Isolation and Biochemical Characterization of the Human Dkk-1 Homologue, a Novel Inhibitor of Mammalian Wnt Signaling*

(Received for publication, February 17, 1999, and in revised form, April 27, 1999)

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In an effort to isolate novel growth factors, we identified a human protein, designated Sk, that co-eluted with Neuregulin during chromatographic separation of conditioned medium from the SK-LMS-1 human leiomyosarcoma cell line. Degenerate oligonucleotides based on amino-terminal sequence analysis of the purified protein were used to isolate the corresponding cDNA from a library generated from this cell line. Sk is a novel 266-amino acid protein that contains a signal peptide sequence and two cysteine-rich domains with no similarity to other known growth factors. A single major 2-kilobase transcript was expressed in several embryonic tissues. Transfection of mammalian cells demonstrated that the protein was secreted and expressed as a doublet of approximately 35 kDa. In vitro translation and endoglycosylase analysis indicated that this doublet, which was also observed in cells expressing the endogenous protein, arises from posttranslational modification. A search of the GenBank™ data base revealed a match of Sk with Dkk-1, which is a novel secreted protein required for head induction in amphibian embryos and a potent Wnt inhibitor. When coexpressed with Wnt-2 in NIH3T3 cells, human Sk/Dkk-1 caused repression of Wnt-2 induced morphological alterations and inhibited the Wnt-2 induced increase in uncomplexed β-catenin levels. These results provide biochemical evidence that human Sk/Dkk-1 antagonizes Wnt signaling upstream of its effect on β-catenin regulation.

Members of the Wnt gene family encode secreted glycoproteins that are required for a variety of developmental processes ranging from cell lineage decisions to control of differentiation of the central nervous system in higher vertebrates (1, 2). In Xenopus embryonic development, Wnt signaling is involved in dorsoventral axial patterning both before and after the midblastula transition (1). Wnts bind and act through the frizzled family of cell surface proteins, a large family of seven membrane-spanning domain receptors (3). A number of downstream components of Wnt signaling have been identified by a combination of genetic and biochemical approaches. Wnts act through the cytoplasmic protein dishevelled to inhibit the activity of the serine threonine kinase glycogen synthetase kinase-3. Glycogen synthetase kinase-3 appears to bind through a bridging molecule, Axin, to the β-catenin/adenomatous polyposis coli complex and phosphorylate β-catenin, causing its rapid degradation. Wnt-induced inhibition of glycogen synthetase kinase-3 leads to β-catenin stabilization resulting in an increased level of the uncomplexed soluble form (4–6). The latter can interact with T-cell factor/LEF transcription factors and, after translocation to the nucleus, activate target genes (7).

There is evidence that activation of Wnt signaling can contribute to the neoplastic process. Inappropriate expression of these ligands due to promoter insertion of the mouse mammary tumor virus (reviewed in Ref. 2) or targeted expression in transgenic mice causes mammary tumor formation (8). Moreover, in cell culture, several Wnt family members have been shown to induce altered morphology and increased saturation density of certain epithelial (9, 10) and fibroblast (11, 12) cell lines. Finally, genetic alterations affecting adenomatous polyposis coli or β-catenin levels, have been observed in human colon cancer (13), melanomas (14), and hepatocellular carcinomas (15), indicating that aberrations of Wnt signaling pathways are critical to the development of these and possibly other human cancers.

Recent studies in Xenopus embryos have identified several families of inhibitors of Wnt signaling. One, designated as frizzled-related protein (FRP) (16), has structural similarities to the frizzled family of Wnt receptors. Wnts interacts with frizzled to induce axis duplication following conjection of early Xenopus embryos (17). In contrast, FRP, also designated as Frzb (18, 19), inhibits Wnt-induced axis duplication. FRP shares with frizzled a cysteine-rich domain, which has been shown to confer Wnt binding properties (3). Thus, FRPs presumably act as Wnt antagonists at the level of receptor binding.

The gene cerberus encodes for a secreted protein that is capable of inducing ectopic head formation in Xenopus embryos (20). It has been recently known that this protein is a multivalent growth factor antagonist capable of binding Nodal, bone morphogenetic protein, and Wnt proteins, inhibiting their effects (21).

Another inhibitor was isolated by an expression cloning strategy for cDNAs able to complement in the formation of a complete secondary axis in Xenopus (22). A novel molecule, designated dickkopf-1 (dkk-1) (German for “big head, stub-

* This project was supported in part by Grants CA71997 (to P. F.) and CA71672 (to S. A. A.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF127563.

