Phosphorylation of the src Epithelial Substrate Trask Is Tightly Regulated in Normal Epithelia but Widespread in Many Human Epithelial Cancers

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Abstract

Purpose: The frequently elevated activities of the c-src and c-yes products in human epithelial tumors suggest that these activated tyrosine kinases have tumorigenic functions analogous to the v-src and v-yes oncogene products. Studies of v-src – transformed fibroblasts have identified many of the effectors of this potent oncogene; however, because c-src and c-yes lack the mutational and promiscuous activities of their retroviral oncogene homologues, their presumptive tumorigenic functions in human epithelial tumors are more subtle, less well-defined, and await identification of possible effectors more directly relevant to epithelial cells.

Experimental Design: We recently identified a transmembrane glycoprotein named Trask that is expressed in epithelial tissues but not fibroblasts and is phosphorylated by SRC kinases in mitotic epithelial cells. In this study, we have surveyed the expression and phosphorylation of Trask in many human epithelial cancer cell lines and surgical tissues and tumors.

Results: Trask is widely expressed in human epithelial tissues, but its phosphorylation is tightly regulated and restricted to detached mitotic cells or cells undergoing physiologic shedding. However, aberrant Trask phosphorylation is seen in many epithelial tumors from all stages including preinvasive, invasive, and metastatic tumors. Trask phosphorylation requires SRC kinases, and is also aberrantly hyperphosphorylated in the SRC-activated PyMT mouse epithelial tumors and dephosphorylated by the SRC inhibitor treatment of these tumors.

Conclusions: The widespread phosphorylation of Trask in many human epithelial cancers identifies a new potential effector of SRC kinases in human epithelial tumorigenesis.

The oncogenic potential of Src kinases has been recognized for more than three decades stemming from the identification of the v-src tyrosine kinase oncogene as the tumorigenic driver of the Rous sarcoma virus (reviewed in ref. 1). v-src or engineered activating mutants of c-src are highly transforming in experimental models (2, 3). Although the mutational activation of Src kinases is extremely rare in human tumors, Src and Yes show increased activity in many human epithelial cancers including cancers of the colon, breast, pancreas, and lung (reviewed in ref. 4). This is recapitulated in mouse models of epithelial cancer where Src and Yes kinases are activated in PyMT-induced and in Neu-induced mammary epithelial tumors (5, 6). The essential role of Src in the PyMT model is confirmed as these tumors are suppressed in a src-null background (7). However, the mechanisms that lead to the activation of Src kinases in human tumors remain undefined, and an essential role for Src kinases in human cancers remains presumptive at this point and awaits further definition.

Numerous lines of evidence suggest that the function of Src kinases may be particularly important for invasion and metastasis in human cancers. The increased invasive and metastatic properties of ErbB2-induced epithelial tumors are associated with increased expression and activity of Src and this can be reverted by pharmacologic inhibitors or dominant-negative mutants of Src kinase (8). Increased invasive properties are similarly conferred to intestinal epithelial cells by overexpression of c-src (9). Treatment of cancer cells with Src-selective inhibitors reduces their invasive and migratory properties with much less effect on their proliferative attributes (10, 11). In mouse orthotopic models of human epithelial cancer, the inactivation of Src kinases identifies a function more important to invasion and metastasis than proliferative growth (12, 13). Cellular substrates of Src that are thought to mediate the invasive and migratory properties conferred by overactive Src kinases are highly expressed in epithelial tissues but their phosphorylation is tightly regulated and restricted to detached mitotic cells or cells undergoing physiologic shedding. However, aberrant Trask phosphorylation is seen in many epithelial tumors from all stages including preinvasive, invasive, and metastatic tumors. Trask phosphorylation requires SRC kinases, and is also aberrantly hyperphosphorylated in the SRC-activated PyMT mouse epithelial tumors and dephosphorylated by the SRC inhibitor treatment of these tumors.

Conclusions: The widespread phosphorylation of Trask in many human epithelial cancers identifies a new potential effector of SRC kinases in human epithelial tumorigenesis.
Src kinases in epithelial tumors not appreciated from fibroblast cell models may provide mechanistic insight into the role of cell detachment and migration. As such, experimental epithelialized mechanisms are most likely involved to allow epithelial require dissociation of these and other structures and special-

include adhesion signaling proteins including certain integrins, focal adhesion complex proteins, and certain extracellular and membrane proteases (reviewed in ref. 14). Much of this evidence comes from the analysis of cellular changes induced by the highly transforming v-src oncogene product.

Although the evidence that Src and Yes are functionally important in human tumors is compelling, mechanistic exploration of this function has proven to be complex and challenging. A large body of evidence has been derived from mechanistic studies of cell transformation by v-src in fibroblast models (reviewed in refs. 14, 15). However, the v-src oncogene product has considerable structural and functional differences from the c-src and c-yes gene products of human tumors and much more potent transforming activity compared with the human proto-oncogenes. In fact, c-src is not transforming, even when overexpressed, and its mutational activation is very rarely seen in human cancers (reviewed in ref. 16). Therefore, the tumor promoting functions of c-src and c-yes may be much more subtle than their viral oncogene homologues. In addition, the fibroblast models do not faithfully represent the full spectrum of human cancers. In fact, the increased activity of Src kinases is mostly seen in the very common human epithelial cancers as well as the much rarer mesenchymal cancers. Trask phosphorylation is uniquely linked with Src inhibitor suppressible protein in detached mitotic epithelial cells (17). Trask is a substrate of Src and Yes kinases and membrane proteases (reviewed in ref. 14). Much of this evidence comes from the analysis of cellular changes induced by the highly transforming v-src oncogene product.

