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Twist Is Up-Regulated in Response to Wnt1 and Inhibits Mouse Mammary Cell Differentiation[1]

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ABSTRACT

Wnt1, initially identified as a mammary oncogene, can activate transcription via β-catenin/TCF complexes. Twist, a transcription factor of the basic helix-loop-helix class, has also been suggested to have oncogenic properties. The aim of this study was to determine whether *Twist* is regulated by Wnt1 and might thus be a novel mediator of Wnt signaling. We found that *Twist* was up-regulated in C57MG and HC11 murine mammary epithelial cells in response to *Wnt1* expression. Additionally, we detected *Twist* expression in normal mammary gland and found elevated *Twist* expression in ~70% of mammary tumors from *Wnt1* transgenic mice. A murine *Twist* promoter fragment was shown to be responsive to β-catenin, and its activity was enhanced by coexpression of c-*jun* and Ets factors of the PEA3 family. Both PEA3 factors and c-*jun* were highly expressed in tumors from *Wnt1* transgenic mice and may therefore contribute to the increased *Twist* expression observed in these tumors. To evaluate functional consequences of *Twist* induction, we examined the effect of Twist on mammary cell differentiation. Strikingly, overexpression of either *Wnt1* or *Twist* in HC11 mammary epithelial cells completely suppressed induction of the milk protein β-casein in response to lactogenic hormones. Additionally, Wnt1, but not Twist, partially abrogated induction of WDNM1, another marker of lactogenic differentiation. Taken together, our data indicate that *Twist* expression is regulated by Wnt/β-catenin signaling and that both Wnt1 and Twist can function as inhibitors of lactogenic differentiation, an effect that could contribute to mammary tumorigenesis.

INTRODUCTION

Wnt proteins are secreted signaling factors that play multiple roles during development, including essential functions in gastrulation, limb patterning, brain morphogenesis, kidney formation, and placental development. Activation of Wnt signaling is also associated with tumorigenesis. *Wnt1*, the founder member of the *Wnt* gene family, was initially identified as a mammary oncogene insertionally activated by mouse mammary tumor virus (1–3). Overexpression of *Wnt1 in vivo* results in mammary tumorigenesis (4) and *in vitro* causes morphological transformation of mammary epithelial cell lines such as C57MG and RAC311 (5, 6). Additionally, several *WNT* genes are misexpressed in human breast cancer (7–9), and mutational activation of the Wnt signaling pathway occurs in numerous human epithelial cancers (8, 10).

The canonical Wnt signaling pathway involves stabilization of a cytoplasmic β-catenin pool (11, 12), which can modulate transcription by interaction with transcription factors, including those of the TCF family (13, 14). Inappropriate β-catenin accumulation with consequent transcriptional activation can result from *Wnt* gene misexpression but also occurs as a consequence of mutation of the β-catenin gene (*CTNNB1*) itself or of other components of the Wnt/β-catenin signaling pathway such as *AXIN* or *APC* (10). Thus, there is considerable interest in identifying transcriptional targets of Wnt/β-catenin signaling, particularly those that contribute to tumorigenesis. Several candidate genes have been identified, including cyclin D1 (15, 16), c-myc (17), Matrilysin (18, 19), PPARδ (20), WISP-1 (21), c-jun, and fra-1 (22). We are interested in identifying additional Wnt transcriptional targets and understanding their roles in Wnt-activated processes.

We focused on *Twist* as a candidate Wnt target gene based on evidence from *Drosophila* developmental genetics. *Twist* was first described in *Drosophila* as a gene essential for dorsoventral polarity (23, 24) and encodes a transcription factor of the bHLH[6] family. Ventral *Twist* expression in early embryos is regulated by the Rel factor Dorsal (25–27). Additionally, *Twist* expression is diminished in *Drosophila* embryos that are deficient in *wingless*, the *Drosophila* homologue of *Wnt1* (28), suggesting that *Twist* might also be regulated by Wnt signaling. In mammals, Twist contributes to morphogenesis of the cranial neural tube: *Twist*-null mice die at E11.5 with unfused cranial neural folds, as well as defects of the head mesenchyme, somites, and limb buds (29). Embryonic *Twist* expression patterns are consistent with contributions to neural and limb development (30–32). Germ-line mutations at the *TWIST* locus in humans cause Saethre-Chotzen syndrome, an autosomal-dominant craniosynostosis syndrome that results in premature closure of the coronal sutures of the skull (33, 34). Additionally, Twist shows some oncogenic properties, promoting colony formation of mouse embryonic fibroblasts in soft agar and antagonizing p53-induced growth arrest (35).

Here we demonstrate that *Twist* is up-regulated in response to *Wnt1* expression in mouse mammary epithelial cell lines and tumors. Analysis of *Twist* promoter regulation revealed responsiveness to β-catenin, c-jun, and Ets factors of the PEA3 family. All of these are elevated in *Wnt1*-expressing mammary tumors and may therefore contribute to the observed *Twist* up-regulation in these tumors. We also found that overexpression of either *Wnt1* or *Twist* abrogated prolactin-stimulated β-casein induction in a cell culture model. Furthermore, up-regulation of an additional lactogenic marker, WDNM1, was also diminished in *Wnt1*-overexpressing cells. Since suppression of terminal differentiation is associated with tumorigenesis, Wnt-mediated suppression of lactogenic differentiation could contribute to Wnt-induced mammary tumorigenesis.

MATERIALS AND METHODS

Plasmids. A single genomic clone containing the *Twist* gene was isolated from a lambda Fix II C3H mouse genomic library (Stratagene, La Jolla, CA). The library was probed with a 268-bp fragment generated by PCR with primers designed to amplify a region of the 3′ untranslated region straddling an intron. Primers were 5′-CGGAGACCTAGATGTCATTGTTTCC-3′ and 5′-GGG-

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[6] The abbreviations used are: bHLH, basic helix-loop-helix; MG, mammary gland; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; DIP, dexamethasone/insulin/prolactin; FBS, fetal bovine serum; WAP, whey acidic protein.

GACACAAACGAGTGTTCAG-3′. A clone of ~9 kb was isolated and subcloned into pBluescript SK+ (Stratagene). A 2-kb *Ngo*MI fragment containing ~70 bases from exon 1 (30) plus 1.9 kb of upstream sequence was subcloned from this construct into *Sma*I-cut pGL2basic (Promega, Madison, WI) to generate the promoter reporter construct Twist-LUC. An additional promoter reporter construct Rev-Twist-LUC was also generated in which the 2-kb promoter fragment was inserted in the reverse orientation. pMV-Twist was constructed by subcloning a PCR product encompassing the entire coding region of *Twist* into the retroviral vector pMV7 (PCR primers; 5′-CCGGAATTCATGATGCAGGACGT-3′ and 5′-CCGGAATTCCTAGTGGGACGCGGA-3′). pSK-Twist was generated by subcloning a blunted 859-bp *Sac*II fragment of genomic *Twist* comprising the entire *Twist* open reading frame into *Not*I-digested pBluescript SK+ using *Not*I linkers. pRcTwist was constructed by subcloning a *Bam*HI-*Avr*II fragment from pSK-Twist containing the entire coding sequence of *Twist* into pRcCMV (Invitrogen, Carlsbad, CA). All constructs were verified by DNA sequencing by the DNA/Protein Technology Center (Rockefeller University, New York, NY). The plasmids used for transfection of 293 cells (pMT23, pMT23β-catenin, pCANΔN89β-catenin, pCANmycPEA3, pcDNA-ER81, pCANmycERM, Ets-1, pSG5-Ets-2, pCMX-c-jun, pRL-TK, and the stromelysin-1 promoter construct p754TR-Luc) were as described previously (18, 36).

Mouse Tissue and Tumor Harvesting. A breeding colony of *Wnt1* transgenic mice (4) was maintained by crossing *Wnt1* transgenic B6/SJL males (Jackson Laboratory) with strain-matched females. Mice were genotyped by PCR analysis of tail-tip DNA, as described previously (36). *Wnt1* transgenic animals were sacrificed when tumors were 1 cm in diameter, and wild-type littermates were simultaneously sacrificed. Tumors and MGs were snap-frozen in liquid nitrogen and stored at −80°C before RNA preparation.