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1 The abbreviations used are: FRP, frizzled-related protein; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; GST, glutathione S-transferase; kb, kilobase.
Isolation of Human Dkk-1, a Novel Inhibitor of Wnt Signaling

born) encoded by the isolated cDNA, contained a signal sequence as well as two cysteine-rich domains and was unrelated to any previous protein. Like FRP, Dkk-1 was shown to possess the ability to inhibit Wnt-induced axis duplication. Genetic analysis indicated further that Dkk-1 acts upstream of the dishevelled pathway to inhibit Wnt signaling (22). In the course of efforts to isolate novel epidermal growth factor-like ligands, we identified a human Dkk-1 homologue, which we have characterized in the present study with respect to its expression pattern and function as an inhibitor of Wnt signaling in mammalian cells.

EXPERIMENTAL PROCEDURES

Purification, Physical Characterization, and Microsequencing—SK-LMS-1 cells were plated onto 175-cm² T flasks and grown to confluence in Dulbecco's modified Eagle's medium (Life Technologies, Inc.) supplemented with 10% (v/v) calf serum (Life Technologies, Inc.). Confluent medium was then generated by two consecutive harvests in serum-free medium for 72 h, alternate by 4 days of culturing with serum-containing medium deoxycholate, and separated by SDS-PAGE (29). Immunodetection, 100 μg of total cell lysates from cultures solubilized in radiolabeled precipitation buffer were resolved by SDS-PAGE and transferred to an Immobilon-P membrane. Specific signals were visualized by ECL analysis (Amersham Pharmacia Biotech) after incubation with horseradish conjugated rabbit anti-mouse HRP antibody (2.6 μg/ml; DAKO).

Enzymatic Deglycosylation—One ml of conditioned medium from cultured SK-LMS-1 cells was immunoprecipitated using the rabbit antihuman Dkk-1 IgG antibody. Immunoprecipitated proteins were resuspended in 40 μl of PBS that contained 0.1% SDS, and 0.5% sodium deoxylate, and separated by SDS-PAGE (29). Immunodetection was then performed by use of 125I-labeled protein A. In vitro transcription and translation was performed with a commercially available kit from Promega (TNT coupled reticulocyte lysate system). Briefly, 0.5 μg of cDNA was used for each reaction with or without the addition of canine pancreatic microsomal membrane (Promega), following the manufacturer's instruction. Translation products were separated by SDS-PAGE and subjected to autoradiography. I1C-labeled marker was used for molecular weight determination. For Wnt-2 HFc detection, 100 μg of total cell lysates from cultures solubilized in radiolabeled precipitation buffer were resolved by SDS-PAGE and transferred to an Immobilon-P membrane. Specific signals were visualized by ECL analysis (Amersham Pharmacia Biotech) after incubation with horseradish conjugated rabbit anti-mouse HFC antibody (2.6 μg/ml; DAKO).

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Purification and Molecular Cloning of a Novel Human Secreted Molecules—In the course of the purification of ligands for the erbB3 receptor, we identified a protein that appeared as a doublet of 35 kDa by silver staining analysis and co-eluted with Neuregulin. Microsequencing of the purified material revealed positive identification for both bands in a single run of several amino acids as follows: TLNSVLNSNAIKNLPPPLGGAAGHPGDAV (X indicates inability to make an amino acid assignment). Comparison of the amino-terminal sequence of the purified protein with those in several databases revealed no significant homology, suggesting that this might be a novel protein.

