

## Sorting Nexin 1 Down-Regulation Promotes Colon Tumorigenesis

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**Abstract Purpose:** Colon cancer is one of the most common human malignancies, yet studies have only begun to identify the multiple mechanisms that underlie the development of this tumor. In this study, we have identified a novel mechanism, dysregulation of endocytic sorting, which promotes colon cancer development.

**Experimental Design:** Immunohistochemical and microarray analyses were done on human colon cancer tissue specimens to determine the levels of one endocytic protein, sorting nexin 1 (SNX1). SW480 cells, a human colon cancer cell line that retains a relatively high level of SNX1 expression, were used to assess the effects of down-regulating this protein by small hairpin RNA. Activation of signal transduction cascades was evaluated in these cells using Western blotting, and multiple functional assays were done.

**Results:** We determined by immunohistochemistry that the level of SNX1 was significantly down-regulated in 75% of human colon cancers. In corroborative studies using microarray analysis, SNX1 message was significantly decreased ( $\log_2$  ratio less than  $-1$ ) for 8 of 19 colon carcinomas. Cell lines with reduced SNX1 levels showed increased proliferation, decreased apoptosis, and decreased susceptibility to anoikis. They also showed increased activation of epidermal growth factor receptor and extracellular signal-regulated kinase 1/2 in response to epidermal growth factor. This increased activation was abolished by inhibition of endocytosis.

**Conclusions:** These data suggest that loss of SNX1 may play a significant role in the development and aggressiveness of human colon cancer, at least partially through the mechanism of increased signaling from endosomes. Further, these findings suggest that dysregulation of endocytic proteins may represent a new paradigm in the process of carcinogenesis.

Despite years of research, colon cancer remains a formidable clinical problem. For the year 2005, colon cancer is estimated to be the third most frequent cause of cancer deaths in both men and women in the United States (1). Most studies investigating the process of carcinogenesis center around signal transduction cascades and their downstream effects on such cellular processes as cell cycle regulation, apoptosis, and cell migration. One cellular process that has received almost no attention from cancer biologists is endocytic sorting. Receptors

internalized from the cell surface proceed first to the early endosome, where each receptor is directed either back to the surface to be reused or to the lysosome for degradation (2, 3). Moreover, the early endosome is itself a location from which activated receptors continue to signal (4–6). Proper receptor sorting at this level ensures appropriate numbers of receptors on the cell surface and accurate intensity of signaling from endosomes and thereby ensures the necessary balance among the signaling cascades downstream of these receptors. Conversely, dysregulation of receptor endocytosis may lead to altered receptor signaling and ultimately to cancer.

In support of this hypothesis, we have evidence that one endocytic protein, sorting nexin 1 (SNX1), is significantly down-regulated during colonic carcinogenesis. The sorting nexins comprise a large family of endocytic proteins that helps determine the fate of internalized receptors as these receptors reach the early endosome (7). Although the precise function of the sorting nexins is not entirely clear, SNX1 may target receptors to the lysosome for degradation (8). SNX1 has been shown to interact with multiple receptor tyrosine kinases [including the epidermal growth factor (EGF) receptor (EGFR), the platelet-derived growth factor receptor- $\beta$ , the insulin receptor, the transferrin receptor, and the long form of the leptin receptor (9)] and multiple receptor serine-threonine kinases (10).

We hypothesized that the decreased SNX1 in colon cancer results in the missorting of cell surface receptors, with consequent alterations in receptor signaling in these cells and

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promotion of tumorigenesis. Immunohistochemical staining of human colon cancers showed significant loss of SNX1 for 75% of the tumors. Microarray analysis of another 19 human colon cancers showed significant down-regulation of SNX1 mRNA in 8 of these 19 cases. Using small hairpin RNA (shRNA) depletion of SNX1 in SW480 cells, which retain SNX1 expression, we found that decreasing SNX1 led to increased proliferation, decreased apoptosis, and decreased anoikis. Moreover, reduction of SNX1 in these same cells resulted in increased EGFR phosphorylation and increased downstream signaling. This increase in mitogen-activated protein kinase (MAPK) signaling in response to EGF could be abolished by inhibition of endocytosis. This constellation of findings suggests that SNX1 plays a significant role in the development and manifestation of human colon cancer.

## Materials and Methods

**Human tissue specimens (immunohistochemistry samples).** Human normal and tumor tissue arrays were obtained (Biomed, Foster City, CA). Institutional Review Board approval was obtained to study archival human colon cancer sections. We selected blocks of formalin-fixed, paraffin-embedded colon carcinomas and normal colonic tissue from 20 recent colonic resections for tumor. The only criteria were that the cases be primary resections of invasive colonic adenocarcinomas with blocks available for staining. No further selection criteria were applied.

**Immunohistochemistry.** Staining was done on 5- $\mu$ m sections of paraffin-embedded human adenocarcinomas. Antigen retrieval was achieved by heating the slides in citrate buffer (pH 6.0; Biogenix, San Ramon, CA). The slides were incubated in 1  $\mu$ g/mL SNX1 antibody (Transduction Laboratories, Lexington, KY) for 45 minutes or in a 1:300 dilution of EGFR antibody (Cell Signaling Technology, Beverly, MA) overnight. The slides were washed and incubated in a biotinylated goat anti-mouse IgG or anti-rabbit IgG secondary antibody (1:200; Vector Laboratories, Burlingame, CA) followed by 3% peroxide block. After washing, the slides were developed using the avidin-biotin complex method/3,3'-diaminobenzidine detection kit (Ventana Medical Systems, Tucson, AZ) followed by methyl green counterstain (DAKO, Carpinteria, CA).