RNA Preparation and Northern Blotting. RNA was prepared from tissue or cells using TRIzol Reagent (Life Technologies, Inc., Grand Island, NY) or RNAzol B (Tel-Test, Inc., Friendswood, TX) according to the manufacturers' instructions. Northern blots and hybridization were performed as previously described (37) using 20 μg of total RNA. The *Twist* probe was a 421-bp *Xba*I-*Eco*RI fragment from the 3′-untranslated region of mouse *Twist* cDNA (30). The murine β-casein probe was prepared by PCR amplification of a 306-bp fragment (bases 437–742 of the mouse genomic DNA), which was subcloned into pBluescript (Stratagene). Murine GAPDH probe was obtained from Alan Ashworth (Institute of Cancer Research, London, United Kingdom). GAPDH was used to control for loading of each lane. c-jun probe (hCJ-1) was obtained from Andrew S. Kraft (University of Colorado Health Sciences Center, Denver, CO). Murine WDNM1 and WAP probes (pBS-WDNM1 and pBS-WAP) were obtained from Gertraud Robinson (National Institute of Diabetes and Digestive and Kidney Diseases, Bethesda, MD). Autoradiographic exposures of Northern blots were quantitated by analysis on a Macintosh computer using the public domain NIH Image program (developed at the United States NIH and available online).[7] Values obtained were normalized to those obtained for GAPDH.

Cell Culture and Transfection. Generation of C57/MV7 and C57/Wnt-1 cell lines, and culture conditions of these and C57MG cells, have been described previously (37, 38). Culture and transfection of 293 human embryonic kidney cells was performed as described previously (36). Lysates were prepared 48 h after transfection, and Firefly and Renilla luciferase activities were measured using a Dual Luciferase Reagent kit (Promega) and a Monolight 2010 luminometer (Analytical Luminescence Laboratory). HC11 mouse mammary epithelial cells (39) were grown in RPMI 1640 containing 8% FBS, 10 ng/ml murine epidermal growth factor, 5 μg/ml bovine insulin, 100 units/ml penicillin, and 100 μg/ml streptomycin. Cells were infected with MV7, MVWnt1, and MVTwist retrovirus using helper-free virus stocks as described previously (40). Approximately 200 G418-resistant colonies were pooled to generate the pooled populations designated HC11/MV7, HC11/Wnt-1, and HC11/Twist. Lactogenic stimulation of HC11-derived cell lines was achieved by maintaining the cells at confluence for 2 days, then incubating for an additional 3 days in RPMI 1640 containing 8% FBS, 1 μM dexamethasone, 5 μg/ml insulin, and 5 μg/ml bovine prolactin. Cos cells were grown in DMEM containing 10% FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin and were transfected with Lipofectamine (Life Technologies, Inc.) according to the manufacturer's instructions.

Generation of Antisera. A peptide corresponding to amino acids 34–46 of Twist was synthesized with an additional COOH-terminal cysteine residue (Peptide Core Facility; Rockefeller University) and conjugated to Keyhole Limpet Hemocyanin using a coupling kit (Pierce, Rockford, IL). Three rabbits were immunized with peptide conjugate and sera collected (Covance Research Products, Philadelphia, PA). Sera were tested for their ability to recognize Twist protein on a Western blot using bacterially produced recombinant protein (glutathione *S*-transferase *versus* glutathione *S*-transferase-Twist) and Twist protein ectopically expressed in Cos cells. Serum from no. 535 was selected for additional use.

Cell Lysate Preparation and Analysis. Crude cell lysates were prepared by removing the cells from the dishes, pelletting, and resuspending in lysis buffer containing 150 mM NaCl, 100 mM Tris (pH 7.4), 1% Tween 20, 1 mM EDTA, 20% glycerol, and Complete Proteinase Inhibitor (Boehringer Mannheim, Indianapolis, IN). Resuspended cell pellets were vortexed briefly and incubated 30 min, 4°C, then sonicated for a total of 30 s. Debris was pelletted by brief centrifugation, and the supernatant was stored at −20°C.

For Western analysis, samples were subjected to SDS-PAGE using 4–15% gradient gels (Bio-Rad, Hercules, CA) and proteins transferred to polyvinylidene fluoride membrane (Immobilon, Millipore, Billerica, MA). Blots were blocked with 3% gelatin in Tris-buffered saline [150 mM NaCl, 10 mM Tris, (pH 8.0)] and incubated 1 h in primary antibody diluted 1:1000 or 1:3000 in 1% gelatin in Tris-buffered saline. Secondary antibody was alkaline phosphatase-conjugated goat antirabbit antibody (Bio-Rad). Blots were developed using a chemiluminescent reagent (NEN, Boston, MA).

RESULTS

***Twist* Expression is Up-regulated in Response to Wnt1.** Our interest in *Twist* as a candidate Wnt-regulated gene was stimulated by the observation that *Twist* expression is diminished in *wingless* mutant *Drosophila* embryos (28). One potential explanation for this observation is that *Twist* is transcriptionally regulated by wingless, the *Drosophila* homologue of Wnt1. Therefore, we asked whether mammalian *Twist* is a target of Wnt/β-catenin signaling. As an initial approach, we evaluated *Twist* expression in *Wnt1*-expressing C57MG cells because C57MG cells have previously proved useful as a model cell line for identifying Wnt target genes relevant to carcinogenesis (21, 37, 41). Northern blot analysis demonstrated significant up-regulation of *Twist* expression in C57/Wnt-1 cells relative to expression levels in control C57/MV7 cells (Fig. 1*A*), and a corresponding increase in Twist protein was observed (Fig. 1*B*). We had previously made the novel observation that *Twist* is expressed in adult murine MG during a PCR-based screen designed to identify bHLH proteins involved in the process of mammary differentiation (data not shown). Therefore, we also examined *Twist* expression in wild-type MG and in mammary tumors from *Wnt1* transgenic mice. Interestingly, we observed increased expression of *Twist* RNA in five of seven tumors tested relative to that observed in normal MG (Fig. 1, *C* and *D*). Taken together, these data suggest that *Twist* is regulated by Wnt signaling.

Coordinate Regulation of the Twist Promoter by β-Catenin, c-jun, and PEA3 Factors. To investigate transcriptional regulation of the *Twist* gene, we generated a *Twist* promoter reporter construct (Twist-LUC) by insertion of a 2.0-kb murine *Twist* promoter fragment into the luciferase reporter construct pGL2basic. Alignment of this promoter fragment with the previously cloned murine *Twist* gene (30), and the human *Twist* gene (42) is shown in Fig. 2*A*. At the 5′ end, our sequence extends the previously characterized murine sequence (30) by ~1280 bases. At the 3′ end, the fragment extends into exon 1 but contains no coding sequence.

Transient transfection experiments were performed using Twist-LUC to test whether the *Twist* promoter was responsive to overexpressed β-catenin. The *Twist* promoter construct Twist-LUC exhibited

[7] Internet address: rsb.info.nih.gov/nih-image.

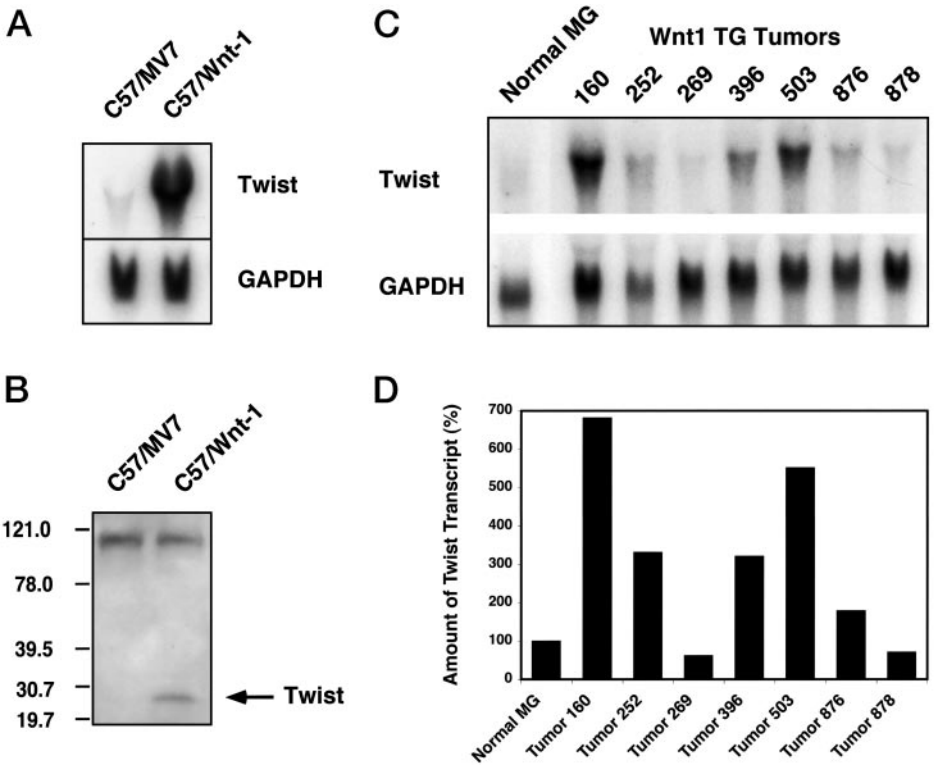


Fig. 1. *Twist* expression is increased in response to *Wnt1*. *A*, total RNA was prepared from C57/MV7 and C57/Wnt-1 cells, and 20 μg of RNA were analyzed by Northern blotting. The blot was probed sequentially with a murine *Twist* probe and a GAPDH probe. Transcript sizes are 1.7 and 1.4 kb for *Twist* and GAPDH, respectively. *B*, cell lysates were prepared from C57/MV7 and C57/Wnt-1 cells, and 50 μg were subjected to SDS-PAGE and Western blotting with anti-Twist antibody. The positions of molecular mass markers (in kDa) are indicated. A Twist protein band was detected in C57/Wnt-1 but not in C57/MV7 cells. *C*, total RNA was prepared from MGs from wild-type mice (normal MG) and from tumors from 7 *Wnt1* transgenic (TG) mice, and 20 μg of RNA were analyzed by Northern blotting for Twist and GAPDH. Low basal *Twist* expression could be detected in normal MG, particularly on longer exposures (data not shown). *D*, Twist signals from the Northern blot in *C* were quantitated using the program NIH Image and normalized to those obtained from GAPDH probing. Values obtained are expressed relative to that obtained from normal MG.

