Wrch-1, a novel member of the Rho gene family that is regulated by Wnt-1

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We report the isolation and cloning of the *Wrch-1* (Wnt-1 responsive Cdc42 homolog) cDNA. *Wrch-1* is a novel gene whose mRNA level increases in response to Wnt-1 signaling in Wnt-1 transformed cells, Wnt-1 transgene induced mouse mammary tumors, and Wnt-1 retrovirus infected cells. *Wrch-1* encodes a homolog of the Rho family of GTPases. It shares 57% amino acid sequence identity with Cdc42, but possesses a unique N-terminal domain that contains several putative PXXP SH3-binding motifs. Like Cdc42, Wrch-1 can activate PAK-1 and JNK-1, and induce filopodium formation and stress fiber dissolution. Active Wrch-1 stimulates quiescent cells to reenter the cell cycle. Moreover, overexpression of Wrch-1 phenocopies Wnt-1 in morphological transformation of mouse mammary epithelial cells. Taken together, Wrch-1 could mediate the effects of Wnt-1 signaling in the regulation of cell morphology, cytoskeletal organization, and cell proliferation.

The Wnt/Wingless signaling pathway is fundamentally important for the development of organisms ranging from nematodes to mammals, where it functions to regulate cell morphology and polarity, modulate cell–cell adhesion and cell motility, specify cell fate, and control cell proliferation (Cadigan and Nusse 1997). Genetic studies in *Drosophila* in combination with biochemical experiments in *Xenopus* and mammalian cells have established a molecular framework for this pathway, which involves interactions between extracellular molecules and membrane receptors, an intracellular signal transduction, and the regulation of gene expression (Dale 1998; Dierick and Bejsovec 1999). In mammals, the Wnt family proteins initiate signaling by binding to their cognate receptors, Frizzled (Fz), on the plasma membrane. The agonist-bound Fz can activate a cytoplasmic protein, disheveled (Dsh), which in turn inhibits the kinase activity of glycogen synthase kinase-3β (GSK-3β). In the absence of Wnt signaling, GSK-3β constitutively phosphorylates the oncoprotein β-catenin (Polakis 1999), which induces a ubiquitin-dependent degradation of β-catenin mediated by a complex of proteins consisting of the tumor suppressor protein, adenomatous polyposis coli (APC), axin (Zeng et al. 1997), and a member of the SCF ubiquitin ligase complex, β-TrCP (Latres et al. 1999). Thus, Wnt signaling results in the stabilization and accumulation of β-catenin in the cytoplasm. The elevated levels of β-catenin promote its oligomerization with a member of the TCF/LEF transcription factor family. The complex of β-catenin and TCF/LEF translocates into the nucleus, where it serves as a functional transcription factor to activate the transcription of downstream genes. In addition to this canonical model of Wnt signaling, a new view has recently emerged, in which a Wnt signal can induce gene expression in a β-catenin independent and protein kinase C (PKC) sensitive manner (Miller et al. 1999; Kuhl et al. 2000; Ziemer et al. 2001). Conceivably, Wnt signaling could also modulate gene expression indirectly by controlling the expression of other transcription factors.

The Wnt signaling pathway is not only developmentally important but also implicated in tumorigenesis (Peifer and Polakis 2000; Polakis 2000). *Wnt-1* was originally identified as an oncogene, whose activation upon the insertion of mouse mammary tumor virus (MMTV) proviral DNA resulted in the formation of mouse mammary adenocarcinoma (Nusse et al. 1984). MMTV–*Wnt-1* transgenic mice develop tumors in both mammary and salivary glands (Tsukamoto 1988). Overexpression of some Wnt-1-related proteins, such as Wnt-2 and Wnt-5A, has been observed in human colon and breast cancers (Lejeune et al. 1995; Vider et al. 1996). In addition, mutations in the *APC* gene that eliminate its ability to promote β-catenin degradation occur in up to 80% of human colon carcinomas (Polakis 1999). Mutations of β-catenin that lead to the stabilization of this oncoprotein are detected in colon cancers, melanomas, hepatocellular carcinomas, and pilomatricomas (Morin

et al. 1997; Rubinfeld et al. 1997; de La Coste et al. 1998; Chan et al. 1999).

The biological effects of Wnt signaling in both development and tumorigenesis are largely mediated by its direct or indirect transcriptional target genes. Recently, an array of genes have been identified as Wnt-1 inducible genes, which include *cyclin D1* (Tetsu and McCormick 1999), *c-myc* (He et al. 1998a), *Wnt-1 Induced Secreted Protein 1* (*WISP-1*) (Pennica et al. 1998), and *c-Jun* (Mann et al. 1999). However, even those newly described genes cannot account for the diverse functions of Wnt signaling. For instance, the molecular mechanisms of Wnt signaling in the regulation of cell morphology, cell–cell adhesion, cell–extracellular matrix interactions, cell migration, and cell proliferation remain elusive.

The Rho family GTPases, including Rho, Rac, and Cdc42, are key regulators of the actin cytoskeleton and play a central role in a wide range of physiological processes, which include cell morphology, cell adhesion, cytokinesis, cell motility, and cell growth (Van Aelst and D'Souza-Schorey 1997; Hall 1998). Overexpression or aberrant activation of these proteins is involved in malignant transformation, tumor formation, and metastasis (Michiels et al. 1995; Qiu et al. 1995; Keely et al. 1997; Clark et al. 2000). While genetic studies in *Drosophila* suggest that RhoA could act downstream of Fz and Dsh to mediate the effect of Wnt signaling in correct establishment of planar polarity in epidermis (Strutt et al. 1997), a biological link of the Wnt pathway and the Rho family proteins in vertebrates has not been observed.

In an attempt to identify additional genes that are directly or indirectly regulated by Wnt-1 signaling and that are relevant to transformation and tumor formation, a cDNA subtraction analysis was carried out between a Wnt-1 expressing mouse mammary epithelial cell line (C57MG/Wnt-1) and the parental cell line (C57MG). From this analysis, we identified a novel gene, *Wrch-1* (Wnt-1 responsive Cdc42 homolog), that is differentially expressed in Wnt-1 expressing cells and MMTV–*Wnt-1* transgenic mouse mammary tumors, and that is rapidly induced by Wnt-1 signaling. Interestingly, *Wrch-1* encodes a structural and functional homolog of the Rho family of GTPases. While most members of the Rho family are constitutively expressed (Ridley 1999), the expression of *Wrch-1* is rapidly induced upon the activation of the Wnt-1 pathway. In addition, an SH3 binding domain at the amino-terminus of Wrch-1 suggests that this protein may be regulated through protein–protein interactions. A number of biological activities of Wrch-1 are demonstrated in this report. Based on these results, Wrch-1 has the potential to mediate the effect of Wnt-1 on cell morphology and to contribute to the ability of Wnt-1 to transform cells and induce tumor formation.

Results

Isolation of Wrch-1 as a putative downstream target gene of Wnt-1

To identify putative downstream target genes of Wnt-1 signaling, a PCR-select cDNA subtraction analysis was

carried out between parental C57MG cells, the mouse mammary epithelial cells that do not express Wnt-1, and C57MG/Wnt-1 cells that stably express Wnt-1 (Pennica et al. 1998). C57MG cells were chosen for this experiment because aberrant activation of Wnt-1 expression in this type of cell in the mouse mammary gland results in hyperplasia, and Wnt-1 can morphologically transform these cells in vitro (Nusse et al. 1984; Jue et al. 1992; Mason et al. 1992). Therefore, C57MG cells provide an in vitro model system for studying Wnt-1 signaling and for identifying genes that are downstream of Wnt-1 and that may be involved in Wnt-1-induced cell transformation. C57MG/Wnt-1 cells were generated by infecting C57MG cells with a Wnt-1-expressing retrovirus (Pennica et al. 1998). Cells that stably express Wnt-1 for three weeks after infection were used in the cDNA subtraction analysis, as it could then allow for the identification of both direct and indirect target genes of Wnt-1 signaling. From this analysis, the *Wrch-1* gene was isolated as one of the genes that was differentially expressed in C57MG/Wnt-1 cells versus the parental C57MG cells (Pennica et al. 1998; Ziemer et al. 2001).