**Patients and tissue samples (microarray samples).** A total of 41 pairs of colorectal cancer tissues and corresponding adjacent noncancerous tissues was obtained from 41 patients with sporadic colorectal adenocarcinoma who underwent surgical resection at the J. Mendez Hospital and M.B. de Martinez Hospital (Buenos Aires, Argentina) between May 2003 and June 2004. Only specimens containing >75% tumor cells were used for analysis. Samples were macrodissected by pathologists and, within 30 minutes of removal, frozen in liquid nitrogen for molecular analysis.

**RNA extraction and cDNA preparation.** Total RNA was extracted from frozen samples using Trizol reagent (Invitrogen Life Technologies, Inc., Carlsbad, CA). Only 38 samples from our tumor bank that yielded high-quality RNA with minimal degradation and clear 28S/18S ribosomal bands were included in the analysis. Twenty-five tumors of different clinical stages, in addition to 13 normal mucosa specimens, were used for microarray experiments. Normal samples were pooled in equimolar amounts to generate a single standard reference RNA used as a control for each microarray hybridization. For half of the microarray hybridizations, 50  $\mu$ g of total RNA isolated from each tumor sample were retrotranscribed and labeled with Cy5-dCTP, and cDNA obtained from 50  $\mu$ g of the standard reference RNA was labeled with Cy3-dCTP; the fluorochromes were inverted in the second half of microarrays to reduce dye bias.

**Microarray hybridization and image acquisition.** Hybridization experiments were done on SS-Human 19Kv7 microarray (University

Health Network Microarray Centre, Toronto, Ontario, Canada). Equivalent amounts of Cy5-cDNA and Cy3-cDNA were combined and hybridized to microarray slides for 18 hours at 37°C. Hybridized slides were scanned using the GenePix 4000a Scanner (Axon Instruments, Foster City, CA), and images were acquired by GenePix Pro v5.0 software.

**Data analysis.** Twenty-five microarray data sets were sorted and loaded on ExpressConverter v1.5 to transform GenePix generated result files to the .mev file format for use with Microarray Data Analysis System v2.18 software. Microarray Data Analysis System was used to preprocess and normalize (within-slide LOWESS normalization) the raw microarray data. The microarray data have been uploaded to the Gene Expression Omnibus repository<sup>4</sup> under the accession number GSE3294.

**Statistical methods.** Using the log<sub>2</sub> ratios of selected genes, the reference list was generated by using Student's *t* statistics calculated on the full set of 21 patients. Significantly different expression between tumor and normal mucosa was estimated using a one-class *t* test; the *P* value of the gene was determined by forming a distribution based on 10,000 random permutations of the data. The *P* value for each gene was scaled by adjusted Bonferroni correction to correct for the large number of observations.

**Cell culture.** SW480, HT29, HCT116, HCT15, Moser, DLD-1, and SW48 human colon cancer cells were grown according to the American Type Culture Collection (Manassas, VA) recommended conditions.

**shRNA preparation.** Sequences to deplete SNX1 were modified from Gullapalli et al. (11) to insert into the pSuperRetro vector. The specific sequence used for the depletion (5'-ACTCTAGTCAACCATAGGA-3') was taken directly from SNX1 siRNA2 from Gullapalli et al. (11). A non-silencing control shRNA oligonucleotide was also obtained (Qiagen, Valencia, CA). Oligonucleotides were ligated into the pSuperRetro vector (OligoEngine, Seattle, WA). The constructs were transfected into SW480 cells. Puromycin (4  $\mu$ g/mL) was used to select multiple stable clones.

**Tritiated thymidine incorporation assay.** SW480 control and SNX1 shRNA-transfected cells were plated in triplicate. Cells were pulsed with 1  $\mu$ Ci/mL tritiated thymidine (Amersham Biosciences, Piscataway, NJ) for 3 hours, precipitated with 10% trichloroacetic acid, and solubilized with 0.5 N sodium hydroxide before quantitation with a scintillation counter.

**Western blotting.** Whole-cell lysates (10  $\mu$ g total protein) were immunoblotted using antibodies against SNX1 (Transduction Laboratories). Whole-cell lysates (5  $\mu$ g total protein) were also immunoblotted using antibodies against (Tyr<sup>1068</sup>) phosphorylated and total EGFR (Cell Signaling Technology, Danvers, MA), phosphorylated extracellular signal-regulated kinase (ERK) 1/2 (Sigma, St. Louis, MO), total ERK 1/2 (12), and  $\gamma$ -tubulin (Sigma).

**Active caspase-3 assay.** Sixteen hours after plating, both adherent and floating cells were collected, and protein lysates were prepared as described previously (13). Protein (100  $\mu$ g) was incubated with the fluorogenic caspase-3 substrate DEVD-AMC (BioMol, Plymouth Meeting, PA) for 1 hour, and enzymatic activity was determined as described previously (13). As a positive control for apoptosis, AML-12 human hepatocyte cells were pretreated for 30 minutes with actinomycin D (250 ng/mL) followed by 15-hour treatment with 20 ng/mL tumor necrosis factor (13).

**Anoikis assay.** The anoikis assay was modified from Frisch and Francis (14). SW480 cells were cultured for 24 hours in plates coated with poly-HEMA (Sigma) to prevent attachment. Nonadherent cells were collected, rinsed with PBS, and lysed. Lysates were quantitated for protein levels, and active caspase-3 was measured.