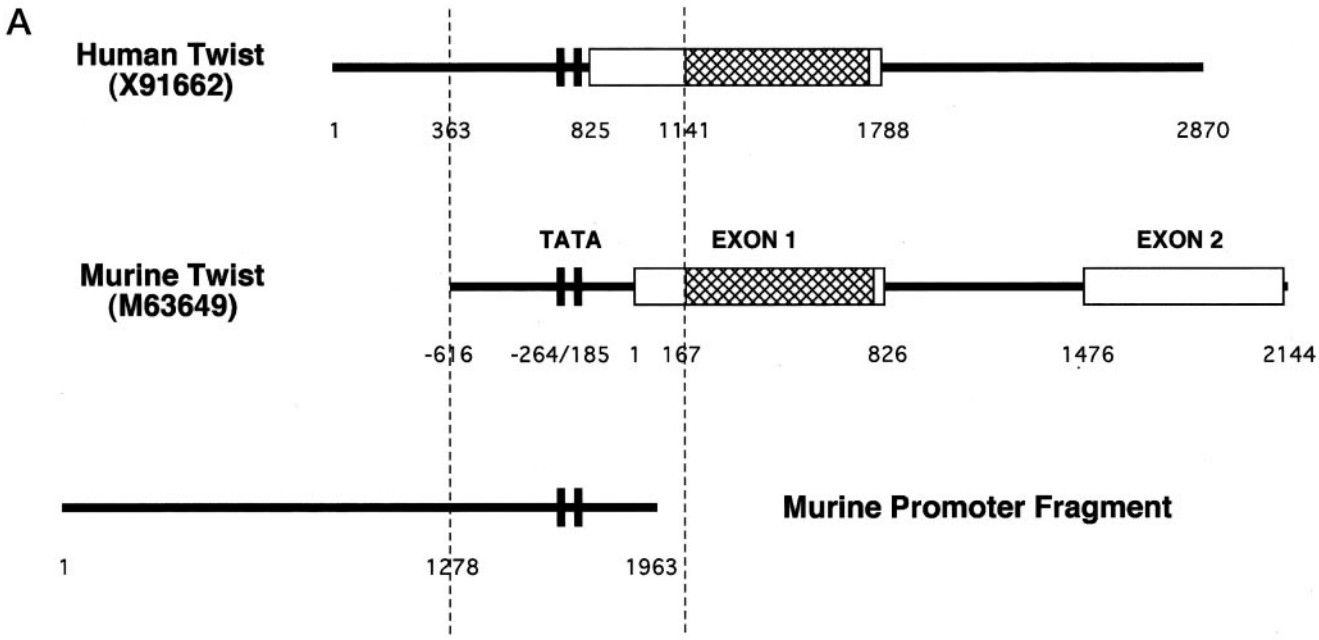
dose-dependent activation in response to β-catenin. Twist-LUC activity increased to ~200% in response to β-catenin overexpression (Fig. 3, *A* and *B*; $P < 0.001$). In contrast, the construct Rev-Twist-LUC in which the *Twist* promoter fragment is inserted in the reverse orientation was unresponsive to β-catenin (Fig. 3*A*), demonstrating specificity of the Twist-LUC response.

Synergistic activation of the Wnt target gene *Matrilysin* by β-catenin, c-jun, and PEA3 family Ets transcription factors has been reported previously (43). Additionally, c-*jun* transcription can be regulated by β-catenin/TCF complexes (22), and we have previously reported that *PEA3* is highly expressed in C57/Wnt-1 cells and in tumors from *Wnt1* transgenic mice (36). Therefore, we also tested the effect of c-jun and PEA3 in combination with β-catenin. PEA3 caused a modest increase in *Twist* promoter activity to 240% of control ($P < 0.001$), whereas c-jun alone failed to stimulate promoter activity (Fig. 3*B*). However, both c-jun and β-catenin synergistically increased Twist-LUC activity when coexpressed with PEA3 ($P < 0.01$). Strikingly, coexpression of all three transcription factors increased *Twist* promoter activity to 840% of control ($P < 0.001$). We also examined the response of the *Twist* promoter to the Ets factors ETS-1 and ETS-2 in comparison with the PEA3 subfamily members PEA3, ER81, and ERM. Of these, PEA3 and ERM were the most potent activators of the *Twist* promoter (Fig. 4). ER81 and ETS-1 elicited weaker responses, and ETS-2 caused no activation. It was not possible to directly compare expression levels of the various factors because the cDNAs were not uniformly epitope-tagged. However, because ETS-2 caused much stronger activation of the stromelysin-1 promoter than did PEA3 in the same experiment (Fig. 4, *right panel*), these data suggest that the *Twist* promoter is preferentially responsive to PEA3 subfamily members.

PEA3 is known to be up-regulated in *Wnt1*-expressing C57MG cells and mammary tumors from *Wnt1* transgenic mice (36). Thus, it seems likely that PEA3 contributes to the increased *Twist* expression observed in response to Wnt1 (Fig. 1). To test the involvement of c-jun, we analyzed c-*jun* expression in *Wnt1*-expressing C57MG cells and mammary tumors. Although c-*jun* transcription can be activated by β-catenin in human colorectal carcinoma cell lines (22), we did not observe increased c-*jun* expression in C57/Wnt-1 relative to control C57/MV7 cells (data not shown). However, c-jun was highly expressed in all six tumors tested (Fig. 5). These data indicate that both c-jun and PEA3 factors may contribute to the *Twist* up-regulation observed in a subset of *Wnt1* transgenic mammary tumors. However, because both c-*jun* and *PEA3* are uniformly expressed at high levels in all tumors tested (Fig. 5; Ref. 36), whereas *Twist* expression was increased in only five of seven tumors examined (Fig. 1, *C* and *D*), the murine *Twist* promoter may also be subject to regulation by other factors yet to be identified.

Twist Expression Antagonizes β-Casein Induction by Lactogenic Hormones. Twist has previously been demonstrated to antagonize differentiation during mammalian myogenesis and osteogenesis (44, 45). Given our novel observation of *Twist* expression in mammary cell lines and tissues, we speculated that Twist might similarly repress mammary cell differentiation. To test this hypothesis, we used HC11, a murine mammary epithelial cell line. HC11 cells exhibit up-regulation of milk protein genes in response to lactogenic hormones, with no requirements for extracellular matrix components or cocultivation with other cell types (39). This makes them a convenient *in vitro* model for studying lactogenic differentiation. HC11 cells overexpressing *Twist* were generated by retroviral infection with MVTwist, and *Twist* overexpression was confirmed by Western blotting (Fig. 6*A*). No Twist protein was detectable in the control infected HC11/MV7 cells or in the parental HC11 cells (Fig. 6*A*).