Degenerate oligos were designed for isolation by PCR of a gene-specific insert that was used for the cloning of the full-length coding sequence from a SK-LMS-1 cDNA library. After three consecutive screening cycles, positive clones were classified into three groups based on their restriction digestion patterns. Sequence analysis of one clone (1.5 kb) demonstrated an open reading frame of 798 base pairs surrounded by 145 base pairs of 5' noncoding sequence and 614 of 3' noncoding sequence (Fig. 1A). The 3' noncoding region contains a poly(A) tail. Two consecutive ATGs, starting at nucleotides 146 and 149, were identified as initiation codons, fulfilling the consensus sequence for a translation initiation codon as defined by Kozak (30). Two upstream in-frame stop codons were also identified. The open reading frame predicted a 266-amino acid protein with an approximately 26-kDa molecular mass and an isoelectric point of 9.67 determined by using the MacVec-tor program (Eastman Kodak). The experimentally determined protein sequence began 32 residues downstream of the first identified methionine. Hydrophilicity analysis (31) of the coding sequence revealed a hydrophobic region at the amino terminus, 31 amino acids upstream of the experimentally determined sequence, which likely functioned as signal peptide for protein secretion (Fig. 1B). Several cysteine residues, clustered into two separate areas, were also identified in the amino acid sequence, consistent with the secreted nature of the protein. However, no similarity was detected with the epidermal growth factor-like family of growth factor or other known growth factors or secreted proteins. A potential site for N-linked glycosylation in the carboxyl-terminal region of the protein was identified at position 256–258, suggesting posttranslational modification. Consistent with this possibility was the size of the isolated doublet, which was larger than the predicted molecular weight. The other clones (2.5 and 1.2 kb) contained the same coding sequence but differed in size from the 1.5-kb clone due to variations in their 3' untranslated regions. The 2.5-kb clone also contained an unspliced intron.

Pattern of Human Sk Tissue Expression—We performed Northern blot analysis to determine expression of Sk in different human tissues using the entire coding sequence as probe. PB, peripheral blood.

**RESULTS**

**Purification and Molecular Cloning of a Novel Human Secreted Molecules**—In the course of the purification of ligands for the erbB3 receptor, we identified a protein that appeared as a doublet of 35 kDa by silver staining analysis and co-eluted with Neuregulin. Microsequencing of the purified material revealed positive identification for both bands in a single run of several amino acids as follows: TLNSVLNSNAIKNLPPPLGGAAGHPGDAV (X indicates inability to make an amino acid assignment). Comparison of the amino-terminal sequence of the purified protein with those in several databases revealed no significant homology, suggesting that this might be a novel protein.

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**Pattern of Human Sk Tissue Expression**—We performed Northern blot analysis to determine expression of Sk in different human tissues using the entire coding sequence as probe. In a panel of poly(A)+ mRNAs from fetal tissues, a strong 2-kb transcript was detected in kidney. A less intense band was present also in liver and brain but not in lung (Fig. 2A). In adult tissues, a similar size transcript was identified in placenta and prostate (Fig. 2B). Hybridization signals were also visible after longer exposure in colon and spleen. A lower level of expression of a higher molecular weight transcript was also
ular weight markers are indicated at the SK-LMS-1 but not from SK-OV-3 or PA-1 cells. The positions of molecular weight markers are indicated at the right. C, Northern blot analysis of the Sk transcript in tumor cell lines. 30 μg of total RNA was probed with the human Sk cDNA probe. The positions of 28S and 18 S ribosomal RNA are shown.

detected both in embryonic and adult tissues. It is conceivable that these larger transcripts may represent signals from unprocessed mRNA. However, we cannot exclude the possibility that they may reflect cross-hybridization with transcripts of related proteins.

Identification and Characterization of the Human Sk Protein—A polyclonal antibody was generated against the full-length human Sk protein, produced as a GST fusion molecule in E. coli. Immunization was monitored by testing the capacity of antisera obtained from successive bleeds to recognize the GST fusion protein and the endogenous molecule in concentrated conditioned medium from SK-LMS-1 cells. As shown in Fig. 3A, the antiserum specifically recognized a major species of approximately 35 kDa in conditioned medium of SK-LMS-1 cells. As shown in Fig. 3A, the antiserum specifically recognized a major species of approximately 35 kDa in conditioned medium of SK-LMS-1 cells. Moreover, the protein was also detected by immunoprecipitation followed by immunoblotting with the same antisera (Fig. 3B). Therefore, the polyclonal antibody generated was capable of identifying the endogenous Sk protein in a native as well as denatured state. A less intense immunoreactive species migrating more slowly was detected under both conditions. In contrast, the same antiserum failed to detect a similar band in culture fluids of SK-OV-3 or PA-1 cells, which lacked any detectable Sk transcript (Fig. 3, B and C).