**Inhibition of endocytosis.** Endocytosis was reduced through either depletion of potassium or incubation in a hypertonic medium as used by Hansen et al. (15).

<sup>4</sup> <http://www.ncbi.nlm.nih.gov/projects/geo/>.

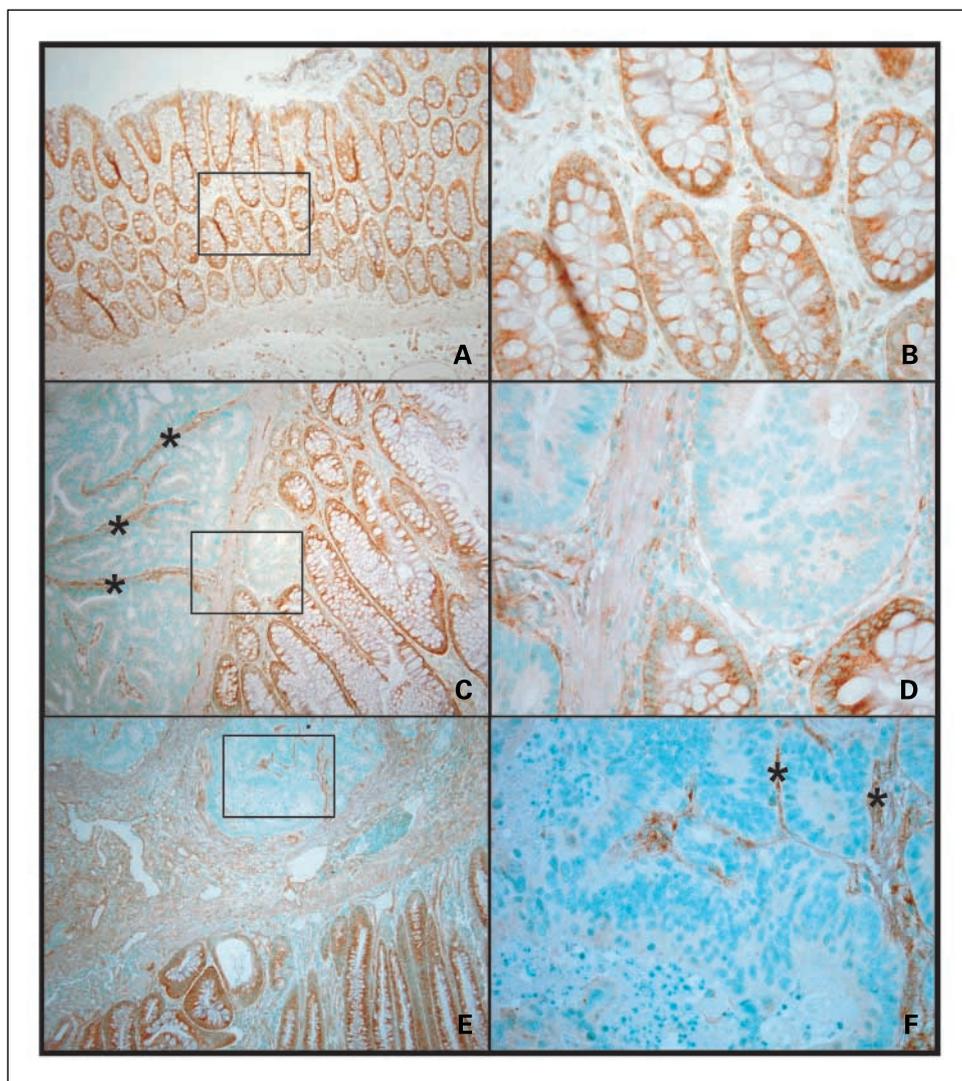
## Results

**SNX1 expression is down-regulated in human colon cancer.** To test the hypothesis that endocytic dysfunction plays a role in tumorigenesis, we used an antibody against SNX1 to immunostain both a normal and a tumor tissue array. Compared with normal colonic tissue, the two colon carcinomas on the array showed marked down-regulation in SNX1 expression (data not shown). To assess larger areas and increased numbers of normal and carcinomatous colon, we stained sections from 20 archival colon carcinomas and the adjacent normal colons for SNX1. The average age of these patients was  $66 \pm 12$  years (Supplementary Table S1). Thirteen of the patients were males and 7 were females. The majority of the tumors were moderately differentiated (13 of 20, 65%), and only one tumor was poorly differentiated (5%). The average tumor size was  $3.6 \pm 2.0$  cm. Lymph node metastases were present in 11 cases (55%) at the time of diagnosis.

The intensity of the SNX1 staining pattern was quantitated as described in Supplementary Table S2. Normal colonic epithelium shows a distinctly vesicular pattern when stained with SNX1 (as is expected for a protein that associates with the