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Materials and Methods

Cell culture and reagents. All cell lines were obtained from the American Type Culture Collection. Cells were grown in a 1:1 mixture of DMEM:F12 media supplemented with 10% heat-inactivated fetal bovine serum, 100 U/mL penicillin, 100 μg/mL streptomycin, and 4 mM L-glutamine, and incubated at 37 °C in 5% CO2. MCF10A cells were grown in DMEM:F12 media supplemented with 5% donor horse serum, 0.5 μg/mL hydrocortisone, 10 μg/mL insulin, 20 ng/mL epidermal growth factor, 100 U/mL penicillin, 100 μg/mL streptomycin, and 4 mM L-glutamine. To force cells into suspension, cells were washed in PBS and exposed to a 0.05% solution of trypsin or a 2 mM/L solution of EDTA in Hank's buffer. When required to maintain cells in suspension and prevent spreading and attachment, cells were spun
down, resuspended in growth media, and cultured in ULC plates (Corning) for growth. To harvest lysates, cells were rapidly scraped on ice at fixed time points and lysed in radioimmunoprecipitation assay buffer. Antiphosphotyrosine antibodies (PY99) were purchased from SantaCruz Biotechnology, Inc. Polyclonal anti-Trask antibodies were generated by immunizing rabbits with a recombinant full-length Trask intracellular domain. Monoclonal anti-Trask antibodies were generated by immunizing mice with a recombinant full-length Trask extracellular domain and recognize both cleaved and uncleaved forms of Trask. Anti–phospho-Trask antibodies were generated against a phospho-peptide immunogen containing sequences centered around phosphorylated tyrosine 743 of Trask in rabbits and affinity purified on a phospho-peptide column. PP1 was purchased from EMD-Calbiochem. Dasatinib was purchased from Bristol-Myers Squibb and purified for use (see Supplementary Methods). pcDNA4-MycTrask was constructed as previously described (17). Total cellular lysates were harvested in modified radioimmunoprecipitation assay buffer [10 mmol/L Na phosphate (pH 7.2), 150 mmol/L NaCl, 0.1% SDS, 1% NP40, 1% Na deoxycholate, protease inhibitors, and 1 mmol/L sodium orthovanadate]. For Western blotting, 50 μg of each lysate were separated by SDS-PAGE, transferred to membrane, and immunoblotted using appropriate primary and secondary antibodies and enhanced chemiluminescence visualization. Immunoprecipitations were done by incubating 300 μg of cellular lysate with the indicated antibodies overnight at 4°C. Immune complexes were precipitated by incubation with protein-G sepharose beads, washed several times in radioimmunoprecipitation assay buffer, and boiled in sample buffer.

**Immunohistochemical studies.** Deparaffinized sections were hydrated and antigen retrieval was done by 15-min incubation in warm trypsin followed by microwave in 10 mmol/L citrate buffer for total of 10 min in 1-min intervals. Slides were then washed and blocked with 3% H2O2 followed by blocking in goat serum and primary incubation at 4°C overnight. Secondary staining was done using biotinylated goat anti-rabbit antibodies (Vector Laboratories) and colorized using Vectastain ABC kit (Vector Laboratories) and 3,3′-diaminobenzidine -H2O2 substrate (Sigma). Slides were then counterstained with hematoxylin, dehydrated through graded alcohols and xylene, and mounted. Slides were studied and imaged under brightfield microscopy. All staining procedures included positive and negative controls. Controls were prepared from formalin-fixed paraffin-embedded cell buttons of cell lines with well-defined expression and phosphorylation of Trask by immunoblotting techniques. For Trask immunostains, the positive control was MDA-MB-468 cells and the negative control was MCF-7 cells. For phospho-Trask immunostains, the positive control was MDA-MB-468 cells and the negative controls were MCF-7 cells and dasatinib-treated MDA-MB-468 cells. MDA-468 cells have abundant expression and phosphorylation of Trask by Western blotting. MCF-7 cells have no expression of Trask by Western blotting or by reverse

Fig. 1. The expression and phosphorylation of Trask were determined and compared in a diverse panel of epithelial cancer cell lines. Total cell lysates were immunoprecipitated with rabbit anti-Trask antibodies (R6674) and immunoblotted with mouse anti-Trask antibodies (M19) or anti-phosphotyrosine antibodies. The MDA-231 cell lysate was included in all blots as a basis to allow comparison of Trask expression and phosphorylation across different blots. The MCF10A cell lysate is also included at the far right as an example of untransformed epithelial cells. Arrows, 140- and 85-kDa forms of Trask. IP, immunoprecipitation; IB, immunoblot.

Fig. 2. A, the HCT116 and DLD-1 (colon cancer), MiaPaCa2 (pancreatic cancer), and PC3 (prostate cancer) epithelial cancer cells were harvested either while adherent (A) or 2 h after being forced into suspension by EDTA (S). Cell lysates were analyzed for the expression and phosphorylation of Trask as indicated. Arrows, 140- and 85-kDa forms of Trask. B, the expression and phosphorylation of Trask was determined and compared in L3.6pl-luc pancreatic cancer cells under different in vitro and in vivo circumstances. These L3.6pl-luc cells express luciferase for use in in vivo imaging. All immunoprecipitates are from equal amounts of cell lysate. Lane 1 and 2, correspond to lysates from adherent and suspended cells, respectively, growing in vitro. These cells were also grown orthotopically within the pancreas of nude mice and their growth monitored by in vivo imaging as described in Materials and Methods. Well-established pancreatic tumors from two different sacrificed mice were harvested, snap frozen, and their lysates used in lanes 3 and 4. A pentoncal metastasis from one of the mice was removed and lysate used in lane 5. These data comparing the same cancer cells in vitro and in vivo show that despite the more restricted phosphorylation of Trask in the in vitro growth model, their tumorigenic growth in vivo is characterized by maximal and constitutive phosphorylation of Trask.