To confirm that *Wrch-1* is regulated by Wnt-1 signaling, C57MG cells were infected with either an empty retroviral vector or a Wnt-1-expressing retrovirus. Six days after infection, RNA was isolated and the expression of *Wrch-1* was determined by Northern blot analysis. As shown in Figure 1A, *Wrch-1* mRNA is up-regulated more than fourfold in C57MG/Wnt-1 cells versus C57MG/vector cells.

Since β-catenin is downstream of Wnt-1 in the Wnt signaling pathway and can mediate transcriptional activation of Wnt-1 induced genes (Polakis 1999), the effect of β-catenin on *Wrch-1* expression was assessed. C57MG cell lines overexpressing two stabilized mutants of β-catenin, 4145 and 4145TV, were generated by transfection (Xu et al. 2000). The mutant 4145 has both Thr 41 and Ser 45 at the N-terminal GSK-3β phosphorylation sites changed to alanines. The other mutant, 4145TV, has the above mutations as well as mutations that change Thr 120 and Val 122 to alanines in the α-catenin binding region, which abrogates its ability to bind α-catenin. Northern blot analysis showed that the *Wrch-1* transcript was not increased in C57MG cells that overexpressed 4145 or 4145TV (Fig. 1A). However, both 4145 and 4145TV dramatically induced the expression of *WISP1*, another Wnt-1 responsive gene, in a similar experiment (Xu et al. 2000). These results suggest that Wnt-1 may up-regulate *Wrch-1* expression in a β-catenin independent manner.

To determine the temporal regulation of *Wrch-1* by Wnt-1 signaling, C57MG cells were cocultured with either parental QT6 cells (quail fibrosarcoma cells) that do not express Wnt-1, or QT6 cells that stably express Wnt-1 (QT6/Wnt-1) (Parkin et al. 1993), for various times. *Wrch-1* mRNA levels were then determined by a Northern blot. This coculture experiment establishes a paracrine signaling system within the tissue culture plate, in which the Wnt-1 molecules produced and secreted by QT6/Wnt-1 cells elicit a response and induce
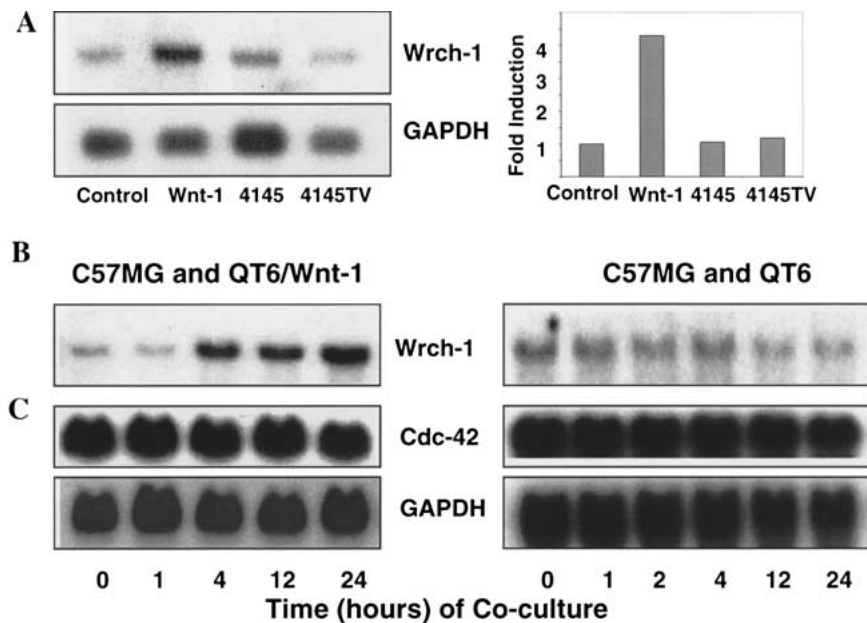
Figure 1. Wnt-1 signaling induces Wrch-1, but not Cdc-42. (*A*) Northern blot analysis of Wrch-1 expression in cells expressing Wnt-1, 4145, or 4145TV was performed using a 393 bp mouse Wrch-1-specific probe (containing codons for amino acids 81–211). Blots were rehybridized with a mouse GAPDH probe for loading control. Fold induction of Wrch-1 over the vector control was represented after normalizing to GAPDH levels. (*B*) C57MG cells were cocultured with either the parental QT6 cells or the QT6/Wnt-1 cells. Total RNA was extracted from the coculture at indicated time points, and Northern blotting was performed using the mouse Wrch-1 probe described above. Levels of GAPDH as a loading control are shown in *C*. (*C*) Cdc42 mRNA levels in the coculture were determined by reprobing the blots described in *B* with a Cdc42 cDNA probe (containing codons for amino acids 1–103).

gene expression in mouse C57MG cells (Parkin et al. 1993; Ziemer et al. 2001). When C57MG cells were co-cultured with QT6/Wnt-1 cells, the transcripts of *Wrch-1* were induced ~sixfold after 4 h of coculture, and sustained at high levels throughout a 24-h coculture experiment (Fig. 1B). However, no induction of *Wrch-1* expression was detected when C57MG cells were cocultured with parental QT6 cells (Fig. 1B). These data show that Wnt-1 can induce *Wrch-1* mRNA levels in cell coculture. RNA isolated from cocultures contains RNA from C57MG cells as well as RNA from QT6 or QT6/ Wnt-1 cells. The mouse *Wrch-1* cDNA probe used in these experiments does not cross hybridize with the quail *Wrch-1* mRNA (data not shown).

In order to determine the biological relevance of *Wrch-1* as a downstream target gene of Wnt-1 signaling, the *Wrch-1* transcript levels were determined in MMTV–*Wnt-1* transgenic mouse mammary tumors (Tsukamoto 1988) as well as in normal, wild-type mouse mammary glands by Northern blot analysis. Although there was a difference in the amount of sample loaded between normal mouse mammary glands and mouse mammary tumors, *Wnt-1* or *Wrch-1* transcripts were not detectable in normal mouse mammary glands (Fig. 2). However, both *Wnt-1* and *Wrch-1* mRNAs were highly expressed in two independent Wnt-1 transgenic mouse mammary tumors. These results establish a strong correlation between overexpression of *Wnt-1* and elevated levels of the *Wrch-1* message in vivo.

Identification of Wrch-1 as a homolog of the Rho family of GTPases

Based on the partial sequence of mouse *Wrch-1* cDNA isolated from PCR-select cDNA subtraction, the full-length mouse and human *Wrch-1* cDNAs were cloned. The cDNAs of human and mouse *Wrch-1* encode proteins of 258 and 261 amino acid residues, respectively,
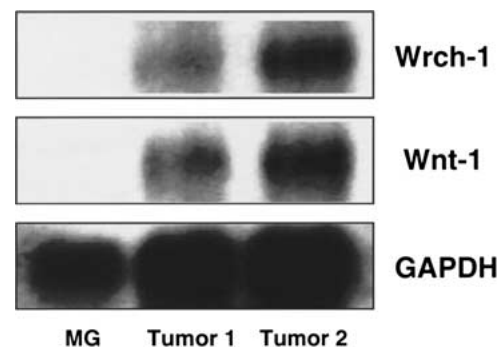

Figure 2. Expression of Wrch-1 and Wnt-1 in Wnt-1 transgenic mouse mammary tumors, but not in normal, wild-type mouse mammary glands. Total RNA was extracted from wild-type mouse mammary glands (MG) and two independent MMTV-Wnt-1 transgenic mouse mammary tumors. Levels of the Wrch-1 transcript were assessed by Northern blot analysis. The blots were reprobed with the full-length mouse Wnt-1 cDNA (2.2 kb) to determine levels of the Wnt-1 mRNA.

which are 92% identical (Fig. 3). Analysis of the deduced amino acid sequence of Wrch-1 identifies it as a homolog of the Rho family of GTPases. The human Wrch-1 protein shares 57% identity and 70% similarity with Cdc42, and 30%–55% similarity with other members of the Rho family. Both human and mouse Wrch-1 contain GTP and GDP binding domains, the effector domain, and a CAAX lipid modification signal at their C termini, all of which are conserved among most members of the Rho family (Fig. 3) (Ridley 1999). In addition, both human and mouse

Wrch-1 possess a unique N-terminal domain that contains several putative PXXP SH3-binding motifs (Fig. 3).

