overexpressing Wnt1 do display some characteristics of morphologic transformation, but they do not display growth factor-independent growth (9) (Fig. 7). The capacity of Wnt1 and MEK1* to drive growth factor-independent S-phase entry supports the notion that the inability of Wnt1 to transform NIH-3T3 cells arises from its failure to activate ERK1 and ERK2 in these cells (9).

The capacity of NIH-3T3 cells overexpressing Wnt1 and activated MEK1 (MEK1*) to enter S phase in the absence of serum-derived growth factors is consistent with the idea that in the presence of active ERKs, Wnt1-dependent signals promote aberrant cellular proliferation. In addition, our data demonstrate that Wnt1-dependent signals can functionally substitute for PI3K to support progression into the DNA synthetic phase of the cell cycle. Sustained activation of the ERKs is necessary for expression of cyclin D1, and co-stimulation of PI3K-dependent pathways is also required for progression through the G₁/S-phase boundary of the cell cycle (17). Mitogen-dependent activation of MEK and PI3K results in the accumulation of high levels of cyclin D1 through MEK1-dependent activation of cyclin D1 expression (15, 17) and PI3K-dependent decreases in cyclin D1 proteolysis via inhibition of GSK-3 β (10). Consistent with previous observations indicating that Wnt-dependent signaling does not utilize the PI3K pathway (26), the PI3K inhibitor wortmannin, did not inhibit Wnt1/MEK1* induced S-phase entry (data not shown).

Our data is consistent with the notion that Wnt1/MEK1*-dependent S-phase entry is a feature of their ability to promote increased expression of cyclin D1 and simultaneously decrease cyclin D1 proteolysis, thereby achieving high levels of cyclin D1-dependent kinase. The ability of a dominant negative cyclin D1 mutant (35), D1-T156A, to block Wnt1/MEK1*-induced proliferation supports this hypothesis. We have previously shown that cyclin D1-T156A acts in a dominant-negative fashion with respect to endogenous cyclin D1 due to its ability to sequester CDK4 into catalytically inactive, cytoplasmic complexes (35).

We also noted that cells expressing Wnt1 and MEK1*, unlike cells expressing only MEK1*, displayed reduced levels of p27^{kip1} (Fig. 6B). The reduced levels of p27^{kip1} may be an indirect result of G₁/S-phase progression (34). Alternatively, Wnt1 may directly induce p27^{kip1} degradation via an as yet uncharacterized pathway. We also noted that activation of

MEK1 was sufficient to induce accumulation of p21^{cip1}. This was demonstrated previously (15) and is consistent with the possibility that p21^{cip1} may participate in the assembly of the active cyclin D1-CDK4 kinase in response to mitogenic stimuli.

Cyclin D1 in Human Cancer—Although cyclin D1 overexpression in human cancer frequently results from gene rearrangements (19, 44), in certain tumors high levels of cyclin D1 accumulation cannot be explained by such mechanisms. In point of fact, cyclin D1 accumulates to high levels in patients containing colorectal adenocarcinoma and mice bearing intestinal adenomas (44, 30), although no genetic abnormalities have been documented in cyclin D1 or components of the cyclin D1, p16^{ink4a}, Rb pathway (19, 45). Inactivating mutations in the adenomatous polyposis coli tumor suppressor occur with a high frequency in familial colon carcinoma (46). Adenomatous polyposis coli, a target of Wnt signaling pathways (47), regulates turnover of β -catenin in a manner that depends upon phosphorylation of β -catenin by GSK-3 β (48, 49). Mutations in adenomatous polyposis coli result in the accumulation of β -catenin and the constitutive activation of TCF/LEF-1-dependent transcription (50, 51). One downstream target of this transcription factor complex is the *cyclin D1* gene (32, 33). Thus, high levels of the β -catenin-TCF-LEF-1 could promote cyclin D1 overexpression in colon carcinoma. In addition, components of the Wnt signaling pathway upstream of GSK-3 β may also be subverted. Events that compromise GSK-3 β activity would result in both β -catenin and cyclin D1 stabilization. Thus, activating mutations in the Wnt-dependent signaling pathway could contribute to cyclin D1 overexpression in colon carcinoma.

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