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transcription-PCR. Staining intensity was scored and agreed upon by two investigators according to the following definitions: 0 indicates no visible expression (similar to negative controls); 1+ indicates expression that is faint but is identifiable and above background and negative controls; 2+ indicates moderate expression that is clearly evident but also clearly less intense than positive controls; 3+ indicates intense expression similar to positive controls. Only intensity was used for scoring. Percentages of cells were not a parameter used for scoring because Trask phosphorylation has focal characteristics in tissues that is relevant to cell behavior, and some degree of focal variability may also be due to uneven fixation artifacts. Immunohistochemically stained tissue sections were viewed and imaged using an Olympus BX41 brightfield microscope fitted with a DP70 digital camera. Images were acquired using the Olympus DP Controller software and y adjusted for optimal representation.

Animal studies. Animals were handled according to protocols approved by the University of California, San Francisco, Institutional Animal Care and Use Committee. Ex vivo infection of mouse mammary epithelial cells was done as previously described (23). Briefly, primary mammary epithelial cells were harvested from 10- to 12-wk-old donor mice and infected in vitro with pMIG-PyMT on 2 successive days and transplanted into cleared mammary fat pads of 3-wk-old recipient mice. Tumors developed in ~3 mo. Tumor-bearing mice were treated with dasatinib or vehicle control administered by Alzet Osmotic Pumps at a rate of 0.4 mg/d of dasatinib. Mice were sacrificed and tumors rapidly dissected and immediately fixed in formalin for immunohistochemical studies or snap frozen for Western blotting studies. Pancreatic orthotopic models were generated as follows. The mouse pancreas was surgically exposed through an abdominal excision under anesthesia, and 250,000 luciferase-expressing L3.6pl tumor cells implanted directly into the pancreas, and the abdomen closed. Tumors were visible by in vivo imaging within 2 to 3 wk and grossly palpable by 4 to 5 wk with a very high take-rate at which time the mice were euthanized and tumors rapidly excised and snap frozen for Western blotting.

Results

Trask expression and frequent phosphorylation in human cancer cell lines. The expression and phosphorylation of Trask was

<table>
<thead>
<tr>
<th>Case</th>
<th>Tissue</th>
<th>Disease subtype</th>
<th>Trask score</th>
<th>P-trask score</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Breast</td>
<td>Normal</td>
<td>2+</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>Breast</td>
<td>Normal</td>
<td>2+</td>
<td>0</td>
</tr>
<tr>
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<td>Normal</td>
<td>2+</td>
<td>0</td>
</tr>
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</tr>
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<td>2+</td>
<td>0</td>
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<td>Breast</td>
<td>Normal</td>
<td>2+</td>
<td>0</td>
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<td>Breast</td>
<td>Normal</td>
<td>2+</td>
<td>0</td>
</tr>
<tr>
<td>8</td>
<td>Breast</td>
<td>Normal</td>
<td>2+</td>
<td>0</td>
</tr>
<tr>
<td>9</td>
<td>Breast</td>
<td>Normal</td>
<td>2+</td>
<td>2+ some areas, hyperplasia</td>
</tr>
<tr>
<td>10</td>
<td>Breast</td>
<td>LCIS</td>
<td>0/1+</td>
<td>No tumor but 2+ in nl&amp;ADH</td>
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<tr>
<td>11</td>
<td>Breast</td>
<td>DCIS+LCIS</td>
<td>2+ LCIS, no DCIS</td>
<td>3+ LCIS, also 3+ col hyp, also in nl</td>
</tr>
<tr>
<td>12</td>
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<td>1+ DCIS, 1+ LCIS</td>
<td>0</td>
</tr>
<tr>
<td>13</td>
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<td>DCIS+IDC+ILC</td>
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<td>0 DCIS, 0 IDC, no tumor in LN</td>
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<td>14</td>
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</tr>
<tr>
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</tr>
<tr>
<td>16</td>
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</tr>
<tr>
<td>17</td>
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<td>2+ IDC</td>
<td>2+ IDC (in emboli)</td>
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<tr>
<td>18</td>
<td>Breast</td>
<td>IDC+DCIS</td>
<td>1+ DCIS, 1+ IDC</td>
<td>2+ DCIS (basal layer), 0 IDC</td>
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<tr>
<td>19</td>
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<td>3+ DCIS (focal), 3+ IDC, 0 LN met</td>
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<td>3+ DCIS, 2+ LCIS, 2+ IDC</td>
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<td>1+ LCIS, 1+ IDC</td>
<td>2+ IDC (invading), 0 LCIS</td>
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<td>0</td>
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<tr>
<td>23</td>
<td>Breast</td>
<td>ILC+LCIS</td>
<td>2+ LCIS, 2+ IDC, 2+ LN met</td>
<td>3+ DCIS, 2+ LCIS, 3+ IDC, 0 LN met</td>
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<td>24</td>
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<td>IDC+ILC</td>
<td>0 LCIS, 1+ IDC</td>
<td>2+ in migrating ILC, 2+ IDC</td>
</tr>
<tr>
<td>25</td>
<td>Breast</td>
<td>IDC</td>
<td>2+</td>
<td>0</td>
</tr>
<tr>
<td>26</td>
<td>Breast</td>
<td>IDC</td>
<td>3+</td>
<td>2+</td>
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<tr>
<td>27</td>
<td>Breast</td>
<td>LN mets</td>
<td>0/1+</td>
<td>2+ LN met</td>
</tr>
<tr>
<td>28</td>
<td>Breast</td>
<td>LN mets</td>
<td>1+</td>
<td>Very rare 2+</td>
</tr>
<tr>
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<td>Breast</td>
<td>Liver mets</td>
<td>2/3+</td>
<td>1+</td>
</tr>
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<td>Breast</td>
<td>Liver mets</td>
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<td>3+</td>
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<tr>
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<td>Breast</td>
<td>Lung met</td>
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<td>3+</td>
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<tr>
<td>32</td>
<td>Breast</td>
<td>Liver mets</td>
<td>3+</td>
<td>Insuff. spec</td>
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<tr>
<td>33</td>
<td>Breast</td>
<td>Liver mets</td>
<td>3+</td>
<td>0</td>
</tr>
<tr>
<td>34</td>
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<td>Liver mets</td>
<td>3+</td>
<td>0</td>
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<tr>
<td>35</td>
<td>Breast</td>
<td>Liver mets</td>
<td>3+</td>
<td>0</td>
</tr>
<tr>
<td>36</td>
<td>Breast</td>
<td>Liver mets</td>
<td>3+</td>
<td>0</td>
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</tbody>
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NOTE. Sections from the indicated archival tumor tissue sources were prepared and stained with anti-Trask and anti–phospho-Trask antibodies. The staining intensity was scored by two observers according to procedures and definitions described in Materials and Methods. All immunostains and their analyses were conducted using well-defined positive and negative controls as described in Materials and Methods. Abbreviations: LCIS, lobular carcinoma in situ; DCIS, ductal carcinoma in situ; IDC, infiltrating ductal cancer; ILC, infiltrating lobular cancer; LN, lymph node; met, metastasis.