Since Wrch-1 is highly homologous to Cdc42, we determined whether the expression of Cdc42 was also regulated by Wnt-1 signaling. The blots used for assessing the effect of Wnt-1 on *Wrch-1* expression were reprobed with a Cdc42 cDNA probe. As shown in Figure 1C, levels of the Cdc42 transcript were constitutively high and were not regulated by Wnt-1 signaling in either the coculture experiment (Fig. 1C) or the retroviral infection (data not
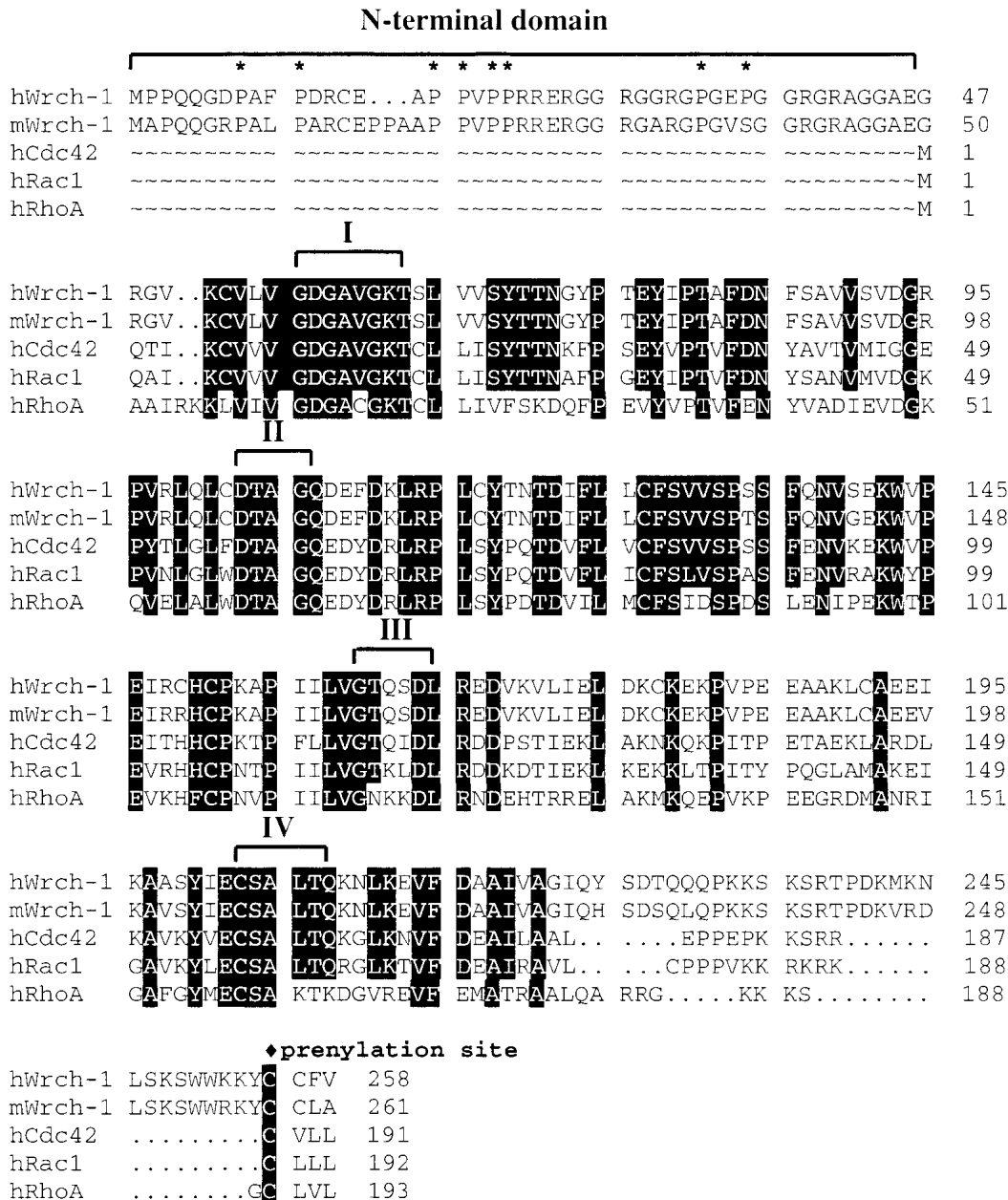
N-terminal domain

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                 *      *          *  *  **              *     *
hWrch-1  MPPQQGDPAF PDRCE...AP PVPPRRERGG RGGRGPGEPG GRGRAGGAEG   47
mWrch-1  MAPQQGRPAL PARCEPPAAP PVPPRRERGG RGARGPGVSG GRGRAGGAEG   50
hCdc42   ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~M    1
hRac1    ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~M    1
hRhoA    ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~M    1

                        I
hWrch-1  RGV..KCVLV GDGAVGKTSL VVSYTTNGYP TEYIPTAFDN FSAVVSVDGR   95
mWrch-1  RGV..KCVLV GDGAVGKTSL VVSYTTNGYP TEYIPTAFDN FSAVVSVDGR   98
hCdc42   QTI..KCVVV GDGAVGKTCL LISYTTNKFP SEYVPTVFDN YAVTVMIGGE   49
hRac1    QAI..KCVVV GDGAVGKTCL LISYTTNAFP GEYIPTVFDN YSANVMVDGK   49
hRhoA    AAIRKKLVIV GDGACGKTCL LIVFSKDQFP EVYVPTVFEN YVADIEVDGK   51
                 II

hWrch-1  PVRLQLCDTA GQDEFDKLRP LCYTNTDIFL LCFSVVSPSS FQNVSBKWVP  145
mWrch-1  PVRLQLCDTA GQDEFDKLRP LCYTNTDIFL LCFSVVSPTS FQNVGBKWVP  148
hCdc42   PYTLGLFDTA GQEDYDRLRP LSYPQTDVFL VCFSVVSPSS FENVKEKWVP   99
hRac1    PVNLGLWDTA GQEDYDRLRP LSYPQTDVFL ICFSLVSPAS FENVRAKWYP   99
hRhoA    QVELALWDTA GQEDYDRLRP LSYPDTDVIL MCFSIDSPDS LENIPEKWTP  101
                         III

hWrch-1  EIRCHCPKAP IILVGTQSDL REDVKVLIEL DKCKEKPVPE EAAKLCAEEI  195
mWrch-1  EIRRHCPKAP IILVGTQSDL REDVKVLIEL DKCKEKPVPE EAAKLCAEEV  198
hCdc42   EITHHCPKTP FLLVGTQIDL RDDPSTIEKL AKNKQKPITP ETAEKLARDL  149
hRac1    EVRHHCPNTP IILVGTKLDL RDDKDTIEKL KEKKLTPITY PQGLAMAKEI  149
hRhoA    EVKHFCPNVP IILVGNKKDL RNDEHTRREL AKMKQEPVKP EEGRDMANRI  151
              IV

hWrch-1  KAASYIECSA LTQKNLKEVF DAAIVAGIQY SDTQQQPKKS KSRTPDKMKN  245
mWrch-1  KAVSYIECSA LTQKNLKEVF DAAIVAGIQH SDSQLQPKKS KSRTPDKVRD  248
hCdc42   KAVKYVECSA LTQKGLKNVF DEAILAAL.. ....EPPEPK KSRR......  187
hRac1    GAVKYLECSA LTQRGLKTVF DEAIRAVL.. ...CPPPVKK RKRK......  188
hRhoA    GAFGYMECSA KTKDGVREVF EMATRAALQA RRG.....KK KS........  188

                      ♦prenylation site
hWrch-1  LSKSWWKKYC CFV  258
mWrch-1  LSKSWWRKYC CLA  261
hCdc42   .........C VLL  191
hRac1    .........C LLL  192
hRhoA    ........GC LVL  193
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Figure 3. Amino acid sequences and domain structure of human and mouse Wrch-1. The deduced amino acid sequences of human and mouse Wrch-1 (hWrch-1 and mWrch-1) were aligned with those of human Cdc42, Rac-1, and RhoA. Identity across these proteins is denoted by black boxes. Nucleotide binding domains I–IV are indicated above the sequences. The proline residues in the unique N-terminal domain of Wrch-1, that are predicted to form PXXP SH3 binding motifs, are indicated by asterisks. The conserved cysteine residues at the C-termini of these proteins that serve as the prenylation site are indicated.

shown). These results are consistent with the previous reports that most members of the Rho family, including Cdc42, are constitutively and widely expressed in many tissues (Ridley 1999). Thus, Wrch-1 is not only structurally unique, but is also regulated by Wnt-1 signaling.