DIP was used as a lactogenic stimulus to induce HC11 differentiation (39). Expression of several milk proteins commonly used as markers of lactogenic differentiation was examined, including WDNM1, β-casein, and WAP (46). We were unable to detect WAP expression in either control or DIP-treated HC11 cells (data not shown). However, both WDNM1 and β-casein were up-regulated in HC11/MV7 cells in response to lactogenic stimulation with DIP (Fig. 6, *B* and *C*). DIP-mediated up-regulation of the *WDNM1* transcript



B

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        10        20        30        40        50        60        70        80        90       100       110       120
CCCGGGGAGGGGGGGGGGGGTCTCTACTCGGATGAAAGTACAGTCGGGTTTACTGGCCTCAGAAGAGAGCTTTCTCTACAAAGGCTGAGTCCTCGGACTAGCCTCAGGCATTCCACACCCC

       130       140       150       160       170       180       190       200       210       220       230       240
AGAGTTTCCGGGACCCAGCTGAGAGCTTCAGCAGGACCCAGCCACGTGGCCTGCCCTGCGTCGCAGGGGCTCCGGCCTCCAGCTTCTCAGGAAGTCGGTGGCCGCGACCTTTGGTCCAAC

       250       260       270       280       290       300       310       320       330       340       350       360
CCAGAAAGGGGCACTTGCTGAGCTGGCAGTTTCCGGGGGATTGGCCAGGGGAGTGGGGATTGGGCGTTTAGCAAGGTCCTCAGCTTGGGAACAGGGCAGGAGTTCGAGACGCTCACCTC

       370       380       390       400       410       420       430       440       450       460       470       480
CACGCCCGCAAACTCTGCCCCTGAAACTCTGGCTACTTCCTCCGGACCCAGAATTGCCTTTCTGAGTAAGAGATCACCTTGTACCGGCTCCCTAAATCCCCATCCTACTTAAAAAACAGA

       490       500       510       520       530       540       550       560       570       580       590       600
AATAAATAAATAAAACATATTTAAAAAATGTAAAGAGCCCTGCAATCACTGGCTTCCATAGCCACTCTCCTTCTTGCTCATTAACCTCTCTGCTTCCACTTTTCCAGAATCCTTTTTGGG

       610       620       630       640       650       660       670       680       690       700       710       720
GTTTCAAGGTATTTGGTAAAAAAAAAAAAAAAAAAAACCAAACCAAAAAAAAAACCAAAAAAAACCGCTTGCAATCAACCAACATGGCTGAGTGCATTTGAGGCACCGGGAGCATAACTA

       730       740       750       760       770       780       790       800       810       820       830       840
CTCACAAGATCTCCTAGGAAGACGAGAATATTGATCCCCCACCTTAAATGTATGGACTACCCAAGGTTAGCATGCAGGGCTTAATTTGGAGTTAGCGGACATGAAATGTTCTCAAGGTTC

       850       860       870       880       890       900       910       920       930       940       950       960
TCCTCCACCTAGGGCTGGCAGCGTGGAGCACCAAGGCTGCTCTATCTTTCCCTAGCTTTGCTCCCTTCAAAAGAACAAGGCTTTTCTCTGTAAAAGAATGAACTCCAGCCTAGGTCCAGC

       970       980       990      1000      1010      1020      1030      1040      1050      1060      1070      1080
ACCTTTGGAAGCACAGTCCTCTGAGAGCAGGGAAACTTTAGATTGGGTCACCGTAGCAGGAAAGGAGAGCCCAGACGTTTCTCCGGGAGGGGGTGACACGGCAGGCCAGGGCGCAGATCCA

      1090      1100      1110      1120      1130      1140      1150      1160      1170      1180      1190      1200
TAGCGCCCACGATGTGGCCACGTCTTGAGAACCGCCCTCGACCACAGGGCCAGGCAGCCGCGGGGAATCCCTTGGGACTAGAGGTTTCCGACTAGAGGTTTCCTCTACCTGACCGTTAGA

      1210      1220      1230      1240      1250      1260      1270      1280      1290      1300      1310      1320
TGGACTCGGTTGTTGAAACTTTTCTTGCCTCGCAGCCCGGGCCCGAGCCACCCCCAAAGGGCCCGGAGAACTCCGAGGGATCCCAGGCGGGATGGGACATCACCCAGAGCGTCGAAGCCG

      1330      1340      1350      1360      1370      1380      1390      1400      1410      1420      1430      1440
CTTGCCACTACTGCTGTCACAGCCACTTGGGGAGGGGGAGACTGCAACAGCGACCCAGACAGTCCCCTGTTTGCGGTGCTGTGCTCGGGTGCGGGGGCGCTGGGCGCGCTCTGGCGCGGA

      1450      1460      1470      1480      1490      1500      1510      1520      1530      1540      1550      1560
AGGAAATCGCCCCGCGTCCTCCCCGAAGCGGAGGGGGAGAGCTGGGGGGGGGAGAGGGAGGGGAGAGGCGGGCGCAGGGGCTGGCCGCTCGGGCCAGGTCGGTTTTGAATGGTTCGCAGG

      1570      1580      1590      1600      1610      1620      1630      1640      1650      1660      1670      1680
ACGAATTGTTAGACCCCGAGCAAGGGGGGAGGGGGCGAGGGAGGGGGGCTGGAAAGAGGAAACTTTCCTATAAAACTTCGAAAAGTCCCTCCTCCCCACGTCAGGCCAATGACACCGCTG

      1690      1700      1710      1720      1730      1740      1750      1760      1770      1780      1790      1800
CCCCCAAACTTTCCGCCCGCACGGAGGTATAAGAGCCGCCAAGTCCACAGCCCGCGCCCAACTCTCAGACACCTCGCGGGCTCGCCAGCACCGGCACCGGCTGCGCCAGGCCGGAACGGG

      1810      1820      1830      1840      1850      1860      1870      1880      1890      1900      1910      1920
GCGCGCTTGCGGCCGTCGGGCGCCTTCTTTTTGGACCTCGGGGCCCTCCACACCGTCCCCTCCCCCGCCCGCCTCCCTCCCCGCCTCCAGCGCCCTCCCCGCGGAGGTCCCCCCACCCCC

      1930      1940      1950      1960
ATCCCGCCTCCCTGCGTCCTCCGGGCCGCACCGCCCGGGCCGG
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Fig. 2. Murine *Twist* promoter. *A*, alignment of 2-kb murine *Twist* promoter fragment with published sequences. Features depicted include: open reading frame (▨), exons (□), and potential TATA boxes (*black rectangles*), of which only the 3′ TATA box is functional in human *Twist* (42). The entire sequence can also be roughly aligned with residues 15391–13419 of the human Twist gene (65). *B*, nucleotide sequence. Three potential TCF binding sites are shown in *bold*. Of these, the site at bases 76–83 corresponds well to a consensus TCF site (5′-CCTTTG,A/T,A/T-3′), but the sites at bases 896–902 and bases 962–970 do not precisely match the consensus TCF binding site. None of these are conserved in the human promoter. Thirty-four Ets binding core sequences (5′-GGA,A/T-3′) are boxed in *gray*.

was unaffected by *Twist* overexpression (Fig. 6*B*). Strikingly, however, induction of β-casein RNA in response to DIP was completely absent in HC11/Twist cells (Fig. 6*C*). Thus, overexpression of *Twist* was sufficient to block hormone-stimulated β-casein expression in HC11 cells.

Ectopic *Wnt1* Expression Antagonizes β-Casein Induction. Taken together, our observations that Twist can regulate β-casein induction and that *Twist* itself is regulated by Wnt signaling suggested that Wnt1 might also modulate cellular responsiveness to lactogenic hormones. To test this possibility, we generated *Wnt1*-expressing HC11 cells (HC11/Wnt-1) and control cells (HC11/MV7) using retroviral expression vectors. Interestingly, *Wnt1* expression in HC11 cells led to morphological transformation (data not shown), as has previously been described for both C57MG and RAC311 cells (5, 6). Expression of *Wnt1* in HC11 cells caused an increase in Twist protein and RNA (Fig. 7, *A* and *B*), consistent with our observations in C57MG-derived cell lines (Fig. 1). The effect of *Wnt1* expression on both β-casein and WDNM1 induction was compared in HC11/MV7 and HC11/Wnt-1 cells, again using DIP as a lactogenic stimulus. Control HC11/MV7 cells showed robust *WDNM1* expression in response to DIP, but *Wnt1* overexpression partially abrogated WDNM1 induction in response to hormonal stimulation (Fig. 7*C*). Additionally, we examined the effect of *Wnt1* overexpression on β-casein induction (Fig. 7*D*). Although β-casein was up-regulated by DIP in control HC11/MV7 cells, there was no induction of β-casein in HC11/Wnt-1 cells. Thus, *Wnt1* expression, similarly to *Twist* overexpression, completely abolished the ability of HC11 cells to activate β-casein transcription in response to DIP. Because Wnt1 causes induction of Twist in these cells, their failure to respond to lactogenic stimuli by activating β-casein transcription may thus be attributable to Twist.