*Luminal > basal.
surveyed in a panel of human epithelial cancer cell lines (Fig. 1). Trask is widely expressed in most of these cancer cell lines, although some lung cancer cell lines lack expression of Trask. Interestingly, many cancer cell lines show aberrant phosphorylation of Trask, some at lower levels (such as MDA-231 or A549), and some at high levels (such as MDA-468 or Colo205). Cancer cell lines vary in their morphologic characteristics in tissue culture. Interestingly, we found a correlation between Trask phosphorylation and the spread or suspended morphology of cells. Certain epithelial cancer cells, which, when cultured in vitro, grow naturally in suspension or in semi-suspension show high phosphorylation of Trask (examples:

### Table 2.

<table>
<thead>
<tr>
<th>Case</th>
<th>Tissue</th>
<th>Disease subtype</th>
<th>Trask score</th>
<th>P-trask score</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>Colon</td>
<td>Normal</td>
<td>2+**</td>
<td>0 crypts (3+ mitoses) /2+ apex</td>
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<tr>
<td>2</td>
<td>Colon</td>
<td>Normal</td>
<td>2+**</td>
<td>0 crypts (3+ mitoses)</td>
</tr>
<tr>
<td>3</td>
<td>Colon</td>
<td>Normal</td>
<td>2+**</td>
<td>0/1 + in crypts/2+ apex</td>
</tr>
<tr>
<td>4</td>
<td>Colon</td>
<td>Normal</td>
<td>2+**</td>
<td>0 crypts (3+ mitoses) /2+ apex</td>
</tr>
<tr>
<td>5</td>
<td>Colon</td>
<td>Normal</td>
<td>2+**</td>
<td>0 crypts/some 2+ apex</td>
</tr>
<tr>
<td>6</td>
<td>Colon</td>
<td>Normal</td>
<td>2+**</td>
<td>0 crypts/1+ apex</td>
</tr>
<tr>
<td>7</td>
<td>Colon</td>
<td>Normal</td>
<td>2+**</td>
<td>0 crypts/2+ apex</td>
</tr>
<tr>
<td>8</td>
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<td>2+**</td>
<td>0 crypts/some 2+ apex</td>
</tr>
<tr>
<td>9</td>
<td>Colon</td>
<td>Normal</td>
<td>2+**</td>
<td>0 crypts (3+ mitoses) /2+ apex</td>
</tr>
<tr>
<td>10</td>
<td>Colon</td>
<td>Normal</td>
<td>2+**</td>
<td>0 crypts/2+ apex</td>
</tr>
<tr>
<td>11</td>
<td>Colon</td>
<td>Normal</td>
<td>2+**</td>
<td>0 crypts/2+ apex</td>
</tr>
<tr>
<td>12</td>
<td>Colon</td>
<td>Normal</td>
<td>2+**</td>
<td>0 crypts/2+ apex</td>
</tr>
<tr>
<td>13</td>
<td>Colon</td>
<td>Normal</td>
<td>2+**</td>
<td>0 crypts/2+ apex</td>
</tr>
<tr>
<td>14</td>
<td>Colon</td>
<td>FAP</td>
<td>3+ patchy</td>
<td>3+ patchy</td>
</tr>
<tr>
<td>15</td>
<td>Colon</td>
<td>FAP</td>
<td>3+ apical/1+ crypts/nl colon = 3+</td>
<td>3+ patchy</td>
</tr>
<tr>
<td>16</td>
<td>Colon</td>
<td>Tubular adenoma</td>
<td>1+</td>
<td>3+</td>
</tr>
<tr>
<td>17</td>
<td>Colon</td>
<td>Tubulovillous adenoma</td>
<td>1+ focal</td>
<td>3+ patchy</td>
</tr>
<tr>
<td>18</td>
<td>Colon</td>
<td>Tubular adenoma</td>
<td>2+</td>
<td>3+</td>
</tr>
<tr>
<td>19</td>
<td>Colon</td>
<td>Tubular adenoma</td>
<td>2+</td>
<td>3+</td>
</tr>
<tr>
<td>20</td>
<td>Colon</td>
<td>Tubular adenoma</td>
<td>2+</td>
<td>3+ scattered</td>
</tr>
<tr>
<td>21</td>
<td>Colon</td>
<td>Tubular adenoma</td>
<td>2+</td>
<td>3+</td>
</tr>
<tr>
<td>22</td>