Expression of Wrch-1 *in adult human tissues and mouse embryos*

Tissue-specific expression of *Wrch-1* in adult human tissues was determined by Northern blot analysis. A hu-man *Wrch-1*-specific probe hybridized to a 4.4 kb mRNA species in several human tissues (Fig. 4A). The highest *Wrch-1* expression was detected in the brain, skeletal muscle, and placenta. Moderate expression was seen in the liver, lung, and heart. Low levels of expression were observed in the colon, spleen, kidney, and small intestine. Little or no expression was detected in the thymus and peripheral blood leukocytes (PBL). Since Wnt-1 is predominantly expressed in the testis of adult mice (Shackleford and Varmus 1987), it is possible that other Wnt-1-related proteins or other signaling molecules could induce the expression of *Wrch-1* in other tissues.
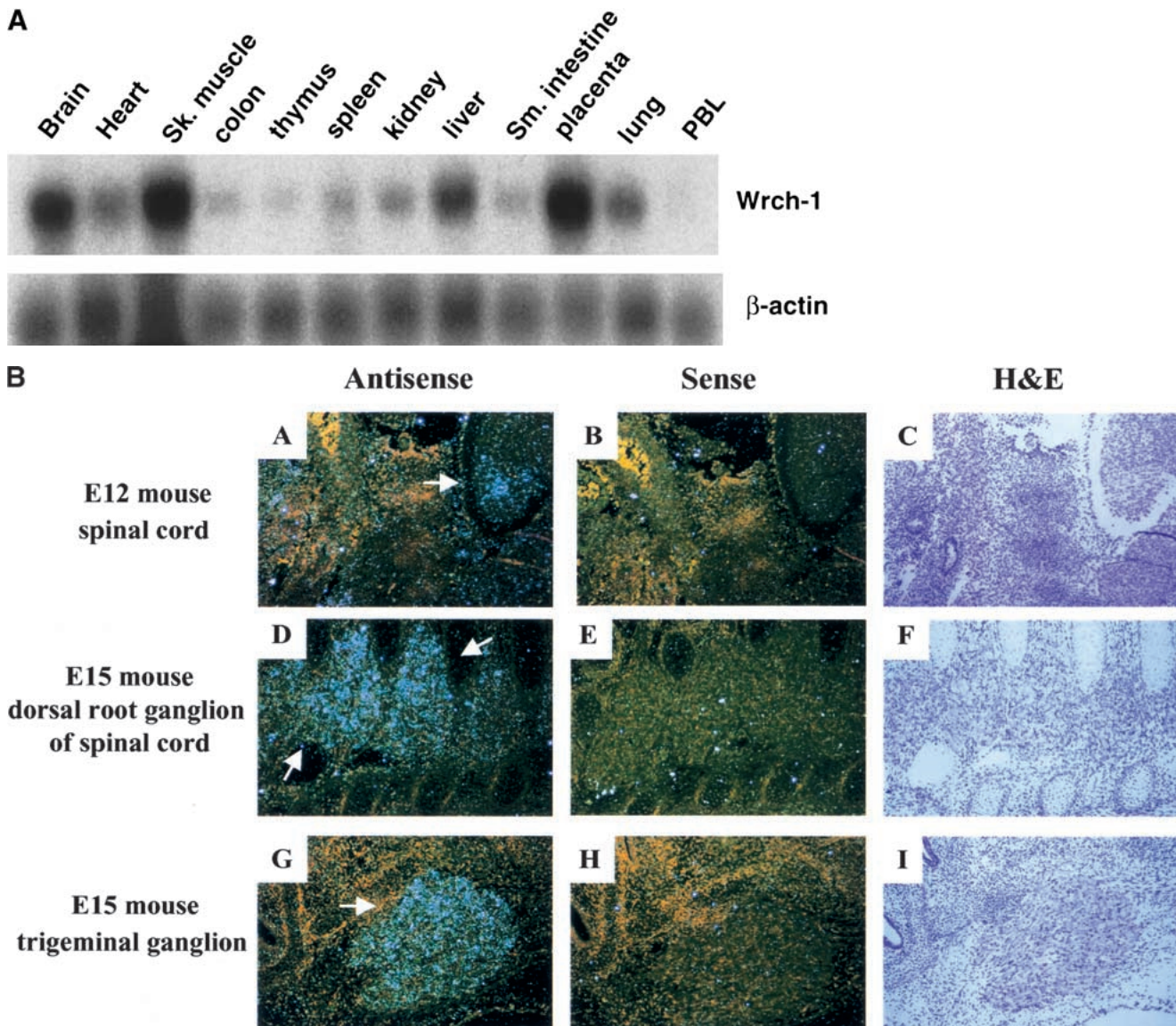
Figure 4. Expression of the Wrch-1 mRNA in adult human tissues and mouse embryos. (*A*) Poly(A)$^+$ RNA blots containing 2 µg of poly(A)$^+$ RNA per lane from multiple human tissues were hybridized with a human *Wrch-1* cDNA probe (containing codons for amino acids 81–207). The same filter was reprobed with human β-actin as a loading control. (*B*) Expression of the *Wrch-1* mRNA in mouse embryos. In situ hybridization was performed on serial sections from various developmental stages using either a mouse *Wrch-1* antisense or sense probe. Sections were also stained with hematoxylin/eosin (H&E) to visualize the tissue. Representative images are shown. Panels *A*, *D*, and *G* show Wrch-1 expression in E12 spinal cord, E15 dorsal root ganglion of the spinal cord, and E15 trigeminal ganglion, respectively. Sites of Wrch-1 expression are indicated by arrows.

Wrch-1 expression in the mouse embryo was assessed by in situ hybridization (Fig. 4B). Strong expression was observed in the nervous system, including the spinal cord and the trigeminal ganglion in the brain, as well as the uterine myometrium (data not shown). Since Wnt-1 signaling is of vital importance during embryonic development of the central nervous system in mice (Shackleford and Varmus 1987; McMahon and Bradley 1990), Wrch-1 could play a role in this process.

Characterization of the biochemical and biological activities of Wrch-1

The high homology between Wrch-1 and the Rho family proteins suggests that Wrch-1 may have similar biological activities. The Rho family proteins include three subfamilies: Rho, Rac, and Cdc42 (Vojtek and Cooper 1995; Van Aelst and D'Souza-Schorey 1997). These small GTPases are involved in many important physiological processes. For instance, Cdc42 and Rac can activate PAK (p21-activated protein kinase) (Manser et al. 1994) and JNK (Jun N-terminal kinase) (Coso et al. 1995; Minden et al. 1995). Cdc42, Rac, and Rho can regulate the actin cytoskeletal organization and stimulate cell cycle progression (Van Aelst and D'Souza-Schorey 1997; Hall 1998). Therefore, we sought to determine whether Wrch-1 can (1) activate PAK-1 and JNK-1; (2) regulate the actin cytoskeletal structure; and (3) stimulate cell cycle progression.