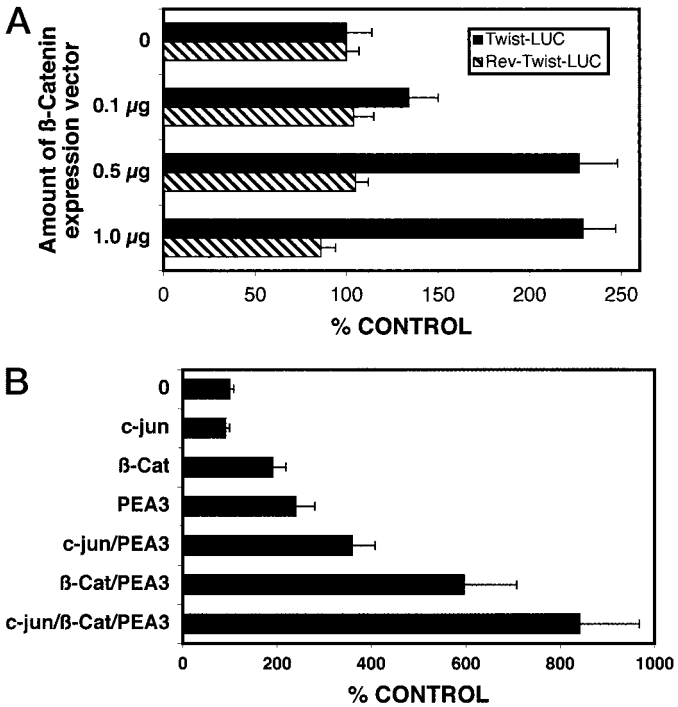

Fig. 3. Regulation of the *Twist* promoter. *A*, β-catenin dose dependence of *Twist* promoter activation. 293 cells were transfected with increasing amounts of β-catenin expression vector, together with either the *Twist* promoter luciferase reporter construct Twist-LUC (■) or the construct Rev-Twist-LUC (▧) in which the *Twist* promoter fragment is inserted in the reverse orientation. pRL-TK was cotransfected as an internal control. Results shown are the mean + SD of 6 replicates from a representative experiment expressed relative to activity in control cells transfected with empty expression vectors. *B*, 293 cells were transfected with combinations of expression vectors encoding c-jun, β-catenin (β-Cat), and PEA3 plus Twist-LUC and pRL-TK, and luciferase assays were performed as described above. Results shown are the mean + SD of 6 replicates.

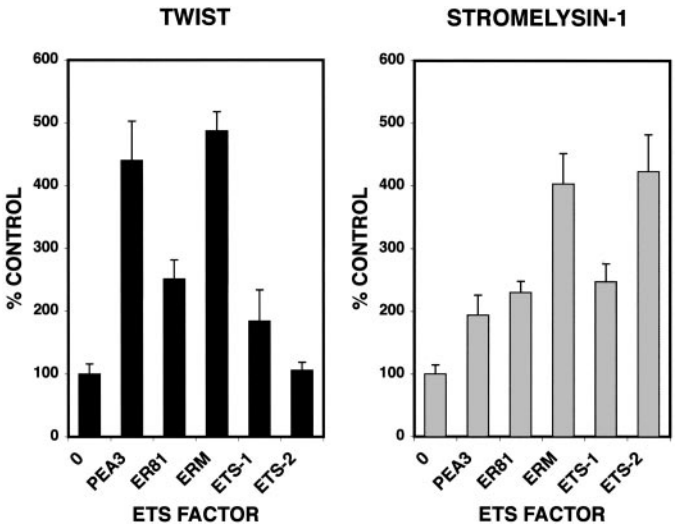

Fig. 4. The *Twist* promoter is selectively activated by PEA3 factors. 293 cells were transfected with expression vectors encoding PEA3, ER81, ERM, ETS-1, or ETS-2, together with pCMX-c-jun, pRL-TK and Twist-LUC (*left panel*). In parallel, cells were transfected with the Ets expression vectors pCMX-c-jun, pRL-TK, and the stromelysin promoter luciferase reporter construct p754TR-Luc (*right panel*). Results shown are the mean + SD of 6 replicates. All factors except ETS-2 caused a significant increase in Twist-LUC activity ($P < 0.01$), and all factors caused a significant increase in stromelysin promoter activity ($P < 0.01$).

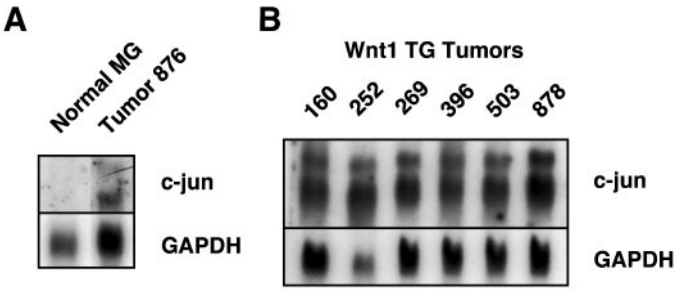

Fig. 5. c-*jun* is highly expressed in tumors from *Wnt1* transgenic mice. *A*, c-*jun* expression is up-regulated in mammary tumor tissue. Total RNA was prepared from a mammary tumor from a *Wnt1* transgenic mouse and also from MG from an age-matched wild-type littermate (normal MG). Twenty μg of RNA were analyzed by Northern blotting for c-jun and GAPDH. *B*, c-*jun* is expressed in all mammary tumors from *Wnt1* transgenic mice. Total RNA was prepared from tumors from 6 *Wnt1* transgenic mice, and 20 μg of RNA were analyzed by Northern blotting for c-jun and GAPDH.

DISCUSSION

Twist Regulation by Wnt1. Here, we report that *Twist* is up-regulated in *Wnt1*-expressing mouse mammary epithelial cell lines (Figs. 1 and 7). Additionally, we have shown that *Twist* is expressed in adult murine MG and that *Twist* expression is significantly increased in mammary tumors from *Wnt1* transgenic mice (Fig. 1). To our knowledge, this is the first demonstration that *Twist* is up-regulated in response to Wnt signaling and that *Twist* is expressed in mammary tissue. Several *Wnt* genes exhibit dynamic expression patterns during mammary development (47–50). In particular, the mammary expression profile of *Wnt2* resembles that which we have observed for *Twist* (48).[8] Wnt2 activates the canonical Wnt/β-catenin signaling pathway (12, 51), and we have observed elevated *Twist* expression in C57/Wnt2 cells (data not shown). Taken together, these data suggest that Wnt2 could contribute to the regulation of endogenous *Twist* expression in the MG.

Having demonstrated Wnt1-mediated *Twist* up-regulation, we next

[8] O. Watanabe and J. Leonard, unpublished data.

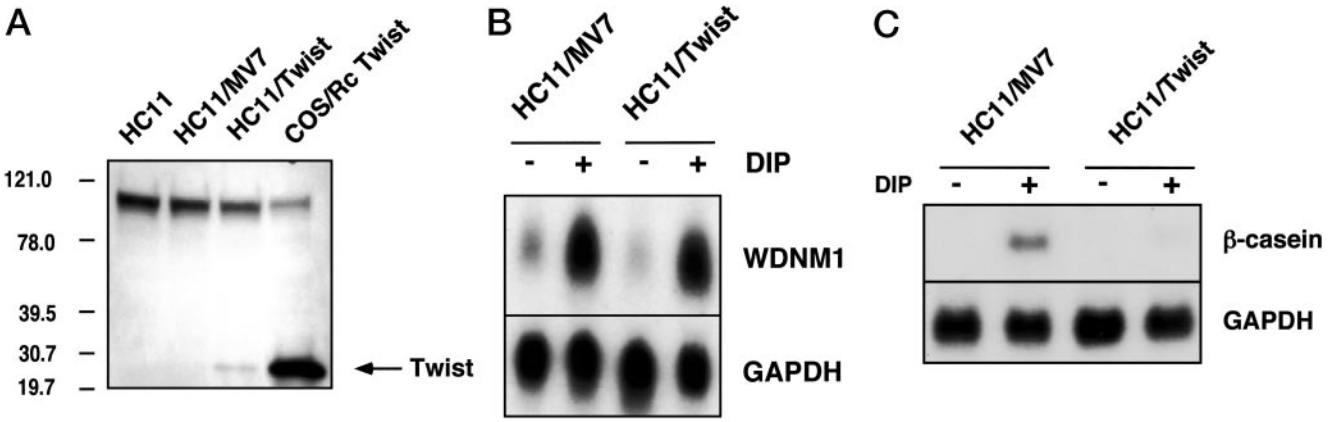

Fig. 6. Effect of *Twist* overexpression on lactogenic marker induction in HC11 cells. *A*, characterization of *Twist*-expressing HC11 cells. Cell lysates were prepared from HC11, HC11/MV7, and HC11/Twist cell lines and also from Cos cells transiently transfected with pRcTwist (COS/Rc Twist) to act as a positive control. Fifty μg of HC11 cell line lysates and 20 μg of Cos cell lysate were subjected to SDS-PAGE and Western blotting with anti-Twist antibody. The positions of molecular mass markers (in kDa) are indicated. A Twist band of ~26 kDa was detected in HC11/Twist cells but not in HC11 or HC11/MV7 cells. *B*, WDNM1 induction in HC11-derived cell lines. HC11/MV7 and HC11/Twist cells were maintained at confluence for 2 days, then incubated for 3 days in medium with or without DIP (+/− DIP). RNA was prepared from the cells and 20 μg analyzed by Northern blotting. The blot was probed sequentially with a murine WDNM1 probe and a GAPDH probe. Induction of WDNM1 was not reduced by *Twist* overexpression. WDNM1 signals were quantitated using the program NIH Image and normalized to those obtained from GAPDH probing. Values obtained are expressed relative to untreated HC11/MV7 cells. HC11/MV7, 100%; HC11/MV7 + DIP, 376%; HC11/Twist, 49%; and HC11/Twist + DIP; 444%. *C*, β-casein induction in HC11-derived cell lines. RNA prepared from HC11/MV7 and HC11/Twist cells treated as described in *B* was analyzed by Northern blotting. The blot was probed sequentially with a murine β-casein probe and a GAPDH probe. *Twist* overexpression totally suppressed hormonal induction of β-casein transcript.