<td>Colon</td>
<td>Tubular adenoma</td>
<td>3+</td>
<td>1+</td>
</tr>
<tr>
<td>23</td>
<td>Colon</td>
<td>Tubular adenoma</td>
<td>3+</td>
<td>1+</td>
</tr>
<tr>
<td>24</td>
<td>Colon</td>
<td>Villous adenoma</td>
<td>Patches of 0, areas of 2+</td>
<td>3+ patchy</td>
</tr>
<tr>
<td>25</td>
<td>Colon</td>
<td>Tubular adenoma</td>
<td>Patches of 0, areas of 2+</td>
<td>3+ patchy</td>
</tr>
<tr>
<td>26</td>
<td>Colon</td>
<td>Tubular adenoma + cancer</td>
<td>2+</td>
<td>3+ in cancer; 0 in adenoma</td>
</tr>
<tr>
<td>27</td>
<td>Colon</td>
<td>Tubular adenoma + cancer</td>
<td>1/2+</td>
<td>3+ cancer, 3+ adenoma</td>
</tr>
<tr>
<td>28</td>
<td>Colon</td>
<td>Cancer</td>
<td>3+</td>
<td>1+ focal</td>
</tr>
<tr>
<td>29</td>
<td>Colon</td>
<td>Cancer</td>
<td>2+</td>
<td>2+ focal</td>
</tr>
<tr>
<td>30</td>
<td>Colon</td>
<td>Cancer</td>
<td>2+</td>
<td>3+ focal</td>
</tr>
<tr>
<td>31</td>
<td>Colon</td>
<td>Cancer</td>
<td>1+</td>
<td>3+</td>
</tr>
<tr>
<td>32</td>
<td>Colon</td>
<td>Cancer</td>
<td>1/2+</td>
<td>3+</td>
</tr>
<tr>
<td>33</td>
<td>Colon</td>
<td>Cancer</td>
<td>2+</td>
<td>3+ focal</td>
</tr>
<tr>
<td>34</td>
<td>Colon</td>
<td>Cancer + LNs</td>
<td>1/2+</td>
<td>2+</td>
</tr>
<tr>
<td>35</td>
<td>Colon</td>
<td>Cancer + LNs</td>
<td>3+ tumor/2+ LN</td>
<td>2+ tumor/2+ LN</td>
</tr>
<tr>
<td>36</td>
<td>Colon</td>
<td>Cancer + LNs</td>
<td>1+ tumor/1+ LN</td>
<td>Mostly 0, scattered 3+</td>
</tr>
<tr>
<td>37</td>
<td>Colon</td>
<td>Liver mets</td>
<td>2+</td>
<td>3+</td>
</tr>
<tr>
<td>38</td>
<td>Colon</td>
<td>Liver mets</td>
<td>1+</td>
<td>3+</td>
</tr>
<tr>
<td>39</td>
<td>Colon</td>
<td>Liver mets</td>
<td>1+</td>
<td>3+</td>
</tr>
<tr>
<td>40</td>
<td>Colon</td>
<td>Liver mets</td>
<td>2+</td>
<td>3+ only very focal</td>
</tr>
<tr>
<td>41</td>
<td>Colon</td>
<td>Liver mets</td>
<td>1+</td>
<td>3+</td>
</tr>
<tr>
<td>42</td>
<td>Colon</td>
<td>Liver mets</td>
<td>1+</td>
<td>3+</td>
</tr>
<tr>
<td>43</td>
<td>Colon</td>
<td>Liver mets</td>
<td>1+</td>
<td>3+</td>
</tr>
<tr>
<td>44</td>
<td>Colon</td>
<td>Liver mets</td>
<td>1+</td>
<td>3+</td>
</tr>
<tr>
<td>45</td>
<td>Colon</td>
<td>Liver mets</td>
<td>2+</td>
<td>2+</td>
</tr>
<tr>
<td>46</td>
<td>Colon</td>
<td>Liver mets</td>
<td>2+</td>
<td>0/rare 3+ cells</td>
</tr>
<tr>
<td>47</td>
<td>Colon</td>
<td>Liver mets</td>
<td>2+</td>
<td>0</td>
</tr>
<tr>
<td>48</td>
<td>Colon</td>
<td>Lung mets</td>
<td>2+</td>
<td>3+ focal</td>
</tr>
<tr>
<td>49</td>
<td>Colon</td>
<td>Lung mets</td>
<td>3+</td>
<td>2+</td>
</tr>
<tr>
<td>50</td>
<td>Colon</td>
<td>Lung mets</td>
<td>1+</td>
<td>0</td>
</tr>
<tr>
<td>51</td>
<td>Colon</td>
<td>Lung mets</td>
<td>2+</td>
<td>2+ focal</td>
</tr>
<tr>
<td>52</td>
<td>Colon</td>
<td>Lung</td>
<td>3+</td>
<td>0</td>
</tr>
<tr>
<td>53</td>
<td>Colon</td>
<td>Liver met</td>
<td>1+</td>
<td>3+</td>
</tr>
<tr>
<td>54</td>
<td>Colon</td>
<td>Liver met</td>
<td>3+</td>
<td>3+</td>
</tr>
<tr>
<td>55</td>
<td>Colon</td>
<td>Liver met</td>
<td>2+</td>
<td>3+</td>
</tr>
</tbody>
</table>