To this end, we made a putative constitutively active mutant of Wrch-1, Wrch-1Q107L, and a putative dominant negative mutant, Wrch-1T63N, by analogy with similar mutants of Cdc42 and Rac (Ridley 1999). The mutant Q107L was predicted to be defective in hydrolysis of GTP and thus it would be locked in the GTP bound form. In contrast, the mutant T63N was presumably unable to bind to nucleotides and it would therefore be locked in the inactive conformation. Moreover, this mutant could function in a dominant negative way by competing for the Wrch-1 nucleotide exchange factor(s).

To determine whether Wrch-1 is able to activate PAK-1, a hemagglutinin (HA) tagged PAK-1 plasmid was cotransfected into COS-7 cells with either an empty vector, or one or more of the constructs encoding wild-type Wrch-1, Q107L, or T63N. Thirty-six hours after transfection, the cells were harvested and the HA-PAK-1 was immunoprecipitated using an anti-HA antibody. Subsequently, an in vitro protein kinase assay was performed. Because PAK-1 can undergo autophosphorylation after its activation (Martin et al. 1995), in this assay PAK-1 was used as both a kinase and a substrate. Figure 5A shows that coexpression of wild-type Wrch-1 stimulated the activation of PAK-1. As predicted, the constitutively active mutant, Q107L, had an augmented activity in the activation of PAK-1, while the dominant negative mutant, T63N, failed to activate PAK-1. Furthermore, coexpression of T63N with wild-type Wrch-1 inhibited Wrch-1-triggered activation of PAK-1 (Fig. 5A). Using a similar assay, the constitutively active mutant Q107L activated JNK-1 (Fig. 5B). However, activation of JNK-1 by wild-
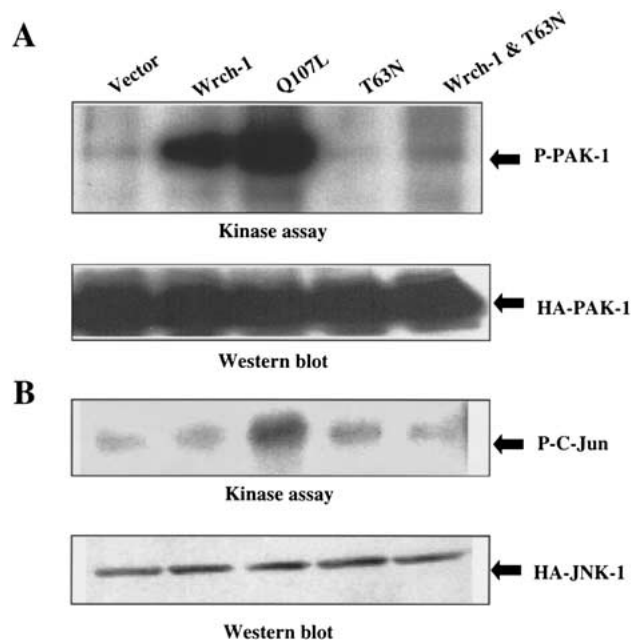

Figure 5. Wrch-1 activates PAK-1 and JNK-1. (*A*) COS-7 cells were cotransfected with the HA-PAK-1 plasmid (3 µg) and either the empty vector or one of the plasmids (3µg) encoding Wrch-1, Q107L, and T63N, or a combination of plasmids encoding Wrch-1 and T63N (1.5 µg each). 36 h after transfection, HA-PAK1 was immunoprecipitated, and the kinase activity of PAK-1 was determined by assessing autophosphorylation of PAK-1 using the immunocomplex kinase assay. Radiolabeled products of the assay were separated by SDS-PAGE and visualized by autoradiography (*upper* panel). Phosphorylated PAK-1 (P-PAK-1) is indicated. Levels of PAK-1 in each sample were determined by Western blotting and shown to be similar (*lower* panel). (*B*) COS-7 cells were cotransfected with a HA-JNK-1 plasmid and the plasmid(s) as described above. 24 h later, cells were serum-starved for 16 h, and then HA-JNK-1 was immunoprecipitated. The activity of JNK-1 was determined by the immunocomplex kinase assay using GST-C-Jun as a substrate. Products of the kinase assay were separated by SDS-PAGE and revealed by autoradiography (*upper* panel). Phosphorylated C-Jun is indicated (P-C-Jun). Levels of HA-JNK-1 were assessed by Western blot analysis and shown to be similar (*lower* panel).

type Wrch-1 was only poorly or not observed at all. This lack of activation by wild-type Wrch-1 may result from a modification of the experimental procedures in assessing JNK-1 activation versus those in assessing PAK-1 activation. To reduce the nonspecific JNK-1 kinase activity in COS-7 cells, the cells must be serum-starved for 16 h before they are assayed, whereas there is no need to serum-starve these cells to test PAK-1 activation. Serum may elicit the activation of ectopically expressed Wrch-1. In addition, the binding of Wrch-1 to PAK-1 was determined in the coimmunoprecipitation assay. Only the constitutively active mutant of Wrch-1, Q107L, but not the dominant negative mutant, T63N, binds to PAK-1 (data not shown). Taken together, these data indicate that like Cdc42 and Rac, Wrch-1 can activate PAK-1 and JNK-1 at least with a constitutively active Wrch-1 mutant. Moreover, these results confirmed the biological

activities of Q107L as a constitutively active mutant and T63N as a dominant negative mutant of Wrch-1.

Next, the effect of Wrch-1 on the actin cytoskeletal structure was determined. For this purpose, Wrch-1 recombinant adenoviruses were made that constitutively expressed the Wrch-1 mutants, Q107L, and T63N, or β-galactosidase (as a control). All of these viruses also expressed green fluorescent protein (GFP), allowing the use of GFP as a marker to identify the infected cells. These viruses were employed to infect Swiss 3T3 cells. Twelve hours after infection, the cells were serum-starved for 24 h, and then fixed and stained for F-actin. The structure of the actin cytoskeleton was analyzed by confocal immunofluorescent microscopy. In cells expressing the constitutively active mutant of Wrch-1, Q107L, there was a dissolution of stress fibers (Fig. 6). In addition, similar to Cdc42-induced filopodia (Kozma et al. 1995; Nobes and Hall 1995), these cells formed the finger-like protrusions that were composed of F-actin. Neither the dominant negative mutant of Wrch-1, T63N, nor β-galactosidase exhibited this phenotype. These data indicate that like Cdc42, Wrch-1 can modulate actin cytoskeletal organization by inducing the formation of filopodia and the dissolution of stress fibers.

The effect of Wrch-1 on cell cycle progression was also determined. Adenoviruses that expressed β-galactosidase, E2F-1, Wrch-1Q107L, and T63N were used to infect quiescent Swiss 3T3 cells that had been serum-starved for 48 h. After infection, cells were cultured under serum-starved conditions for 48 h before they were collected. Their distribution in the cell cycle was determined by measuring the DNA content of cells using FACS analysis. As shown in Figure 7, cells infected with the adenoviruses expressing β-galactosidase show only a slight increase in the fraction at the S-phase, as compared to the profile of noninfected cells. This may be a result of the virus infection. Consistent with previous observations, overexpression of E2F-1 stimulated cell cycle progression through the G_1 phase and DNA synthesis, and at the same time induced apoptosis (note the increase in the fraction of cells containing subdiploid DNA) (Shan and Lee 1994). Similarly, overexpression of the active form of Wrch-1, Q107L, promoted cell cycle progression through the G_1-phase and increased the number of cells undergoing apoptosis. However, T63N, the dominant negative mutant, had no effect on stimulating cell cycle progression, but its overexpression did increase cell death at a small amount. These results suggest that Wrch-1 can stimulate cell cycle progression through the G_1-phase and DNA synthesis.