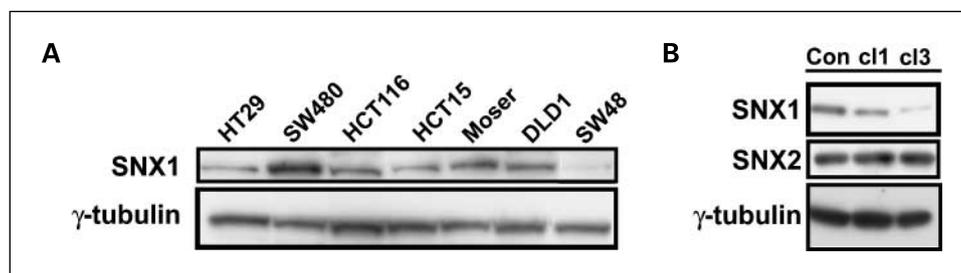
early endosome; Fig. 1A and B). Normal smooth muscle and vascular endothelium show only minimal staining. Reactive endothelial cells and macrophages stain strongly, with reactive stroma often outlining malignant glands (Fig. 1C-F, *asterisks*). Tumors (15 of 20, 75%) showed down-regulation of SNX1 staining (score of 0) when compared with the adjacent normal epithelium (Supplementary Table S1). Figure 1 shows two examples of this staining pattern, each example containing an internal control of normal colonic epithelium. In Fig. 1C, the left half of the image is composed entirely of malignant glands, whereas the right half contains normal epithelium (except for a single malignant gland that is apparent immediately adjacent to the epithelium). In Fig. 1E, normal colon is present along the bottom of the picture, whereas invasive tumor nodules are notable within the submucosa.

Analysis of the relationships in Supplementary Table S1 illustrates more significant findings. Decreased SNX1 immunoreactivity correlated strongly with younger age (the average age of the patients with a SNX1 score of 0 was 62, whereas the average age of the patients with a SNX1 score of  $\geq 1$  was 77;  $P < 0.01$ , two-tailed  $t$  test). Similarly, decreased immunoreactivity for SNX1 correlated with male sex of the patient ( $P = 0.03$ ,



**Fig. 1.** Loss of SNX1 staining in colon cancers. *A* and *B*, vesicular staining pattern of SNX1 in normal colonic epithelium. *C* to *F*, two separate colon cancers are represented. Normal epithelium shows vesicular brown cytoplasmic SNX1 staining. This staining is nearly entirely absent from the tumor tissue. *B*, *D*, and *F*, higher magnification views of the boxes in (*A*), (*C*), and (*E*), respectively. Asterisks, SNX1 staining of reactive stroma and activated endothelium. Original magnification,  $\times 100$  (*A*, *C*, and *E*) or  $\times 400$  (*B*, *D*, and *F*).

**Fig. 2.** Down-regulation of SNX1 in colon cancer cell lines. *A*, seven cell lines were assessed by Western blotting for their expression of SNX1. *B*, stable cell lines were generated using a construct to deplete SNX1 (*cl lanes*) and a nonsilencing control shRNA construct (*Con lanes*). Two clones were selected for further experiments: clones 1 and 3 (*cl1* and *cl3*). Western blotting was done to assess the expression of SNX1 and SNX2.  $\gamma$ -Tubulin was used as a loading control in all Western blotting.



Fisher's exact test). These two associations may suggest that decreased SNX1 immunoreactivity identifies a distinct subset of human colon carcinomas. No association was noted between SNX1 staining and tumor size or grade. No correlation was identified between SNX1 immunoreactivity and the presence of lymph node metastases.

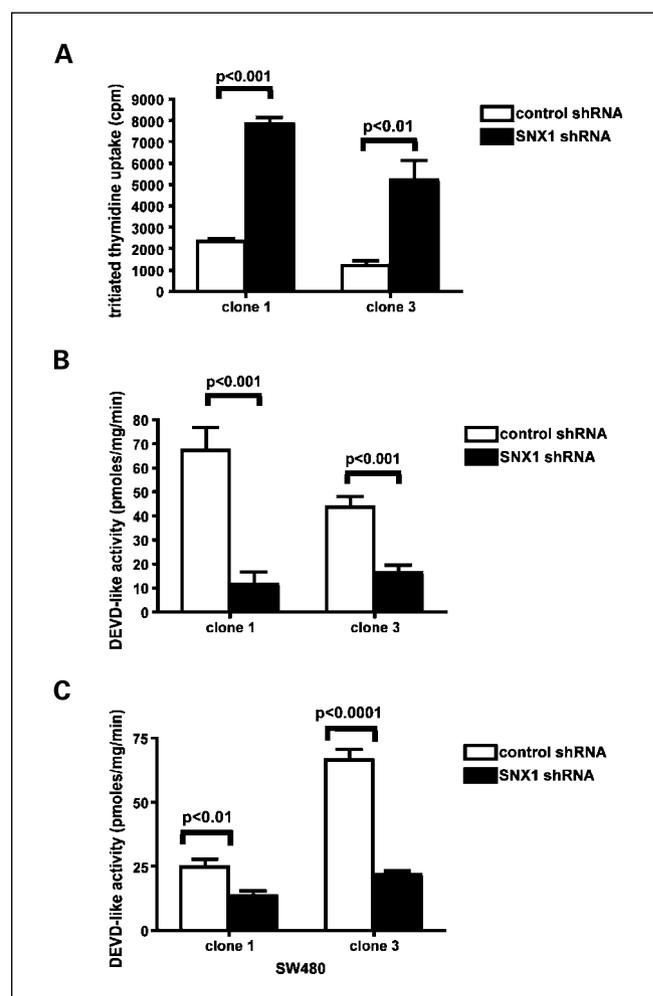
**Microarray analysis confirms SNX1 down-regulation in human colon cancer.** To confirm our immunohistochemical results using a different modality, we compared samples of human colon cancer with normal colonic epithelium by microarray analysis. Tumor SNX1 message expression was less than control for 18 of the 19 tumors. For 8 of these tumors, the  $\log_2$  ratio was less than  $-1$ . The average  $\log_2$  ratio was  $-0.876$ , which was significant at the level of  $P < 0.01$ .