investigated the mechanism by which Wnt1 transcriptionally activates *Twist*. A *Twist* promoter reporter construct was generated containing 2.0 kb of 5′ untranslated sequence from the murine *Twist* gene (Fig. 2) linked to a luciferase reporter gene. This promoter fragment contains three potential TCF binding sites (Fig. 2*B*). In addition, we identified 34 potential Ets binding sites based on the presence of the core binding site 5′-GGA,A/T-3′, of which approximately one-third were conserved between mouse and human. Analysis of the sequence using MatInspector[9] revealed several AP1 sites and numerous other potential transcription factor binding sites. Luciferase assays demonstrated that this *Twist* promoter construct was responsive to β-catenin (Fig. 3). Additionally, Ets factors of the PEA3 subfamily activated the *Twist* promoter, and c-jun enhanced the responses to both β-catenin and PEA3 (Figs. 3 and 4). The synergy between β-catenin, PEA3, and c-jun is particularly striking because similar findings have recently been reported with respect to the *Matrilysin* promoter, an additional target of Wnt/β-catenin signaling (18, 43). We have also shown that PEA3 factors activate transcription of the *cyclooxygenase 2* gene (36), another gene that is responsive to Wnt signaling (37, 41). Taken together, these findings suggest that PEA3 factors may contribute to regulation of multiple target genes of the Wnt/β-catenin pathway. Consistent with this hypothesis, the promoters of several genes known to be β-catenin-responsive also contain consensus Ets binding sites. For example, the *Drosophila* gene *Even-skipped* is coordinately regulated via TCF and Ets binding sites (52), and the *cyclin D1* promoter can be activated cooperatively by PEA3, β-catenin, and c-jun[10]. Interestingly, the promoter of the *Drosophila Twist* gene also contains both Ets and TCF binding sites,[11] suggesting that there may be a role for β-catenin and Ets factors in regulating its expression. This would represent a novel mechanism of *Drosophila Twist* activation, in addition to the previously established *Dorsal*-mediated regulation (25–27).

Both Wnt1 and Twist Inhibit Mammary Cell Differentiation. Twist and Wnt1 have previously been shown to antagonize the differentiation of certain cell lineages (44, 45, 53, 54). Therefore, we were interested in determining whether either Twist or Wnt1 might repress lactogenic differentiation in mammary cells. To address this possibility, responsiveness to lactogenic hormones was examined in HC11 cells, using the milk protein transcripts β-casein and WDNM1 as markers of lactogenic differentiation. Overexpression of *Wnt1*, but not of *Twist*, diminished *WDNM1* up-regulation in response to lactogenic stimuli (Figs. 6 and 7). In contrast, β-casein induction was effectively abolished by either *Wnt1* or *Twist* (Figs. 6 and 7). Interestingly, the expression profile of *Twist* during postnatal murine mammary development is consistent with a role for Twist in suppressing β-casein expression *in vivo*. Mammary *Twist* expression is constant during early gestation but diminishes during mid pregnancy, exhibiting a reciprocal expression pattern with the milk protein β-casein.[8] Taken together, these observations raise the possibility that Twist may function *in vivo* to negatively regulate β-casein expression.

The simplest interpretation of the ability of Twist to antagonize β-casein induction is that Twist is acting as a transcriptional repressor. Several mechanisms have been proposed to account for transcriptional repression mediated by mammalian Twist, mostly through analysis of its role in myogenic differentiation (55–57). Additionally, the ability of Twist to bind to and inhibit histone acetyl transferases may be important for Twist-mediated transcriptional repression (58).

Suppression of Differentiation and Tumorigenesis. The ability of Wnt1 to inhibit differentiation, as evidenced by diminished induction of lactogenic markers, may be a contributory factor to Wnt-mediated tumorigenesis. Suppression of terminal differentiation is thought to favor tumorigenesis, a paradigm particularly well illustrated by the HLH-containing Id proteins. Originally identified as inhibitors of myogenic differentiation, Id proteins are now known to function as dominant negative regulators of cell lineage commitment and differentiation in multiple cell types (59, 60). However, Id proteins are also associated with tumorigenesis (61–63), and indeed, expression of an *Id1* transgene can induce formation of intestinal adenomas (64). By analogy, the ability of Wnt1 to suppress lactogenic differentiation may be important for its function as a mammary oncogene. This effect of Wnt1 may be mediated in part via *Twist* up-regulation because overexpressed *Twist* completely abolishes β-casein induction. However the failure of Twist to repress *WDNM1*

[9] Internet address: transfac.gbf.de/.
[10] C. Messier and J. A. Hassell, personal communication.
[11] V. Cox and M. K. Baylies, personal communication.

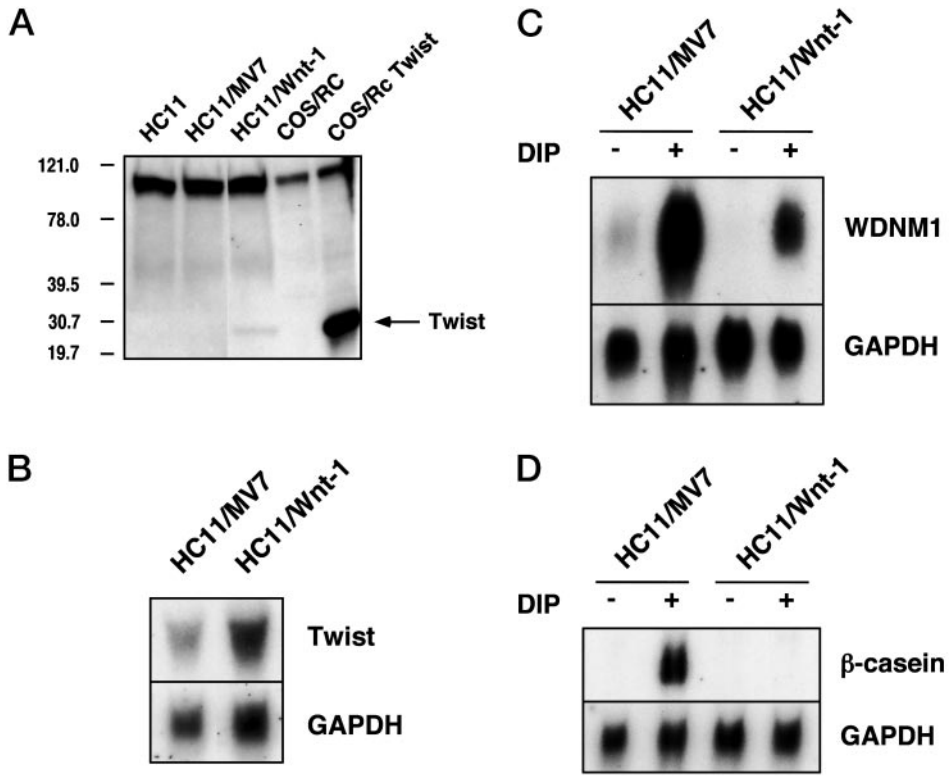

Fig. 7. Ectopic *Wnt1* expression in HC11 cells up-regulates *Twist* and inhibits lactogenic differentiation. *A*, Twist protein is present in HC11 cells expressing *Wnt1*. Cell lysates were prepared from HC11, HC11/MV7, and HC11/Wnt-1 cell lines and also from Cos cells transfected with pRcTwist (COS/Rc Twist) or empty vector pRcCMV (COS/RC). Fifty μg of lysate from HC11 cell lines and 20 μg of Cos cell lysates were subjected to SDS-PAGE and Western blotting with anti-Twist antibody. The positions of molecular mass markers (in kDa) are indicated. *B*, *Twist* RNA is increased in HC11/Wnt-1 cells. Total RNA was prepared from HC11/MV7 and HC11/Wnt-1 cells and 20 μg of RNA analyzed by Northern blotting. The blot was probed sequentially with a murine *Twist* probe and a GAPDH probe. *Twist* signals were quantitated using the program NIH Image and normalized to those obtained from GAPDH probing. Values obtained are expressed relative to HC11/MV7 cells. HC11/MV7, 100%; HC11/Wnt-1, 181%. *C*, WDNM1 induction in HC11-derived cell lines. HC11/MV7 and HC11/Wnt-1 cells were maintained at confluence for 2 days, then incubated for 3 days in medium in the presence or absence of DIP (+/− DIP). RNA was prepared from the cells and 20 μg analyzed by Northern blotting with a murine WDNM1 probe and a GAPDH probe. *Wnt1* overexpression substantially reduced hormonal induction of *WDNM1*. WDNM1 signals were quantitated using the program NIH Image and normalized to those obtained from GAPDH probing. Values obtained are expressed relative to untreated HC11/MV7 cells. HC11/MV7, 100%; HC11/MV7 + DIP, 557%; HC11/Wnt-1, 16%; and HC11/Wnt-1 + DIP, 259%. *D*, β-casein induction in HC11-derived cell lines. RNA prepared from HC11/MV7 and HC11/Wnt-1 cells treated as described in *C* was analyzed by Northern blotting. The blot was probed sequentially with a murine β-casein probe and a GAPDH probe. *Wnt1* overexpression totally suppressed hormonal induction of β-casein transcript.

induction clearly suggests a requirement for other factors to mediate this effect. In addition to altered differentiation, enhanced cellular proliferation and diminished apoptosis are frequently prerequisites for carcinogenesis. Of the Wnt/β-catenin transcriptional targets thus far identified, several are likely to contribute to these processes (15–17, 20–22). Thus, suppression of terminal differentiation by Wnt1 is only one of a panoply of responses that may contribute to Wnt-mediated tumorigenesis.