Expression of Wrch-1 mimics the effect of Wnt-1 in C57MG cells

As mentioned before, overexpression of Wnt-1 in mouse mammary epithelial cells (C57MG) by retroviral infection morphologically transforms these cells, which manifests as an increased refractility, elongated mor-
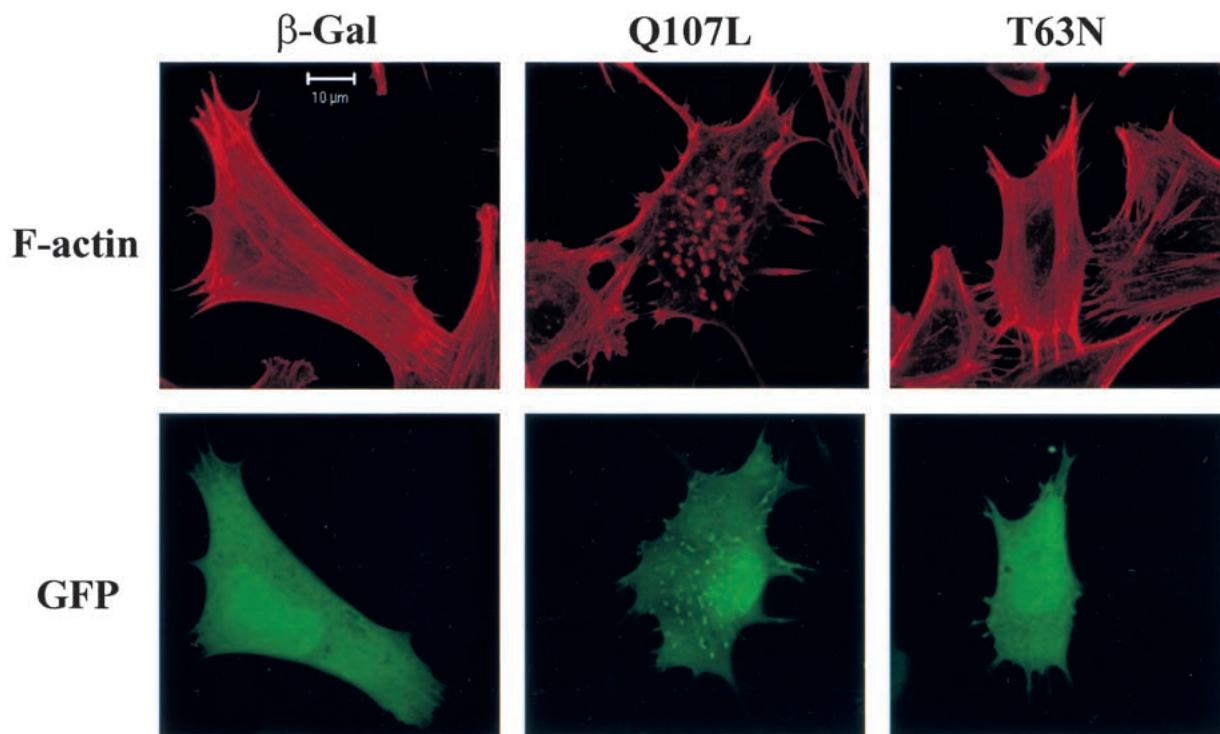

Figure 6. Wrch-1 induces filopodium formation and stress fiber dissolution in Swiss 3T3 cells. Swiss 3T3 cells were infected with the recombinant adenoviruses expressing β-galactosidase (β-gal), Q107L, or T63N. All of these viruses express GFP. Twelve hours after infection, cells were serum-starved for 24 h, and then fixed and stained for F-actin. The structure of F-actin and expression of GFP were detected by confocal immunofluorescent microscopy. A scale bar is shown in the image.
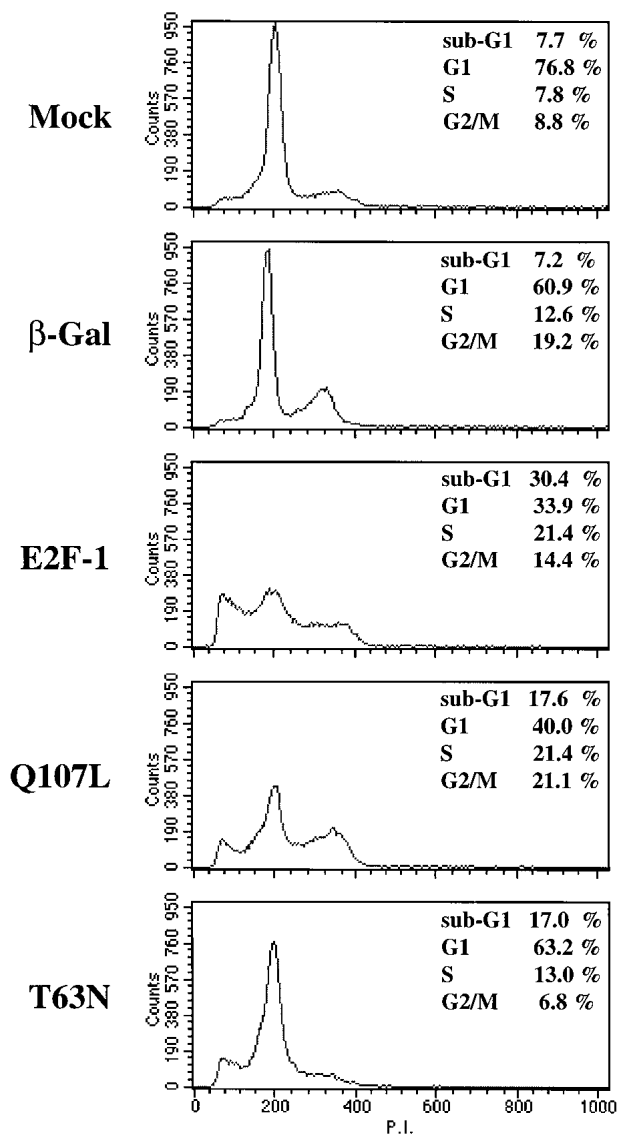
Figure 7. Wrch-1 stimulates quiescent Swiss 3T3 cells to re-enter the cell cycle. Swiss 3T3 cells were rendered quiescent by culturing in the absence of serum for 48 h. They were then infected with recombinant adenoviruses expressing β-galactosidase, E2F-1, Q107L, or T63N. Mock-infected cells were included as a control. After culturing without serum for 48 h, cells were fixed, stained with P.I., and analyzed by flow cytometry. The histogram plots of cell number versus DNA content are presented. The percentages of cells in different phases of cell cycle based on their DNA contents are indicated.

phology, higher saturation density, and a conversion from a monolayer to a multilayered growth (Mason et al. 1992). All of these phenotypes result from alterations in the organization of the actin cytoskeleton, cadherin-based cell–cell adhesion and integrin-dependent cell–matrix (substratum) interactions. Because Wrch-1 is a Cdc42-like small GTPase that could modulate all these processes and is differentially expressed in Wnt-1–transformed C57MG cells, Wrch-1 could mediate the effect of Wnt-1 in the morphology of these cells. To test this hy-

pothesis, the effect of Wrch-1 on the morphology of C57MG cells was determined. C57MG cells were infected with either the retroviruses expressing Wrch-1Q107L or T63N, or the empty vector retrovirus. Over-expression of Q107L, the constitutively active mutant of Wrch-1, causes the cells to become elongated and more refractile, and to have a higher saturation density (Fig. 8). These morphological alterations resemble the morphological transformation phenotype induced by Wnt-1 retroviral infection (Ziemer et al. 2001). In contrast, over-expression of T63N, the dominant negative mutant of Wrch-1, does not have such a phenotype. These results indicate that Wrch-1 can mimic the effect of Wnt-1 in morphological transformation of C57MG cells.

Discussion

Three lines of evidence demonstrate that *Wrch-1* induction is correlated or associated with the expression of *Wnt-1*: (1) After retroviral infection of cells, *Wrch-1* is selectively expressed in Wnt-1-expressing cells but not in control cells infected with an empty viral vector (Fig. 1A); (2) in the coculture assay, *Wrch-1* transcripts are strongly and rapidly induced by Wnt-1 signaling (Fig. 1B); and (3) *Wrch-1* is expressed in Wnt-1-induced mouse mammary tumors but not in wild-type mouse mammary glands (Fig. 2). These data do not distinguish between the possibility that the *Wrch-1* gene is regulated by Wnt-1 signaling directly or indirectly (i.e., by a transcription factor regulated by Wnt-1). One approach to address this issue would be to determine whether *Wrch-1* is up-regulated by Wnt-1 signaling in the absence of new protein synthesis. However, treatment with cycloheximide (an inhibitor of protein synthesis) itself induces *Wrch-1* gene expression in C57MG cells (data not shown), eliminating this approach.