To further confirm that the cDNA isolated encoded for the expected protein, we transfected 293 cells with the human Sk gene after removing its 5′- and 3′-untranslated regions and subcloning in pCEV 29 vector (25). Northern blot analysis was first performed in such cells to determine transfection efficiency. Fig. 4A shows that the levels of Sk transcripts in transfected 293 cells were much higher than present in SK-LMS-1 cells and appeared as two specific bands that migrated more slowly than the endogenous 2-kb transcript present in SK-LMS-1. To correlate RNA levels with protein expression, conditioned medium from transiently transfected cells was analyzed by immunoblotting for the presence of Sk immunoreactive protein. As shown in Fig. 4B, immunoreactive bands in the conditioned medium of Sk transfected 293 cells showed similar sizes to two species identified in SK-LMS-1 medium, and were not present in mock transfectants. The stronger signal identified in 293 cells correlates well with the higher transcript levels shown by Northern analysis.

Our findings that the doublet expressed in 293 cells was encoded by a single cDNA, strongly supported the possibility that posttranslational modification was responsible for these two immunoreactive species. To test this hypothesis, we performed an in vitro transcription and translation analysis. As shown in Fig. 4C, a single radiolabeled species of approximately 35 kDa was observed in the absence of microsomal membranes. In their presence, an additional less intense band of slightly higher molecular weight became evident as well. These species resembled the appearance of the two immunoreactive bands in conditioned medium of SK-LMS-1 or 293 transfecants cells, consistent with the conclusion that Sk undergoes posttranslational modification. Because a potential site for N-linked glycosylation is present in the carboxyl-terminal region of the protein we tested whether glycosylation was indeed responsible for the appearance of the doublet. Sk was immunoprecipitated from conditioned medium and subjected to enzymatic deglycosylation using N-glycosidase F, which releases all types of asparagine bound N-glycans, as described under “Experimental Procedures.” This analysis (Fig. 4D) revealed that after treatment, Sk appeared as a single major immunoreactive band of similar molecular weight to the lower species
in the untreated control. These results demonstrate that N-glycosylation is responsible for the higher molecular weight species.

Although our discovery of Sk was based on the co-elution with neuregulin during chromatographic purification, subsequent experiments with the recombinant protein, indicated that Sk was not a ligand for erbB molecules (data not shown), consistent with the absence of any similarity with epidermal growth factor-like proteins. Due to the novel features of the Sk protein, we continued to search the GenBank™ data base for related molecules in an effort to obtain insights into Sk biological function. In 1997, significant similarity was observed with a protein of unknown function identified in chicken (GenBank™ accession number 517093). A more recent report of a novel secreted protein from the Spemann organizer in amphibian embryos (22) was instrumental in identifying Sk function.

**SK Is the Human Homologue of Dickkopf-1—**Dickkopf-1 (dkk-1) was identified as a secreted protein that is required for head formation during Xenopus embryogenesis (22). By search of the expressed sequence tag-sequence data base, and a mouse cDNA library screening, it was further shown that Xenopus dkk-1 is a member of a new family of genes, composed of at least three members in humans, and present in different species (22). Comparison of the Sk sequence with the dkk-1 gene, revealed that Sk was the human homologue of dkk-1. In fact, Sk was identical in the region of overlap with the human dkk-1 gene, identified as partial clone in the expressed sequence tag-sequence data base, and a mouse cDNA library screening. The consensus sequence is as reported by Glinka et al. (22).

![Fig. 5](image_url)

**Fig. 5.** A, schematic representation of the Sk/Dkk-1 protein. **SP**, signal peptide; C1, cysteine 1 domain; C2, cysteine 2 domain; N, N-glycosylation site. Percentage of identity between human and mouse or Xenopus cysteine-rich domains is shown. B, pile-up of Dkk-1 sequences from human (AF127563), mouse (AF030433), and Xenopus (AF030434) proteins in the two cysteine-rich domains. The consensus sequence is as reported by Glinka et al. (22).

Evidence indicates that Wnts act through dishevelled to regulate β-catenin stability (9). In Xenopus, genetic analysis has indicated that Dkk-1 inhibits Wnt signaling upstream of dishevelled (33). The morphologic effects of transforming Wnts in mammalian cells such as NIH3T3 are associated with an increase in β-catenin stability as measured by the level of uncomplexed β-catenin using a GST-E cadherin binding assay (11). To assess the effect of human Sk/Dkk-1 to revert Wnt-2-induced alterations in the growth of these cells (11). Stable transfectants were obtained by double marker selection and grown postconfluence. As shown in Fig. 6, and quantitated in Table 1, Wnt-2 expressing cells grew to higher cell density then the vector control. Coexpression of Sk/Dkk-1 with Wnt-2 essentially blocked this effect such that human Dkk-1/Wnt-2 co-transfectants were indistinguishable from the controls. Moreover, in the carboxyl-terminal region, the last nine amino acids were identical in all three sequences analyzed. Thus, Sk was the human homologue of Dkk-1.