The combination of these immunohistochemical and microarray data thus shows that down-regulation of SNX1 can be detected in a substantial subset of human colon carcinomas at the level of both transcription and protein expression. The detection of this down-regulation through two unrelated modalities argues strongly against the possibility of a technical artifact and instead documents the validity of these results in human colon tumors.

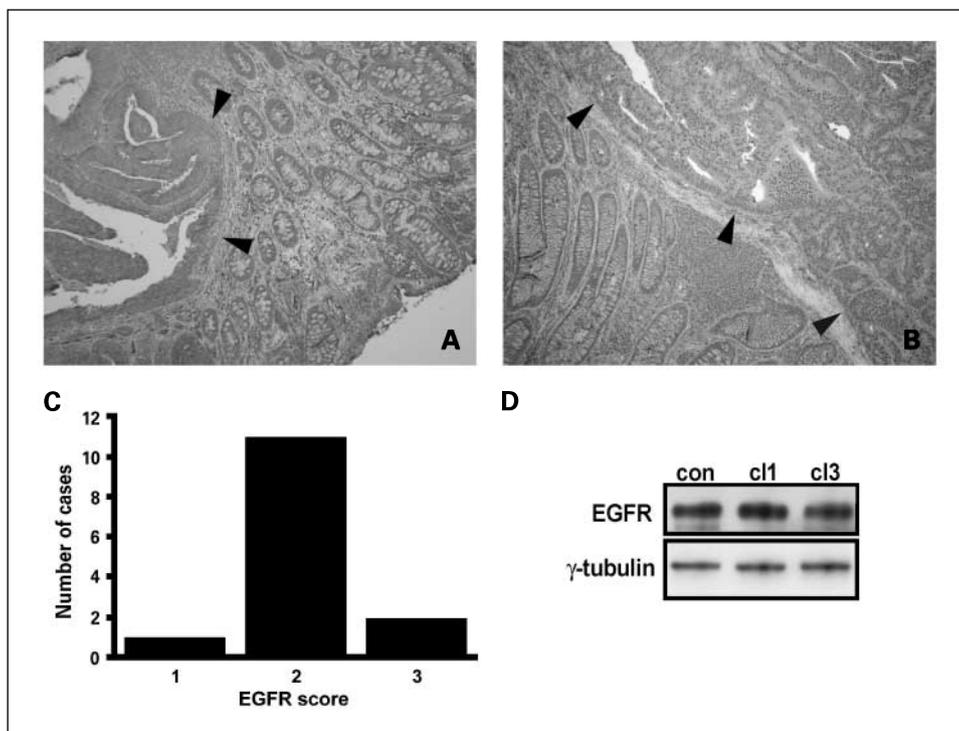
**shRNA constructs deplete SNX1 in human colon cancer cells.** Mere down-regulation of a protein within a tumor does not establish the significance of the protein for the development of the tumor. To test the hypothesis that down-regulation of SNX1 promotes colon tumorigenesis, we sought to determine the effects of SNX1 depletion on colon tumor cells. We screened seven colon cancer cell lines (SW480, HT29, HCT116, HCT15, Moser, DLD-1, and SW48) for endogenous SNX1 by Western blotting. We found that, of these cell lines, SW480 cells contain the highest level of SNX1 (Fig. 2A). HT29, HCT116, HCT15, Moser, and DLD-1 cells contain low but detectable levels of SNX1 (Fig. 2A). SW48 cells contain only minimal levels of SNX1 (Fig. 2A). Comparison with the results of a tumor cell line microarray (Gene Expression Omnibus accession GSD89) confirmed the SNX1 Western blotting (data not shown). We then chose to continue our experiments in SW480 cells using the only cell line that retained relatively high levels of SNX1 expression. SNX1 was sequenced in this cell line and was free of mutations (data not shown). Stable shRNA-mediated depletion of SNX1 was established, and two clones were selected for further experiments. Approximately 70% inhibition was apparent in SW480 clone 1, and SW480 clone 3 showed even greater inhibition ( $\sim 90\%$ ) compared with cells stably transfected with a control shRNA construct (Fig. 2B). Levels of SNX2, the most closely related sorting nexin to SNX1, were unchanged by the depletion of SNX1 (Fig. 2B).

**SNX1 down-regulated cells show increased proliferation, decreased apoptosis, and decreased anoikis.** To determine if

down-regulation of SNX1 affects the proliferation rate of these tumor cells, the SW480 cell lines were assessed for their rates of tritiated thymidine incorporation. The SW480 SNX1 shRNA clones both showed significantly increased rates of proliferation,



**Fig. 3.** Increased proliferation, decreased apoptosis, and decreased anoikis in SNX1-depleted cells. *A*, proliferation was assessed through the incorporation of tritiated thymidine. Representative studies from four separate experiments for clone 1 and two separate experiments for clone 3. *B*, apoptotic rates were assessed using a fluorescent assay to measure the activity of active caspase-3. Activity was calculated as picomoles of cleaved fluorescent substrate per milligram cell lysate protein per minute. Average results from three separate experiments for clone 1 and two separate experiments for clone 3. *C*, anoikis assay. The ability of the cells to survive in the absence of attachment was assessed. Poly-HEMA was used to prevent adherence of the cells to substrate, and active caspase-3 activity was quantitated after 24 hours. White columns, control shRNA cells; black columns, SNX1 shRNA cells. Bars, SE. Statistical significance was determined using the Student's *t* test (two tailed).