The induction of downstream genes by the Wnt-1 signaling pathway could occur by different mechanisms. In the canonical model, Wnt-1 signaling elicits target gene transcription through β-catenin/TCF-LEF transcription factors by stabilizing β-catenin (Cadigan and Nusse 1997; Polakis 1999). Most of the identified Wnt-1-responsive genes are induced by this mode (Behrens et al. 1996; He et al. 1998a; van der Heyden et al. 1998; Gradl et al. 1999; Tetsu and McCormick 1999). Alternatively, the accumulated β-catenin in the cytoplasm could stimulate the activation of other transcription factors, such as CREB, through the cAMP/PKA pathway (Xu et al. 2000), or associate with other transactivation partners, such as *Teashirt*, to induce gene expression (Gallet et al. 1999). In addition, Wnt-1 could induce gene expression independently of β-catenin (Ziemer et al. 2001). Recently, the promoter of *BTEB2*, one of the genes isolated in the same screen as *Wrch-1*, was shown to be responsive to Wnt-1 signaling in a PKC-sensitive and β-catenin-independent manner (Ziemer et al. 2001). Indeed, some Wnt family proteins, such as *Xenopus* Wnt-5A, use the PKC/calcium pathway rather than β-catenin/TCF–LEF pathway to activate their target gene expression (Slusarski et al. 1997a,b). Interestingly, *Wrch-1* was up-regu-
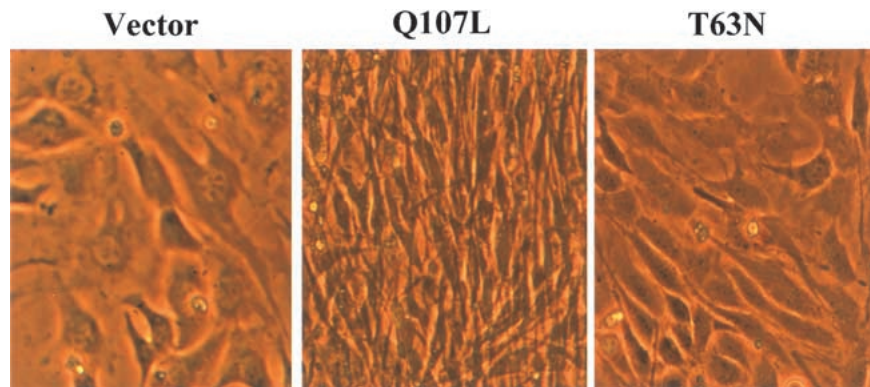
Vector	Q107L	T63N

Figure 8. Wrch-1 morphologically transforms C57MG cells. C57MG cells were infected with either a retrovirus expressing Wrch-1Q107L or Wrch-1T63N, or an empty retroviral vector. Cells stably expressing each of these proteins were selected for 4 d. The cell morphology was examined by microscopy.

lated in Wnt-1–expressing cells but not in cells expressing the stabilized, transactivation-competent mutants of β-catenin (Fig. 1A). These results suggest that Wnt-1 might regulate Wrch-1 expression independently of β-catenin. The isolation and analysis of the *Wrch-1* promoter would be required to confirm that *Wrch-1* is a direct target of Wnt-1 signaling, and to elucidate the mechanism(s) by which *Wrch-1* transcription is regulated.

Identification of Wrch-1 as a Wnt-1-regulated homolog of the Rho family of GTPases is likely to fill a gap between upstream Wnt-1 signaling and its effects on cell morphology, motility, and growth. The Rho-like GTPases act as molecular switches. In response to extracellular signals, they elicit coordinate changes in the organization of the actin cytoskeleton and in gene expression to regulate a variety of physiological processes, including morphogenesis, cell migration, axonal guidance, and cell cycle progression (Van Aelst and D'Souza-Schorey 1997; Hall 1998). Wrch-1 could regulate these processes in response to Wnt-1 signaling. Consistent with this hypothesis, Wrch-1 is predominantly expressed in the embryonic mouse nervous system (Fig. 4B), where Wnt-1 is also predominantly expressed (Shackleford and Varmus 1987). It has been shown that Wnt-1 plays a vital role in the development of the mouse nervous system. Homozygous deletion of the *Wnt-1* alleles results in the absence of midbrain and cerebellum, and the *Wnt-1*$^{-/-}$ mice die within 24 h after birth (McMahon and Bradley 1990). In this scenario, Wrch-1 might act downstream of Wnt-1 signaling to control neural migration and axonal growth.

Wnt-1 is not normally expressed in the mouse mammary gland. However, aberrant activation of Wnt-1 expression in this tissue leads to tumor formation (Nusse et al. 1984). Detection of *Wrch-1* expression in Wnt-1 induced mouse mammary tumors, but not in wild-type mouse mammary glands, is consistent with the involvement of Wrch-1 in Wnt-1-induced transformation and tumor formation. The observation that Wrch-1 can mimic the effect of Wnt-1 in morphological transformation of C57MG cells further supports this notion (Fig. 8). Indeed, overexpression or aberrant activation of Rho, Rac, or Cdc42 results in cell transformation, tumor for-

mation, and metastasis (Olson et al. 1995; Qiu et al. 1995, 1997; Keely et al. 1997; Clark et al. 2000).

Most of the Rho family GTPases are constitutively expressed in many tissues (Ridley 1999). Their biological activities are induced by extracellular cues that activate their corresponding nucleotide exchange factors (Van Aelst and D'Souza-Schorey 1997). However, expression of several Rho family members, such as RhoB and RhoG, is growth factor inducible, indicating an additional mechanism that regulates their activities, that is, controlling their transcription (Jahner and Hunter 1991; Vincent et al. 1992). *Wrch-1* appears to be regulated by both mechanisms. Conceivably, Wnt-1 could elicit Wrch-1 activities by inducing its expression and activating its nucleotide exchange factor(s) simultaneously. Alternatively, the expressed Wrch-1 could be activated by other signaling molecules. Of note is the SH3 binding domain of Wrch-1. This domain could either mediate the association of Wrch-1 with an activator or effector protein, or target Wrch-1 to a specific subcellular compartment. Interestingly, another novel protein has been identified in a yeast two-hybrid screen that can specifically bind to Wrch-1 (E.J. Hsieh, W. Tao, and A. J. Levine, unpubl.). This protein contains three putative SH3 domains and might act as an adaptor for Wrch-1 to target it to an activator or effector. Elucidation of the pathways upstream and downstream of Wrch-1 should lead to a better understanding of the fundamental processes controlled by Wnt-1 signaling.

Materials and methods

Suppressive subtraction hybridization

Suppressive subtraction hybridization was performed using the PCR-select cDNA Subtraction kit (Clontech) as previously described (Pennica et al. 1998; Ziemer et al. 2001). The mRNA from C57MG/Wnt-1 cells that had stably expressed Wnt-1 for 3 wk was used to synthesize the Tester cDNA, while the Driver cDNA was made from the parental C57MG cell mRNA.

cDNA library screening

Clones encoding full-length mouse Wrch-1 were obtained by screening a λgt10 mouse embryo cDNA library (Clontech) with

a 297-bp probe from the original partial clone 65.11 (codons for amino acids 207–306 of Wrch-1). Clones encoding full-length human Wrch-1 were isolated by screening λgt10 lung and fetal cDNA libraries with the same probe at low stringency.

Plasmids

The wild-type *Wrch-1* cDNA was subcloned into pcDNA3 (Invitrogen) between *Hin*dIII and *Bam*H1 restriction sites by polymerase chain reaction (PCR) to generate pcDNA3-*Wrch-1*. The constructs encoding Wrch-1Q107L and Wrch-1T63N were generated by introducing mutations in pcDNA3-*Wrch-1* using the QuikChange kit for site-directed mutagenesis (Stratagene). The human PAK-1 cDNA was cloned by reverse transcription polymerase chain reaction (RT–PCR) using total RNA from HeLa cells and primers based upon published sequences. The construct encoding the HA-tagged PAK-1 (HA-PAK-1) was generated by subcloning PAK-1 cDNA into the plasmid, pCGN, between *Xba*I and *Bam*H1 restriction sites. The plasmid for HA-JNK-1 was a gift from Dr. Ze'en Ronai. The sequences of all constructs were verified.