Abbreviation: FAP, familial adenomatous polyposis.

*Uniform throughout.
Trask expression and frequent phosphorylation in human cancers. Trask is expressed in most epithelial cancers that we surveyed. In addition, Trask phosphorylation is seen in many cancers. Trask phosphorylation is seen in early preinvasive cancers, such as tubular adenomas of the colon and rare cases of ductal carcinoma in situ of the breast (Figs. 4A and Figs. 5D), as well as invasive primary tumors, and tumors at sites of distant metastases (Fig. 5A-D). In some tubular adenomas, there is abundant tumor cell shedding with high Trask phosphorylation (Fig. 4A). These Trask-phosphorylated detached cells are not mitotic cells as confirmed by p-HH3 staining (Fig. 4B), and they are not at the apices of the villi where physiologic shedding is seen in normal colons. Rather, they reveal an abnormal interphase detachment and shedding in these preinvasive cancers. In some tumors, Trask phosphorylation is seen in focal areas and in a patchy distribution, whereas other tumors show a more widespread phosphorylation of Trask.

Table 3.

<table>
<thead>
<tr>
<th>Lung tissue and cancer</th>
<th>Case</th>
<th>Tissue</th>
<th>Disease subtype</th>
<th>Trask score</th>
<th>P-trask score</th>
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<tbody>
<tr>
<td>1</td>
<td>Lung</td>
<td>Normal lung</td>
<td>3+*</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Lung</td>
<td>Normal lung</td>
<td>2+*</td>
<td>0, 3+ detached/mitotic cells</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Lung</td>
<td>Normal lung</td>
<td>2+</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Lung</td>
<td>Normal lung</td>
<td>3+</td>
<td>0, 3+ rare detached cells</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Lung</td>
<td>Normal lung</td>
<td>2+*</td>
<td>0, 3+ in mitoses</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Lung</td>
<td>Normal lung</td>
<td>3+*</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>Lung</td>
<td>Normal lung</td>
<td>3+</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>Lung</td>
<td>Normal lung</td>
<td>2+*</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>Lung</td>
<td>Normal lung</td>
<td>3+*</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>Lung</td>
<td>Normal lung</td>
<td>2+*</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>Lung</td>
<td>Adenoca</td>
<td>3+</td>
<td>Patchy 1+</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>Lung</td>
<td>Adenoca</td>
<td>2+</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>Lung</td>
<td>Adenoca</td>
<td>2+</td>
<td>3+</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>Lung</td>
<td>Adenoca</td>
<td>3+</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>Lung</td>
<td>Adenoca</td>
<td>2+</td>
<td>0/1+</td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>Lung</td>
<td>Adenoca</td>
<td>3+</td>
<td>Patchy 2+</td>
<td></td>
</tr>
<tr>
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<td>Lung</td>
<td>Adenoca</td>
<td>3+</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>Lung</td>
<td>Adenoca/BAC</td>
<td>2+</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>19</td>
<td>Lung</td>
<td>BAC</td>
<td>2+</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>Lung</td>
<td>BAC</td>
<td>0/1+</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>21</td>
<td>Lung</td>
<td>BAC</td>
<td>0/1+</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>22</td>
<td>Lung</td>
<td>Larger cell</td>
<td>3+</td>
<td>2+</td>
<td></td>
</tr>
<tr>
<td>23</td>
<td>Lung</td>
<td>Squamous cell</td>
<td>3+</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>Lung</td>
<td>Squamous cell</td>
<td>1+</td>
<td>1+</td>
<td></td>
</tr>
<tr>
<td>25</td>
<td>Lung</td>
<td>Squamous cell</td>
<td>3+</td>
<td>Patchy 3+</td>
<td></td>
</tr>
<tr>
<td>26</td>
<td>Lung</td>
<td>Squamous cell</td>
<td>3+</td>
<td>3+</td>
<td></td>
</tr>
<tr>
<td>27</td>
<td>Lung</td>
<td>Squamous cell</td>
<td>3+</td>
<td>Patchy 3+</td>
<td></td>
</tr>
<tr>
<td>28</td>
<td>Lung</td>
<td>Squamous cell</td>
<td>2+</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>29</td>
<td>Lung</td>
<td>Squamous cell</td>
<td>3+</td>
<td>3+</td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>Lung</td>
<td>Small-cell ca</td>
<td>1+</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>31</td>
<td>Lung</td>
<td>Small-cell ca</td>
<td>1+</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>32</td>
<td>Lung</td>
<td>Small-cell ca</td>
<td>1+</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>33</td>
<td>Lung</td>
<td>Small-cell ca</td>
<td>2+</td>
<td>2/3+</td>
<td></td>
</tr>
<tr>
<td>34</td>
<td>Lung</td>
<td>Small-cell ca</td>
<td>1+</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>35</td>
<td>Lung</td>
<td>Small-cell ca</td>
<td>1+</td>
<td>3+</td>
<td></td>
</tr>
<tr>
<td>36</td>
<td>Lung</td>
<td>Small-cell ca</td>
<td>1+</td>
<td>3+</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: Adenoca, adenocarcinoma; BAC, bronchoalveolar carcinoma; small-cell ca, small-cell carcinoma.
*Bronchioles.
Trask phosphorylation is also seen in lymphatic tumor emboli (Fig. 5D). The widespread phosphorylation of Trask seen in tumors is not due to the mitotic phase, as evidenced by the counterstain that shows that most of the tumor cells are in interphase. Intense phosphorylation of Trask is seen in some cancers (Fig. 5).