**Fig. 4.** No change in EGFR levels in colon cancers lacking SNX1. *A* and *B*, two separate colon cancers. Arrowheads, outline the regions of tissue involved by tumor. Original magnification,  $\times 100$ . *C*, distribution of EGFR staining scores for those tumors with a SNX1 score of 0 (no to minimal SNX1 staining). *D*, control shRNA cells (*Con* lane), clone 1 cells (*cl1* lane), and clone 3 cells (*cl3* lane) were assessed by Western blotting for their expression of the EGFR.

with clone 1 cells tripling and clone 3 cells more than quadrupling their rate of tritiated thymidine incorporation when compared with control shRNA cells (Fig. 3A).

Active caspase-3 assays were used to assess the steady-state rates of apoptosis of these same stable cell lines. Control cells had a moderately high basal rate of apoptosis, and these rates were substantially reduced in both the clone 1 and clone 3 SNX1-depleted cells (Fig. 3B).

To assess the effect of SNX1 reduction on the ability of these cells to survive in the absence of attachment to an underlying substrate, an anoikis assay was done. SW480 control cells had a moderately high basal rate of anoikis, and this rate was substantially reduced in both SNX1-depleted clones (Fig. 3C).

**Loss of SNX1 does not correlate with increased EGFR expression.** Because overexpression of SNX1 has been shown to result in increased degradation of the EGFR (8), we initially hypothesized that the corollary also holds true—that is, loss of SNX1 results in decreased receptor degradation and therefore increased receptor expression. To test this hypothesis, we stained the cohort of tumor samples with an antibody against the EGFR. The intensity of EGFR staining was scored according to the scoring criteria described in Supplementary Table S2. The results are detailed in Supplementary Table S1. For the majority of the tumors (12 of 17), the intensity of the EGFR staining was equal between the tumor and the surrounding normal epithelium (Supplementary Table S1). Figure 4A and B shows two representative sections of tumor and adjacent normal epithelium in which the staining patterns are comparable. Most significantly, the majority (11 of 14) of the tumors that showed marked down-regulation of SNX1 (a SNX1 score of 0) showed no change in EGFR levels (Fig. 4C). This finding suggests that down-regulation of SNX1 in human tumors typically does not lead to increased EGFR expression.

**shRNA-mediated knockdown of SNX1 leads to increased EGFR phosphorylation and increased ERK 1/2 signaling.** Our functional *in vitro* results (increased proliferation with decreased apoptosis and anoikis following down-regulation of SNX1) suggest that depletion of SNX1 increases the strength of EGF signaling. In our tumor samples, however, EGFR levels did not correlate with loss of SNX1. We similarly tested our SW480 SNX1-depleted cells, and they too showed no change in EGFR levels compared with control shRNA cells (Fig. 4D). We then hypothesized that reduction of SNX1 could still affect EGFR signaling in the absence of increased receptor levels by altering the dynamics of EGFR endosomal transport and signaling. To begin to test this hypothesis, we first assessed both EGFR phosphorylation and downstream signaling through the ERK 1/2 pathway. For both clone 1 (Fig. 5A) and clone 3 (Fig. 5B), reduced SNX1 levels resulted in increased EGFR phosphorylation. For confirmation, we used ERK 1/2 phosphorylation as a sensitive marker for activation of this receptor tyrosine kinase pathway. Cells with decreased SNX1 levels (both clone 1 and clone 3) had a more robust and prolonged activation of this kinase compared with cells stably expressing a control shRNA construct (Fig. 5C and D). For each clone, quantitation of the ratio of total ERK 1/2 to the loading control ( $\gamma$ -tubulin) showed a slightly higher level of total ERK 1/2 in the SNX1-depleted cells than in the control cells. This difference was far less than the difference in ERK phosphorylation between these cell lines, however.

**Reduction in endocytosis eliminates the differences in ERK 1/2 signaling.** To test more definitively the hypothesis that reduction of SNX1 alters the dynamics of EGFR endosomal transport and signaling, we used two methods, potassium depletion and use of a hypertonic medium (15), to minimize the endocytosis of EGF-stimulated receptors from the cell surface. We hypothesized that the downstream signaling we

then observed would derive from cell surface but not endosomally localized EGFRs. Because endosomally internalized EGFR has been shown to signal preferentially through MAPK/ERK cascades (16, 17), we further hypothesized that inhibition of endocytosis would both decrease overall ERK 1/2 activation and equalize the activation of the pathway between our cell lines. Both techniques decreased EGF-stimulated ERK 1/2 activation for both clones (Fig. 6A and B for hypertonic sucrose and Fig. 6C and D for potassium depletion). Moreover, both techniques also eliminated the increased EGF-stimulated ERK 1/2 activation previously observed. Following inhibition of endocytosis, both clones showed either equal or lesser ERK 1/2 activation than control cells.

### Discussion

Among the most studied mechanisms underlying the process of colon carcinogenesis are those affecting signaling cascades, especially the Wnt, transforming growth factor- $\beta$ , ras, and EGF pathways (18, 19). Dysregulation of these pathways can occur through several different mechanisms, including inactivating mutations (transforming growth factor- $\beta$  pathway), activating mutations (Wnt and ras pathways), and increased surface receptor levels (EGF pathway; refs. 18, 19). Alternatively, changes in more general cellular processes, such as endocytosis, which enhance receptor signaling pathways, may also facilitate carcinogenesis. Here, we postulate that dysregulation of endocytic sorting by loss of SNX1 contributes to colon cancer.