Tissue culture, cell lines, retroviral infection, and transfection

C57MG, COS-7, Swiss3T3, and 293 cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS). The C57MG cell lines expressing one of the stabilized mutants of β-catenin, 4145, and 4145TV were generated by transfection as described (Xu et al. 2000).

To generate Wrch-1Q107L and T63N retroviruses, the cDNAs encoding Q107L and T63N were subcloned into the retroviral vector pBabePuro at the *Eco*RI site. The retroviruses expressing Wrch-1Q107L and T63N were generated by transfection of the packaging cell line BOSC23 with pBabePuro containing their cDNA inserts as described (Ziemer et al. 2001).

C57MG cells were infected with an empty vector (pBabePuro) retrovirus or a Wnt-1 retrovirus as described (Xu et al. 2000; Ziemer et al. 2001). Forty-eight hours after infection, cells were split into the medium containing puromycin (2.5 μg/mL) and selected for 4 d before the RNA was extracted.

Similarly, C57MG cells were infected with an empty vector retrovirus or a retrovirus expressing Wrch-1Q107L or Wrch-1T63N, and selected in puromycin for 4 d before the cell morphology was examined by Normarski microscopy.

All transfections were performed using the lipofectamine reagent (GIBCO BRL) according to manufacturer's protocol.

Northern blot analysis

Total RNA was isolated from culture cells using the RNeasy Mini kit (QIAGEN) or from tissues using Trizol reagent (GIBCO BRL) as directed by the manufacturer. A human multiple tissue Northern blot was purchased from Clontech. Northern blotting was performed as previously described on 10 μg total RNA from each sample (Xu et al. 2000; Ziemer et al. 2001). The signals were visualized by autoradiography and quantitated using a PhosphorImager.

Coculture assay

The cell lines QT6 and QT6/Wnt-1 (a gift from Dr. Harold Varmus, Sloan Kettering Cancer Center, New York) were derived from a quail fibrosarcoma. Wnt-1 expression in the QT6/Wnt-1 cell line was driven by the MMTV promoter. In this assay, QT6 and QT6/Wnt-1 cells were plated on 10-cm tissue culture dishes at such a density that after culturing for 48 h they would be about 30%–40% confluent. The identical number of C57MG cells was added to dishes containing either QT6 or QT6/Wnt-1 cells. Cells were harvested after different time periods of coculture. Total RNA was extracted and subjected to Northern blot analysis.

In situ hybridization

In situ hybridization was done as previously described using the ^{32}P-labeled sense and anti-sense riboprobes were transcribed from a 279-bp PCR product corresponding to the codons for amino acids 207–306 of mouse Wrch-1.

In vitro PAK-1 assay

Forty-eight hours after transfection, COS-7 cells were lysed by supervortex in lysis buffer containing 40 mM Hepes (pH 7.4), 1% Nonidet P40, 2 mM EDTA, 150 mM NaCl, 20 mM NaF, 10 mM β-glycerophosphate, 1 mM Na$_3$VO$_4$, 1 mM DTT, and a combination of protease inhibitors (Complete from Boehringer). HA-PAK-1 was immunoprecipitated from the cleared lysates with equal amounts of total proteins (500 μg) by incubation with an anti-HA monoclonal antibody 16B12 (BAbCO) for 1 h at 4°C. Protein-G conjugated agarose beads were used to precipitate the immune complexes. The immunoprecipitates were washed twice with the lysis buffer and once with the kinase reaction buffer that contained 20 mM Hepes (pH 7.4), 20 mM MgCl$_2$, 20 mM NaF, 1 mM Na$_3$VO$_4$, 10 mM β-glycerophosphate, and 1 mM DTT. The PAK-1 activity in the immunoprecipitates was determined in 30 μL kinase reaction buffer with an addition of 20 μM cold ATP and 10 μCi [γ-^{32}P]ATP using HA-PAK-1 in the immunoprecipitates as a substrate. After 20 min at 30°C, reactions were terminated by addition of the protein loading buffer (1% SDS, 10 mM β-mercaptoethanol, 0.02% bromophenol blue). Samples were subject to electrophoresis on a SDS-10% polyacrylamide gel and detected by autoradiography. Cell lysates containing 60 μg of total protein were subjected to Western blot analysis using a PAK-1-specific antiserum (PharMingen).

In vitro JNK-1 assay

Transfected COS-7 cells were cultured in medium containing 10% FBS for 24 h and then serum-starved for 12 h. The HA-tagged JNK-1 was immunoprecipitated and the activity of JNK-1 in the immunoprecipitates was determined as described above using the GST-c-Jun protein (1 μg) (a gift from Dr. Yigong Shi, Princeton University, NJ) as a substrate. Parallel samples containing 60 μg of total protein were processed for Western blot analysis using a JNK-1-specific antiserum (Santa Cruz).

Western blot analysis

Proteins in the cell lysates from each sample were separated by 10% SDS–polyacrylamide gel electrophoresis (SDS-PAGE) (60 μg per lane) and then transferred to nitrocellulose membrane. PAK-1 and JNK-1 were detected using specific antibodies (Santa Cruz). Immunoblotting was performed as described previously (Tao et al. 1997).

Recombinant adenoviruses

Recombinant adenoviruses were generated using the AdEasy system (a gift from Dr. Bert Vogelstein, Johns Hopkins Medical School, Baltimore, MD) as described previously (He et al.

1998b). The cDNAs for Wrch-1Q107L and Wrch-1T63N from the pcDNA3–Wrch-1Q107L and pcDNA3-Wrch-T63N (described above) were subcloned into the plasmid pADTrack-CMV between the *Hin*dIII and *Xba*I sites. An *E2F-1* cDNA from pCMVE2F-1 was subcloned as a *Bam*H1 fragment into the *Bgl*II site of pADTrack-CMV, and the *LacZ* gene from pSVB (Clontech) was subcloned as a *Not*I fragment into the *Not*I site of pADTrack-CMV. In the pADTrack-CMV, the expression of both GFP and the gene of interest is driven by its own CMV promoter. Thus, the recombinant adenoviruses express GFP and the protein of interest. Recombination in *Escherichia coli* BJ5183 and transfection of the recombinant plasmids into 293 cells were performed as described. CsCl-banded recombinant adenovirus stocks were prepared as described, diluted with an equal volume of 2X storage buffer (50% glycerol, 4 mM MgCl$_2$, 20 mM Tris at pH 8.0), and stored at –20°C. Viral titers were determined by optical absorbance.

Immunofluorescent analysis

Swiss 3T3 cells (3×10^5) growing on a 1.8 × 1.8-cm glass coverslip in a 35-mm dish were infected with the recombinant adenoviruses [Multiple of Infection (MOI): 4000 particle/cell]. Twelve hours postinfection, these cells were serum-starved for 24 h. The cells were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) followed by permeabilization with 0.2% Triton X-100 in PBS. After treatment with the blocking solution (PBS containing 0.5% BSA, 10% horse serum and 0.05% Tween-20) for 20 min, the cells were incubated with Alexa568 conjugated philloidin (3 units/mL in the blocking solution) for 30 min at room temperature. Finally, the cells were mounted with Aqua Poly/Mount (Polysciences) and visualized by confocal microscopy.

Flow cytometry

Quiescent Swiss 3T3 cells (serum-starved for 48 h) were infected with adenoviruses (MOI: 20,000) and then incubated in medium containing 0.1% FBS for 48 h. Both attached and floating cells were collected, fixed with ethanol, stained with propidium iodide (PI), and analyzed by fluorescence-activated cell sorting (FACS) as previously described (Kalejta et al. 1999).

Acknowledgments

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