**Trask is a unique Src substrate.** The phosphorylation of Trask is tightly linked with Src kinases, and in contrast to many of the well-known substrates of Src kinases, Trask may be a unique substrate of Src kinases or at minimum, uniquely dependent on Src kinases for tyrosine phosphorylation. Although the transfection and overexpression of Trask in all cells that we have tested induces its phosphorylation, its overexpression in SYF (Src/Yes/Fyn null) cells shows no evidence of phosphorylation (Fig. 6A), suggesting that Src kinases are essential for Trask phosphorylation. The cotransfection of c-src or c-yes into SYF cells restores Trask phosphorylation, further confirming the essential role of src kinases in Trask phosphorylation (Fig. 6A).

To further confirm the link between Src and Trask phosphorylation in vivo, we studied the phosphorylation of Trask in a model of Src-induced epithelial tumorigenesis. Mouse mammary tumors induced by the expression of the Polyoma middle T (PyMT) antigen are driven by the PyMT-induced activation of Src (7). We established an orthotopic model of PyMT-driven mouse by ex vivo PyMT retroviral infection of mouse mammary epithelium and reimplantation into the mammary fat pad. Trask expression and phosphorylation were studied in these tumors and their lung metastases by immunohistochemical methods. Trask is expressed in the normal mammary epithelium but not phosphorylated (Fig. 6B). However, PyMT-induced mammary tumors show significant phosphorylation of Trask, particularly in lung metastases (Fig. 6B). The phosphorylation of Trask in these tumors is due to the activation of Src kinases by PyMT and can be suppressed by treating the mice with Src inhibitors (Fig. 6B). The phosphorylation of Trask is not seen in all parts of the tumor but rather in a patchy and focal distribution. The growth of these tumors is highly suppressible by Src inhibitors as expected from their Src-driven biology (Fig. 6C).

**Discussion**

A principal function of epithelial cells is adherence to tissue level architecture. At its most basic level, this architecture consists of monolayers of attached cells against a basement

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*Fig. 3. A, sections from normal colon biopsies were prepared and stained with anti–phospho-Trask antibodies. The staining intensity was scored by two observers according to procedures and definitions described in Materials and Methods. All immunostains and their analyses were conducted using well-defined positive and negative controls as described in Materials and Methods. Trask phosphorylation is seen in occasional mitotic cells. This is shown here in the mitotically active crypt regions of normal colonic mucosa. The detaching cells in the crypts were confirmed to be epithelial cells and not extrinsic cells as shown by staining with the intestinal epithelial differentiation marker CDX2 and confirmed to be mitotic cells by the mitotic marker phospho-histone H3. B, trask phosphorylation is also seen in some clusters of cells at the apices of colonic villi where shedding commonly occurs.*
membrane. This highly structured organization in epithelial tissues allows them to serve as protective barriers and function to interface with the luminal or outside environment. Certain cellular functions require disengagement from substratum, including functions such as cell migration during development or wound repair, mitotic cell division, and physiologic shedding. Disengagement from substratum requires the activation of autonomous signaling mechanisms not regulated by tissue architecture. The activation of such anchorage-independent programs and the ability to defy tissue-level imposed programs are also important parts of epithelial tumorigenesis. Much of the signaling that underlies the anchorage-dependency and independency of epithelial cells occurs through tyrosine phosphorylation. Here, we report that a common signaling pathway that characterizes the anchorage-independent growth of epithelial tumor cells is the Src family kinase phosphorylation of their transmembrane substrate Trask.

In untransformed epithelial cells, the phosphorylation of Trask is tightly regulated and Trask phosphorylation is only seen during circumstances of mitotic detachment or physiologic shedding such as seen at the apex of the intestinal villi. Trask phosphorylation can be induced in vitro by forced detachment and growth in suspension. In contrast to the tightly regulated phosphorylation of Trask in untransformed epithelial cells and tissues, we find that the Src phosphorylation of Trask is commonly seen in many epithelial cancers and cancer cell lines. We see Trask phosphorylation in all stages of cancer including preinvasive cancers such as tubular adenomas of the colon, as well as invasive, and metastatic cancers. As such, the aberrant phosphorylation of Trask is not an activity acquired in later stages of invasive or metastatic cancers. It is possible that Trask phosphorylation in preinvasive cancers identifies those preinvasive tumors that are at high risk for invasion and dissemination. Future studies may attempt to test that hypothesis; however, this may be too simplistic a hypothesis, considering what we know thus far about the functions of Trask. It is likely that both the phosphorylation and the dephosphorylation of Trask are important functions in
migrating cells and likely also in invading and metastasizing cells and some level of regulation would be optimal for these tumorigenic functions. As such, Trask phosphorylation may be a marker of cellular activities at certain points in time or certain tumor areas. Consistent with this, we see only focal phosphorylation of Trask in many tumors. The spacial pecularity we seen in the immunostains may merely reflect a snapshot in time captured at the moment of tissue acquisition and fixation, and may be evidence of a wider temporal plasticity in Trask phosphorylation reflecting the dynamic nature of tumor cell adhesion and migration characteristics \textit{in vivo} that we are unable to appreciate in these snapshot analyses. The cellular