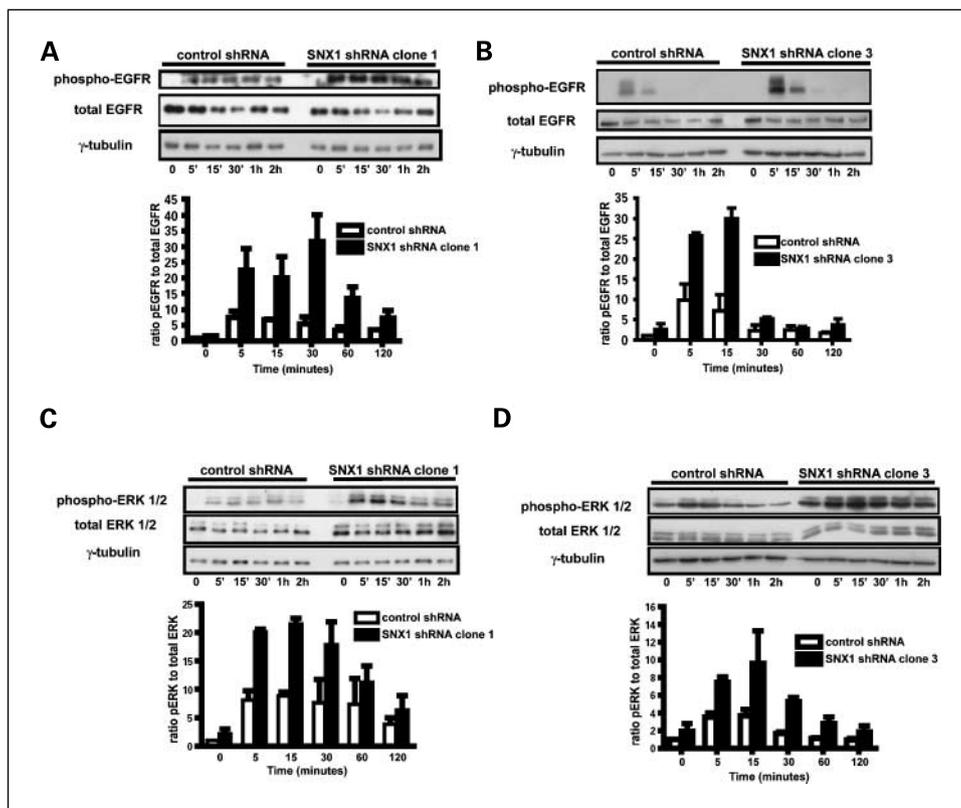
Our evidence strongly suggests that down-regulation of SNX1 is a significant factor in the development of the majority of human colon cancers. We found that 75% of these tumors showed immunohistochemical evidence of significant SNX1

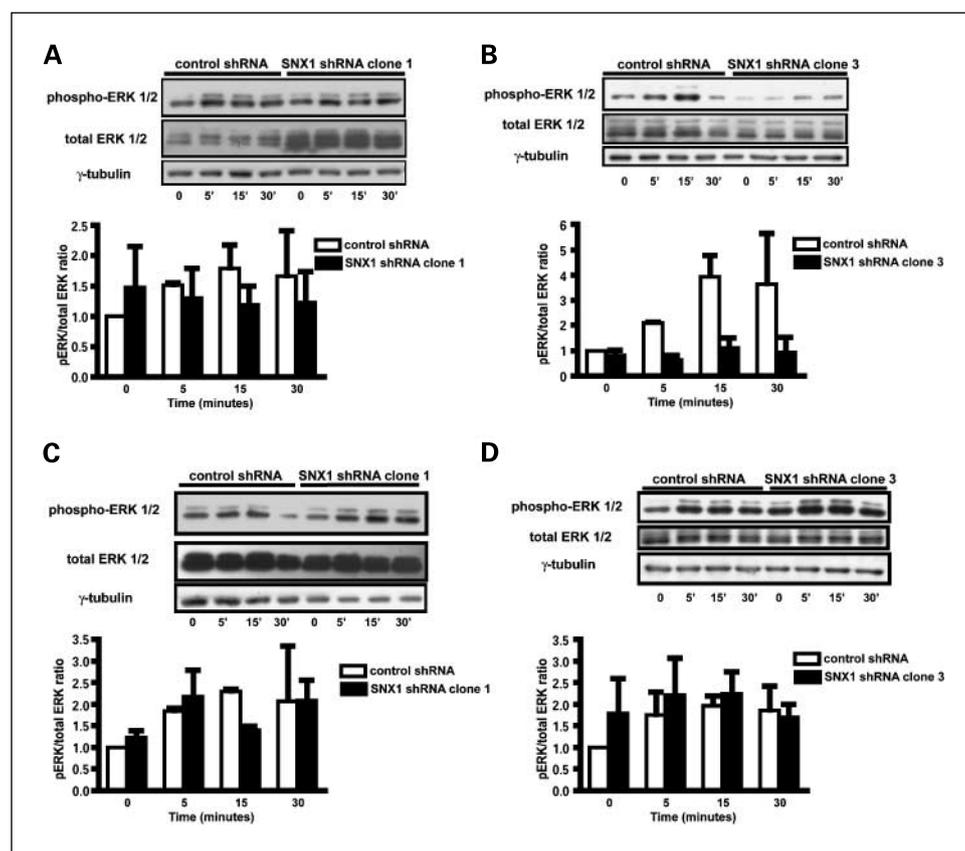
reduction (Fig. 1). We then confirmed these immunohistochemical results with microarray data that also showed significant down-regulation of SNX1 mRNA in at least 8 of 19 human colon cancers. When human colon cancer cells retaining SNX1 expression were depleted of this protein, they showed multiple alterations, suggesting that they had developed a more aggressive phenotype. Down-regulation of SNX1 both increased proliferation (Fig. 3A) and decreased apoptosis (Fig. 3B) in these tumor cells. In addition, decreased SNX1 protein resulted in decreased susceptibility to anoikis (Fig. 3C). Taken together, these changes *in vivo* likely synergize to increase the rate of tumor growth and may promote the ability of these tumor cells to invade and metastasize.