**Human SK/Dkk-1 Inhibits Wnt-induced Morphological Transformation and Signaling—**It has been previously shown that injection of mRNA encoding Dkk-1 in Xenopus embryos is sufficient and necessary to cause head induction (22). This effect seems to be mediated by the ability of Dkk-1 to antagonize Wnt signaling, as demonstrated by the ability of dkk-1 to rescue the secondary-axis duplication induced by Xwnt-8 (22). To assess the ability of human Sk to function as a Wnt inhibitor in a mammalian system, we transfected NIH3T3 cells with human Wnt-2 and human Sk/Dkk-1, individually or in combination, to determine the capacity of Sk/Dkk-1 to revert Wnt-2-induced alterations in the growth of these cells (11). Stable transfectants were obtained by double marker selection and grown postconfluence. As shown in Fig. 6, and quantitated in Table 1, Wnt-2 expressing cells grew to higher cell density then the vector control. Coexpression of Sk/Dkk-1 with Wnt-2 essentially blocked this effect such that human Dkk-1/Wnt-2 co-transfectants were indistinguishable from the controls.

**Isolation of Human Dkk-1, a Novel Inhibitor of Wnt Signaling**

19469
DISCUSSION

In this report we describe the identification and characterization of a novel human protein Sk, isolated as a heparin-binding protein that co-purified with Neuregulin in conditioned medium from the SK-LMS-1 human leiomyosarcoma cell line. A continued search of the GenBank™ data base for related molecules revealed a sequence match with the *Xenopus* Dkk-1 protein recently reported (22). Dkk-1 was identified by an expression cloning strategy in an effort to identify novel genes that function as head inducers in *Xenopus* embryo development (22). Co-injection experiments also revealed that Dkk-1 is a potent inhibitor of Wnt signaling, as indicated by its ability to completely rescue the formation of a secondary axis induced by *XWnt-8*. These findings encouraged us to investigate human Sk/Dkk function with respect to Wnt signaling in mammalian cells. We demonstrated that Sk/Dkk, when coexpressed in NIH 3T3 cells, is able to cause reversion of Wnt-induced morphological transformation. Moreover, this inhibition correlated with its ability to cause a dramatic decrease in Wnt-induced accumulation of uncomplexed β-catenin. These biochemical findings complement genetic analysis in *Xenopus* indicating that Sk/Dkk-1 acts upstream of dishevelled to inhibit Wnt signaling (22).

The endogenously expressed human Sk/Dkk-1 protein appeared as a secreted doublet of approximately 35 kDa on SDS-PAGE either after chromatographic purification or when detected by immunoblot in conditioned medium. Two similar sized species were identified in transfected 293 cells, implying that a single cDNA encoded both forms. In *Xenopus*, exogenous Dkk-1 was expressed as two species, of which the higher molecular weight form was predominantly secreted (22). All of these findings suggest that this molecule undergoes posttranslational modification. Our *in vitro* transcription and translation analysis of Sk cDNA proved that in addition to a lower molecular weight species of around 35 kDa, a slower migrating protein was synthesized in the presence of microsomal membranes, consistent with the latter arising from posttranslational modification. Enzymatic deglycosylation of immunopurified Sk/Dkk-1 from conditioned medium of SK-LMS-1 cells, confirmed that the higher molecular weight species represented the N-glycosylated form of the protein.

Northern blot analysis revealed that Sk/Dkk-1 is broadly expressed in embryonic tissues. The presence of the Sk transcript in human fetal kidney, liver, and lung suggests that Sk may play a role in the development of these organs, consistent with the reported expression of Dkk-1 in foregut endoderm of mouse embryos (22). The limited expression of Sk detected in several human adult tissues does not exclude a possible role of this protein in later development/differentiation. However, more sensitive methods of analysis will be required to establish whether expression can be demonstrated in specific cell types. Moreover, because Sk was isolated as a secreted molecule from a tumor cell line, analysis for Sk expression in tumor tissues...
will be of interest in testing whether Sk may be commonly up-regulated under such conditions.