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REFERENCES

1. Nusse, R., and Varmus, H. E. Many tumors induced by the mouse mammary tumor virus contain a provirus integrated in the same region of the host genome. Cell, *31:* 99–109, 1982.
2. Peters, G., Brookes, S., Smith, R., and Dickson, C. Tumorigenesis by mouse mammary tumor virus: evidence for a common region for provirus integration in mammary tumors. Cell, *33:* 369–377, 1983.
3. Nusse, R., van Ooyen, A., Cox, D., Fung, Y. K., and Varmus, H. Mode of proviral activation of a putative mammary oncogene (int-1) on mouse chromosome 15. Nature (Lond.), *307:* 131–136, 1984.
4. Tsukamoto, A. S., Grosschedl, R., Guzman, R. C., Parslow, T., and Varmus, H. E. Expression of the *int-1* gene in transgenic mice is associated with mammary gland hyperplasia and adenocarcinomas in male and female mice. Cell, *55:* 619–625, 1988.
5. Brown, A. M. C., Wildin, R. S., Prendergast, T. J., and Varmus, H. E. A retrovirus vector expressing the putative mammary oncogene int-1 causes partial transformation of a mammary epithelial cell line. Cell, *46:* 1001–1009, 1986.
6. Rijsewijk, F., van Deemter, L., Wagenaar, E., Sonnenberg, A., and Nusse, R. Transfection of the int-1 mammary oncogene in cuboidal RAC mammary cell line results in morphological transformation and tumorigenicity. EMBO J., *6:* 127–131, 1987.
7. Brown, A. M. C. Wnt signaling in breast cancer: have we come full circle? Breast Cancer Res., *3:* 351–355, 2001.
8. Smalley, M. J., and Dale, T. C. Wnt signaling and mammary tumorigenesis. J. Mamm. Gland Biol. Neoplasia, *6:* 37–52, 2001.
9. Wong, S. C. C., Lo, S. F. E., Lee, K. C., Yam, J. W. P., Chan, J. K. C., and Hsiao, W. L. W. Expression of frizzled-related protein and Wnt-signalling molecules in invasive human breast tumours. J. Pathol., *196:* 145–153, 2002.
10. Polakis, P. Wnt signaling and cancer. Genes Dev., *14:* 1837–1851, 2000.
11. Papkoff, J., Rubinfeld, B., Schryver, B., and Polakis, P. Wnt-1 regulates free pools of catenins and stabilizes APC-catenin complexes. Mol. Cell. Biol., *16:* 2128–2134, 1996.
12. Shimizu, H., Julius, M. A., Giarre, M., Zheng, Z., Brown, A. M. C., and Kitajewski, J. Transformation by Wnt family proteins correlates with regulation of β-catenin. Cell Growth Differ., *8:* 1349–1358, 1997.
13. Bullions, L. C., and Levine, A. J. The role of β-catenin in cell adhesion, signal transduction, and cancer. Curr. Opin. Oncol., *10:* 81–87, 1998.
14. Cadigan, K. M., and Nusse, R. Wnt signaling: a common theme in animal development. Genes Dev., *11:* 3286–3305, 1997.
15. Tetsu, O., and McCormick, F. β-Catenin regulates expression of cyclin D1 in colon carcinoma cells. Nature (Lond.), *398:* 422–426, 1999.
16. Shtutman, M., Zhurinsky, J., Simcha, I., Albanese, C., D'Amico, M., Pestell, R., and Ben-Ze'ev, A. The *cyclin D1* gene is a target of the β-catenin/LEF-1 pathway. Proc. Natl. Acad. Sci. USA, *96:* 5522–5527, 1999.