Overall, our results strongly suggest that down-regulation of SNX1 is a significant factor in the development and phenotypic expression of human colon cancer, and they further suggest that defects in endocytic sorting may represent an emerging mechanism underlying the process of carcinogenesis. For example, one proto-oncogene, Cbl, whose function has been unknown until recently, has been found to regulate endocytic sorting. The Cbl proto-oncogene has been shown to control the sorting of endocytosed receptors through monoubiquitination (20–22). Activating mutations of Cbl, which alter the endocytic pathways taken by internalized receptors, are oncogenic.

Considerable evidence has developed recently suggesting that endocytic processes directly regulate signaling pathways (4, 5). Signaling is modulated when changes in receptor trafficking alter one of two cellular properties. The first is cell surface receptor levels; increased receptor levels, in the presence of the appropriate ligand, lead to increased signaling. Because overexpression of SNX1 has previously been shown to increase the degradation of the EGFR (8), we initially hypothesized that our

**Fig. 5.** Increased EGFR phosphorylation and ERK 1/2 activation in SNX1-depleted cells. *A* and *B*, control shRNA and clone 1 (*A*) or clone 3 (*B*) cells were treated for the indicated times with 20 ng/mL EGF. Activation of EGFR was assessed using a phosphorylation-specific antibody against Tyr<sup>1068</sup>, which becomes a docking site for growth factor receptor binding protein 2 when phosphorylated and thereby promotes activation of the MAPK/ERK pathway. *C* and *D*, control shRNA and clone 1 (*C*) or clone 3 (*D*) cells were treated for the indicated times with 20 ng/mL EGF. Activation of ERK 1/2 was assessed using phosphorylation-specific antibodies.  $\gamma$ -Tubulin was used as a loading control for all Western blotting. The intensity of the Western blot signals was quantitated using ImageJ software. White columns, control shRNA cells; black columns, SNX1 shRNA cells. Columns, average results from two independent experiments for each clone; bars, SE.





**Fig. 6.** Equalization of ERK 1/2 activation following inhibition of endocytosis. Hypertonic sucrose medium (*A* and *B*) or depletion of potassium (*C* and *D*) was used to inhibit endocytosis following EGF stimulation. Control shRNA and clone 1 (*A* and *C*) or clone 3 (*B* and *D*) cells were treated for the indicated times with 20 ng/mL EGF. Activation of ERK 1/2 was assessed using phosphorylation-specific antibodies.  $\gamma$ -Tubulin was used as a loading control for all Western blotting. The intensity of the Western blot signals was quantitated using ImageJ software. White columns, control shRNA cells; black columns, SNX1 shRNA cells. Columns, average results from two independent experiments for each clone; bars, SE.

observation of a loss of SNX1 on colon tumors would correlate with an increase in EGFR expression. We tested this hypothesis by staining our colon cancer sections for EGFR expression. We saw increased EGFR expression in 4 of 17 (24%) of the tumors that we stained, a percentage on par with recently reported results for EGFR expression (23). When we particularly examined the 14 of these cancers that showed down-regulation of SNX1, EGFR expression was increased only for 2 (Fig. 4C). Although immunohistochemistry is only a crude measure of surface receptor levels, these data accord with our *in vitro* findings in our SNX1-depleted cells (Fig. 4D) and with previous *in vitro* studies in HeLa cells finding no change in EGFR levels with SNX1 down-regulation (11, 24). In aggregate, these results suggest that down-regulation of SNX1 increases EGFR signaling through a mechanism independent of increased surface EGFR expression.

The second property that endocytic sorting can modulate to influence signaling is the degree of signaling that takes place on endocytic vesicles. It is now widely accepted that signaling occurs not only from cell surface receptors but also from activated receptors after they have been endocytosed (4–6). Changes in endocytic trafficking may affect signaling (and therefore tumorigenesis) not only by altering cell surface receptor levels but also by modifying the transit of these proteins through the cytoplasm, with resulting modulation in their intracytoplasmic

signaling characteristics, independent of the number of surface receptors. That is, cells with the same number of cell surface receptors may show different signal fluxes for a given ligand due to differences in the degree of endosomal signaling. Our studies clearly show that down-regulation of SNX1 leads to increased EGFR phosphorylation (Fig. 5A and B) and increased ERK 1/2 signaling (Fig. 5C and D) in response to EGF. To test the significance of the signaling from endosomes, we inhibited endocytosis in our cell lines. Limitation of EGF stimulation to cell surface receptors reduced overall ERK 1/2 signaling and specifically equalized ERK 1/2 signaling between our control and SNX1-depleted cells (Fig. 6), suggesting that the previously observed increase in ERK 1/2 activation in SNX1-depleted cells was derived from increased endosomal EGFR signaling.

In summary, we have identified SNX1 as a protein markedly down-regulated in the majority of human colon cancers. shRNA-mediated depletion of this protein from a human colon cancer cell line results in a more aggressive tumor phenotype, including increased proliferation with decreased susceptibility to apoptosis and anoikis. These data suggest that loss of SNX1 may play a significant role in the development and manifestation of human colon cancer, and they further suggest that dysregulation of endocytic proteins may represent a new paradigm in the process of carcinogenesis.

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