There is evidence that members of the Wnt family play important roles in a variety of developmental processes (1, 2). Thus, it can be postulated that the simultaneous expression of Sk/Dkk-1 might be required to regulate Wnt function during development. According to their behavior following ectopic expression in Xenopus embryos and in mammalian cells, the Wnt genes have been divided into two functional classes (10, 34–36). The first, which includes Wnt-2, induces a secondary axis in embryos, causes transformation of cells in culture, and signals through the β-catenin/T-cell factor pathway or in-terferes with the frizzled/Wnt binding interaction (37). Another Wnt inhibitor is represented by the secreted protein Cerberus, which has been shown to bind and inhibit Wnts, among other proteins (21). More recently WIF-1, or Wnt inhibitory factor 1, has been described. This secreted protein, which contains five epidermal growth factor-like repeats in its sequence, was shown to inhibit axis induction by Wnts in Xenopus embryos (38).

The Sk/Dkk proteins contain two related cysteine-rich domains that are distinct from the FRP cysteine-rich domain but are highly conserved among members of the Dkk family. The mechanism by which Sk inhibits Wnt has not yet been elucidated. In the Xenopus embryo system, Dkk-1 and Frzb exhibit similar patterns of expression, and both antagonize Wnt signaling upstream of dishevelled, suggesting that both may act at an extracellular level (22). When compared with Frzb, Dkk-1 appeared to be a more potent head inducer and possibly a more effective Wnt antagonist (22). Consistent with these findings, when we compared the ability of Dkk-1 and FRP to interfere with Wnt signaling in NIH3T3 cells, Dkk-1 was a more potent Wnt inhibitor. Further studies will be required to determine whether Sk/Dkk binds Wnt, like FRP/Frzb and Cerberus, or antagonizes Wnt signaling indirectly through an independent inhibitory pathway.

Acknowledgments—We thank J. Rubin for helpful advice in protein purification and S. Tronick for help in cDNA sequencing.