17. He, T. C., Sparks, A. B., Rago, C., Hermeking, H., Zawel, L., da Costa, L. T., Morin, P. J., Vogelstein, B., and Kinzler, K. W. Identification of c-MYC as a target of the APC pathway. Science (Wash. DC), *281:* 1509–1512, 1998.
18. Crawford, H. C., Fingleton, B. M., Rudolph-Owen, L. A., Goss, K. J., Rubinfeld, B., Polakis, P., and Matrisian, L. M. The metalloproteinase matrilysin is a target of β-catenin transactivation in intestinal tumors. Oncogene, *18:* 2883–2891, 1999.
19. Brabletz, T., Jung, A., Dag, S., Hlubek, F., and Kirchner, T. β-Catenin regulates the expression of the matrix metalloproteinase-7 in human colorectal cancer. Am. J. Pathol., *155:* 1033–1038, 1999.
20. He, T. C., Chan, T. A., Vogelstein, B., and Kinzler, K. W. PPARδ is an APC-regulated target of nonsteroidal anti-inflammatory drugs. Cell, *99:* 335–345, 1999.
21. Xu, L., Corcoran, R. B., Welsh, J. W., Pennica, D., and Levine, A. J. WISP-1 is a Wnt-1- and β-catenin-responsive oncogene. Genes Dev., *14:* 585–595, 2000.
22. Mann, B., Gelos, M., Siedow, A., Hanski, M. L., Gratchev, A., Ilyas, M., Bodmer, W. F., Moyer, M. P., Riecken, E. O., Buhr, H. J., and Hanski, C. Target genes of β-catenin-T cell-factor/lymphoid-enhancer-factor signaling in human colorectal carcinomas. Proc. Natl. Acad. Sci. USA, *96:* 1603–1608, 1999.
23. Thisse, B., el Messal, M., and Perrin-Schmitt, F. The twist gene: isolation of a Drosophila zygotic gene necessary for the establishment of dorsoventral pattern. Nucleic Acids Res., *15:* 3439–3453, 1987.
24. Thisse, B., Stoetzel, C., Gorostiza-Thisse, C., and Perrin-Schmitt, F. Sequence of the twist gene and nuclear localization of its protein in endomesodermal cells of early Drosophila embryos. EMBO J., *7:* 2175–2183, 1988.
25. Jiang, J., Kosman, D., Ip, Y. T., and Levine, M. The dorsal morphogen gradient regulates the mesoderm determinant twist in early Drosophila embryos. Genes Dev., *5:* 1881–1891, 1991.
26. Pan, D. J., Huang, J. D., and Courey, A. J. Functional analysis of the Drosophila twist promoter reveals a dorsal-binding ventral activator region. Genes Dev., *5:* 1892–1901, 1991.
27. Thisse, C., Perrin-Schmitt, F., Stoetzel, C., and Thisse, B. Sequence-specific transactivation of the Drosophila twist gene by the dorsal gene product. Cell, *65:* 1191–1201, 1991.
28. Bate, M., and Rushton, E. Myogenesis and muscle patterning in Drosophila. C. R. Acad. Sci. III, *316:* 1047–1061, 1993.
29. Chen, Z. F., and Behringer, R. R. twist is required in head mesenchyme for cranial neural tube morphogenesis. Genes Dev., *9:* 686–699, 1995.
30. Wolf, C., Thisse, C., Stoetzel, C., Thisse, B., Gerlinger, P., and Perrin-Schmitt, F. The M-twist gene of Mus is expressed in subsets of mesodermal cells and is closely related to the Xenopus X-twi and the Drosophila twist genes. Dev. Biol., *143:* 363–373, 1991.
31. Fuchtbauer, E. M. Expression of M-twist during postimplantation development of the mouse. Dev. Dyn., *204:* 316–322, 1995.
32. Gitelman, I. Twist protein in mouse embryogenesis. Dev. Biol., *189:* 205–214, 1997.
33. El Ghouzzi, V., Le Merrer, M., Perrin-Schmitt, F., Lajeunie, E., Benit, P., Renier, D., Bourgeois, P., Bolcato-Bellemin, A. L., Munnich, A., and Bonaventure, J. Mutations of the *TWIST* gene in the Saethre-Chotzen syndrome. Nat. Genet., *15:* 42–46, 1997.
34. Howard, T. D., Paznekas, W. A., Green, E. D., Chiang, L. C., Ma, N., Ortiz de Luna, R. I., Garcia Delgado, C., Gonzalez-Ramos, M., Kline, A. D., and Jabs, E. W. Mutations in TWIST, a basic helix-loop-helix transcription factor, in Saethre-Chotzen syndrome. Nat. Genet., *15:* 36–41, 1997.
35. Maestro, R., Dei Tos, A. P., Hamamori, Y., Krasnokutsky, S., Sartorelli, V., Kedes, L., Doglioni, C., Beach, D. H., and Hannon, G. J. Twist is a potential oncogene that inhibits apoptosis. Genes Dev., *13:* 2207–2217, 1999.
36. Howe, L. R., Crawford, H. C., Subbaramaiah, K., Hassell, J. A., Dannenberg, A. J., and Brown, A. M. C. PEA3 is up-regulated in response to Wnt1 and activates the expression of cyclooxygenase-2. J. Biol. Chem., *276:* 20108–20115, 2001.
37. Howe, L. R., Subbaramaiah, K., Chung, W. J., Dannenberg, A. J., and Brown, A. M. C. Transcriptional activation of cyclooxygenase-2 in Wnt-1-transformed mouse mammary epithelial cells. Cancer Res., *59:* 1572–1577, 1999.
38. Vaidya, A. B., Lasfargues, E. Y., Sheffield, J. B., and Coutinho, W. G. Murine mammary tumor virus (MuMTV) infection of an epithelial cell line established from C57BL/6 mouse mammary glands. Virology, *90:* 12–22, 1978.
39. Ball, R. K., Friis, R. R., Schoenenberger, C. A., Doppler, W., and Groner, B. Prolactin regulation of β-casein gene expression and of a cytosolic 120-kd protein in a cloned mouse mammary epithelial cell line. EMBO J., *7:* 2089–2095, 1988.
40. Bradley, R. S., and Brown, A. M. C. A soluble form of Wnt-1 protein with mitogenic activity on mammary epithelial cells. Mol. Cell. Biol., *15:* 4616–4622, 1995.
41. Haertel-Wiesmann, M., Liang, Y., Fantl, W. J., and Williams, L. T. Regulation of cyclooxygenase-2 and periostin by Wnt-3 in mouse mammary epithelial cells. J. Biol. Chem., *275:* 32046–32051, 2000.
42. Wang, S. M., Coljee, V. W., Pignolo, R. J., Rotenberg, M. O., Cristofalo, V. J., and Sierra, F. Cloning of the human twist gene: its expression is retained in adult mesodermally-derived tissues. Gene (Amst.), *187:* 83–92, 1997.
43. Crawford, H. C., Fingleton, B., Gustavson, M. D., Kurpios, N., Wagenaar, R. A., Hassell, J. A., and Matrisian, L. M. The PEA3 subfamily of Ets transcription factors synergizes with β-catenin/LEF-1 to activate matrilysin transcription in intestinal tumors. Mol. Cell. Biol., *21:* 1370–1383, 2001.
44. Hebrok, M., Wertz, K., and Fuchtbauer, E. M. M-twist is an inhibitor of muscle differentiation. Dev. Biol., *165:* 537–544, 1994.
45. Lee, M. S., Lowe, G. N., Strong, D. D., Wergedal, J. E., and Glackin, C. A. TWIST, a basic helix-loop-helix transcription factor, can regulate the human osteogenic lineage. J. Cell. Biochem., *75:* 566–577, 1999.
46. Robinson, G. W., McKnight, R. A., Smith, G. H., and Hennighausen, L. Mammary epithelial cells undergo secretory differentiation in cycling virgins but require pregnancy for the establishment of terminal differentiation. Development (Camb.), *121:* 2079–2090, 1995.
47. Lane, T. F., and Leder, P. Wnt-10b directs hypermorphic development and transformation in mammary glands of male and female mice. Oncogene, *15:* 2133–2144, 1997.
48. Weber-Hall, S. J., Phippard, D. J., Niemeyer, C. C., and Dale, T. C. Developmental and hormonal regulation of Wnt gene expression in the mouse mammary gland. Differentiation, *57:* 205–214, 1994.
49. Gavin, B. J., and McMahon, A. P. Differential regulation of the Wnt gene family during pregnancy and lactation suggests a role in postnatal development of the mammary gland. Mol. Cell. Biol., *12:* 2418–2423, 1992.
50. Buhler, T. A., Dale, T. C., Kieback, C., Humphreys, R. C., and Rosen, J. M. Localization and quantification of Wnt-2 gene expression in mouse mammary development. Dev. Biol., *155:* 87–96, 1993.
51. Gazit, A., Yaniv, A., Bafico, A., Pramila, T., Igarashi, M., Kitajewski, J., and Aaronson, S. A. Human frizzled 1 interacts with transforming Wnts to transduce a TCF dependent transcriptional response. Oncogene, *18:* 5959–5966, 1999.
52. Halfon, M. S., Carmena, A., Gisselbrecht, S., Sackerson, C. M., Jimenez, F., Baylies, M. K., and Michelson, A. M. Ras pathway specificity is determined by the integration of multiple signal-activated and tissue-restricted transcription factors. Cell, *103:* 63–74, 2000.
53. Rudnicki, J. A., and Brown, A. M. C. Inhibition of chondrogenesis by Wnt gene expression *in vivo* and *in vitro*. Dev. Biol., *185:* 104–118, 1997.
54. Ross, S. E., Hemati, N., Longo, K. A., Bennett, C. N., Lucas, P. C., Erickson, R. L., and MacDougald, O. A. Inhibition of adipogenesis by Wnt signaling. Science (Wash. DC), *289:* 950–953, 2000.
55. Hamamori, Y., Wu, H. Y., Sartorelli, V., and Kedes, L. The basic domain of myogenic basic helix-loop-helix (bHLH) proteins is the novel target for direct inhibition by another bHLH protein, Twist. Mol. Cell. Biol., *17:* 6563–6573, 1997.
56. Hebrok, M., Fuchtbauer, A., and Fuchtbauer, E. M. Repression of muscle-specific gene activation by the murine Twist protein. Exp. Cell Res., *232:* 295–303, 1997.
57. Spicer, D. B., Rhee, J., Cheung, W. L., and Lassar, A. B. Inhibition of myogenic bHLH and MEF2 transcription factors by the bHLH protein Twist. Science (Wash. DC), *272:* 1476–1480, 1996.
58. Hamamori, Y., Sartorelli, V., Ogryzko, V., Puri, P. L., Wu, H. Y., Wang, J. Y., Nakatani, Y., and Kedes, L. Regulation of histone acetyltransferases p300 and PCAF by the bHLH protein twist and adenoviral oncoprotein E1A. Cell, *96:* 405–413, 1999.
59. Norton, J. D. ID helix-loop-helix proteins in cell growth, differentiation and tumorigenesis. J. Cell Sci., *113:* 3897–3905, 2000.
60. Benezra, R., Davis, R. L., Lockshon, D., Turner, D. L., and Weintraub, H. The protein Id: a negative regulator of helix-loop-helix DNA binding proteins. Cell, *61:* 49–59, 1990.
61. Kleeff, J., Ishiwata, T., Friess, H., Buchler, M. W., Israel, M. A., and Korc, M. The helix-loop-helix protein Id2 is overexpressed in human pancreatic cancer. Cancer Res., *58:* 3769–3772, 1998.
62. Lin, C. Q., Singh, J., Murata, K., Itahana, Y., Parrinello, S., Liang, S. H., Gillett, C. E., Campisi, J., and Desprez, P. Y. A role for Id-1 in the aggressive phenotype and steroid hormone response of human breast cancer cells. Cancer Res., *60:* 1332–1340, 2000.
63. Kebebew, E., Treseler, P. A., Duh, Q. Y., and Clark, O. H. The helix-loop-helix transcription factor, Id-1, is overexpressed in medullary thyroid cancer. Surgery (St. Louis), *128:* 952–957, 2000.
64. Wice, B. M., and Gordon, J. I. Forced expression of Id-1 in the adult mouse small intestinal epithelium is associated with development of adenomas. J. Biol. Chem., *273:* 25310–25319, 1998.
65. Krebs, I., Weis, I., Hudler, M., Rommens, J. M., Roth, H., Scherer, S. W., Tsui, L. C., Fuchtbauer, E. M., Grzeschik, K. H., Tsuji, K., and Kunz, J. Translocation breakpoint maps 5 kb 3′ from TWIST in a patient affected with Saethre-Chotzen syndrome. Hum. Mol. Genet., *6:* 1079–1086, 1997.

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