REFERENCES
1. Cadigan, K. M., and Nusse, R. (1997) Genes Dev. 11, 3296–3305
2. Nusse, R., and Varmus, H. E. (1992) Cell 68, 1073–1087
3. Bhanot, P., Brink, M., Samos, C. H., Hsieh, J. C., Wang, Y., Macke, J. P., Andrew, D., Nathans, J., and Nusse, R. (1996) Nature 382, 225–230
4. Gumbiner, B. M. (1995) Curr. Opin. Cell Biol. 7, 634–640
5. Miller, J. R., and Moon, R. T. (1996) Genes Dev. 10, 2527–2539
6. Nakamura, C., Weiss, J. B., and Williams, L. T. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 3020–3025
7. Behrens, J., von Kries, J. P., Kuhl, M., Bruhn, L., Wedlich, D., Grosschedl, R., and Birchmeier, W. (1996) Nature 382, 635–642
8. Lin, T. P., Guzman, R. C., Oshara, R. C., Thordarson, G., and Nandi, S. (1992) Cancer Res. 52, 4413–4419
9. Brown, A. M., Wildin, R. S., Prendergast, T. J., and Varmus, H. E. (1986) Cell 46, 1091–1099
10. Wong, G. T., Gavin, B. J., and McMahon, A. P. (1994) Mol. Cell. Biol. 14, 6278–6286
11. Bafico, A., Gazit, A., Wu-Morgan, S. S., Yaniv, A., and Aaronson, S. A. (1998) Oncogene 16, 2519–2525
12. Young, C. S., Kitamura, M., Hardy, S., and Kitajewski, J. (1998) Mol. Cell. Biol. 18, 2474–2485
13. Morin, P. J., Sparks, A. B., Korinek, V., Barker, N., Clevers, H., Vogelstein, B., and Kinzler, K. W. (1997) Science 275, 1787–1790
14. Rubinfeld, B., Robbins, P., El-Gamil, M., Albert, I., Porfiri, E., and Polakis, P. (1997) Science 275, 1790–1792
15. de la Coste, A., Romagnolo, B., Biliardt, P., Renard, C. A., Andrew, D., Nathans, J., and Nusse, R. (1996) Nature 382, 225–230
16. Leys, L., Bouwmeester, T., Kim, S. H., Piccolo, S., and De Robertis, E. M. (1997) Cell 88, 747–756
17. Wang, S., Krinks, M., Lin, K., Layten, F. P., and Moos, M. J. (1997) Cell 88, 757–766
18. Bouwmeester, T., Kim, S., Sasai, Y., Leys, L., and De Robertis, E. M. (1999) Nature 391, 595–601
19. Piccolo, S., Agius, E., Leys, L., Bhattacharyya, S., Grunz, H., Bouwmeester, T., and De Robertis, E. M. (1999) Nature 397, 707–710
20. Glinka, A., Wu, W., Delius, H., Monaghan, A. P., Blumenstock, C., and Niehrs, C. (1998) Science 275, 1557–1564
21. Sakanaka, C., Weiss, J. B., and Williams, L. T. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 8847–8851
22. Finch, P. W., He, X., Kelley, M. J., Uren, A., Schaudies, R. P., Popescu, N. C., Rudikoff, S., Aaronson, S. A., Varmus, H. E., and Rubin, J. S. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 6770–6775
23. He, X., Jean-Jeanet, J. P., Wang, Y., Nathans, J., and Varmus, H. E. (1997) Science 275, 2474–2485
24. Moret, J. P., Sparks, A. B., Korinek, V., Barker, N., Clevers, H., Vogelstein, B., and Kinzler, K. W. (1997) Science 275, 1787–1790
25. de la Coste, A., Romagnolo, B., Biliardt, P., Renard, C. A., Andrew, D., Nathans, J., and Nusse, R. (1996) Nature 382, 225–230
26. Leys, L., Bouwmeester, T., Kim, S. H., Piccolo, S., and De Robertis, E. M. (1997) Cell 88, 747–756
27. Wang, S., Krinks, M., Lin, K., Layten, F. P., and Moos, M. J. (1997) Cell 88, 757–766
28. Bouwmeester, T., Kim, S., Sasai, Y., Leys, L., and De Robertis, E. M. (1999) Nature 391, 595–601
29. Piccolo, S., Agius, E., Leys, L., Bhattacharyya, S., Grunz, H., Bouwmeester, T., and De Robertis, E. M. (1999) Nature 397, 707–710
30. Glinka, A., Wu, W., Delius, H., Monaghan, A. P., Blumenstock, C., and Niehrs, C. (1998) Science 275, 1557–1564
31. Alimandia, M., Romano, A., Curia, M. C., Muraro, R., Fedi, P., Aaronson, S. A., Di Fiore, P. P., and Kraus, M. H. (1995) Oncogene 10, 1813–1821
32. Miki, T., Fleming, T. P., Crescenzi, M., Malloy, C. J., Blum, S. B., Reynolds, S. H., and Aaronson, S. A. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 5167–5171
33. Miki, T., and Aaronson, S. A. (1995) Methods Enzymol. 254, 196–206
34. Kraus, M. H., and Aaronson, S. A. (1991) Methods Enzymol. 200, 546–556
35. Kraus, M. H., Fedi, P., Starks, V., Muraro, R., and Aaronson, S. A. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 2900–2904
36. Fuchs, P. W., Rubin, J. S., Miki, T., Ben, D., and Aaronson, S. A. (1989) Science 245, 752–755

2 A. Bafico and S. A. Aaronson, unpublished observations.
29. Fedi, P., Pierce, J. H., di Fiore, P. P., and Kraus, M. H. (1994) *Mol. Cell. Biol.* **14**, 492–500
30. Kozak, M. (1989) *J. Cell Biol.* **108**, 229–241
31. Kyte, J., and Doolittle, R. F. (1982) *J. Mol. Biol.* **157**, 105–132
32. Aravind, L., and Koonin, E. V. (1998) *Curr. Biol.* **8**, R477–R478
33. Glinka, A., Wu, W., Onichtchouk, D., Blumenstock, C., and Niehrs, C. (1997) *Nature* **389**, 517–519
34. Du, S. J., Purcell, S. M., Christian, J. L., McGrew, L. L., and Moon, R. T. (1995) *Mol. Cell. Biol.* **15**, 2625–2634
35. Torres, M. A., Yang-Snyder, J. A., Purcell, S. M., DeMarais, A. A., McGrew, L. L., and Moon, R. T. (1996) *J. Cell Biol.* **133**, 1125–1137
36. Bradbury, J. M., Niemeyer, C. C., Dale, T. C., and Edwards, P. A. (1994) *Oncogene* **9**, 2597–2603
37. Lin, K., Wang, S., Julius, M. A., Kitajewski, J., Moos, M., Jr., and Luyten, F. P. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94**, 11196–11200
38. Hsieh, J. C., Kodjabachian, L., Rebbert, M. L., Rattner, A., Smallwood, P. M., Samos, C. H., Nusse, R., Dawid, I. B., and Nathans, J. (1999) *Nature* **398**, 431–436