![Image of immunostains showing phosphorylation in different types of cancer](https://example.com/immunostains)

**Fig. 5.** Representative images from phospho-Trask immunostains are shown for a number of subtypes of epithelial cancers as indicated.
attributes that Trask phosphorylation may be reporting could be disrupted cell-stromal or cell-cell interactions, changes in cytoskeletal signaling, or activation of anoikis resistance. We have evidence that Trask phosphorylation is regulated by cell-cell and cell-matrix engagement. Several studies have reported the activation of Src kinases when epithelial cells detach from

Fig. 6. A, SYF cells were transfected with a pcDNA4-MycTrask expression vector and either Src or Yes-expressing vectors, and the expressed Trask protein was immunoprecipitated with antimyc antibodies and immunoblotted with antiphosphotyrosine or anti-Trask antibodies. Lanes correspond to (1) untransfected cells, (2) cells transfected with pcDNA4-MycTrask and pCMV6 vector, (3) cells transfected with pcDNA4-MycTrask and pCMV6-src, (4) cells transfected with pcDNA4-MycTrask and pCMV6-yes. Lane 5 contains the same lysate as lane 4 but was immunoprecipitated with IgG control. B, PyMT-induced mammary tumors were generated in mice as described in Materials and Methods and allowed to grow to ~ 2.5 cm in size. Mice were sacrificed and tumor tissues were fixed in formalin, and lung metastases were dissected, identified, and fixed. Sections of normal mammary tissue from control mice as well as sections from PyMT-induced tumors, and their lung metastases were studied by immunohistochemical analyses to determine the expression and phosphorylation of Trask. Tumors from mice treated with the Src inhibitor dasatinib for 1 wk were also harvested for analysis. Trask phosphorylation is induced in this tumor model and dephosphorylated by Src inhibitor therapy. C, mice were orthotopically implanted with PyMT-induced syngeneic tumors, and after tumors were established at ~ 1 mo, 30 tumor-bearing mice were randomized to 2 arms and treated with dasatinib or DMSO control, and tumor sizes were measured twice weekly.

7 Manuscript in submission.
matrix and its function in averting anoikis when detached (24–26). The specific role of Trask/CDCP1 in promoting anoikis resistance was recently shown in a lung cancer cell line (27).

The apparent deregulation of Trask in preinvasive epithelial tumors is interesting but not altogether surprising. Indeed, increased activity of both Src or Yes is an early event in colon neoplasia and seen very frequently in preinvasive lesions of the colon (28–30). The finding of Trask phosphorylation in preinvasive tumors suggests that some of the molecular and possibly phenotypic attributes of invasive or metastatic epithelial cancers are already present in preinvasive tumors. Consistent with this, the activation of Src and Yes in preinvasive tumors of the colon is seen predominantly in tumors at highest risk for progression to invasive cancer (28, 29). The deregulation of Trask in preinvasive tumors could have phenotypic consequences, but these would likely be clinically undetectable with little symptomatic consequence. These could include phenotypes such as shedding of tumor cells. However, due to the intact nature of the basement membrane in preinvasive tumors, the shedding would be entirely into the epithelial lumen, which would be of little clinical or symptomatic consequence to affected patients. Indeed, we do find significant interphase tumor cell shedding in tubular adenomas, which we do not see in normal colon tissues (Fig. 4A). Trask phosphorylation could also increase the survival of luminaly shed cells. Consistent with this, exfoliated tumor cells are detected much more successfully in the stools of patients with tubular adenomas or colon cancers compared with the stools of patients with inflammatory diseases of the colon or normal colons (31, 32), and exfoliated breast epithelial cells are detected in nipple aspirates of women with preneoplastic lesions of the breast (33). Although not clinically significant in preinvasive disease, the phenotypic consequence of deregulated Trask phosphorylation may be much more significant after progression to invasive disease because the ability to survive and migrate within connective tissues, lymphatics, and the bloodstream can be mediated through Trask phosphorylation. Consistent with this, we do see Trask phosphorylation in tumor emboli (Fig. 5D).

When we use the same tumor cells to compare in vitro and in vivo growth, we find that Trask phosphorylation in the in vivo model resembles the anchorage-deprived state of the in vitro model, not the adherent monolayer growth (Fig. 2B). This is not surprising because it is widely recognized that the growth of tumor cell lines in flattened monolayers on tissue culture–treated plasticware represents an artificial state with many differences from their growth in vivo.

The mechanisms by which Trask may promote survival in suspended epithelial cells are currently unknown. Trask/CDCP1 was found to bind PKC δ in a phosphorylation-dependent manner through the C2 domain of PKC δ (34). PKC δ is thought to regulate apoptosis, although its role may be complex and both proapoptotic and antiapoptotic functions have been attributed to PKC δ (35). The functions of PKC δ may be context dependent. In murine mammary epithelial cells, PKC δ activation promotes the ability to survive and grow in an anchorage-independent manner (36). Almost surely Trask regulates functions other than survival because we have previously shown that the forced overexpression and overphosphorylation of Trask retains detached epithelial cells in suspension and prevents them from respreading on substrate, which suggests that Trask phosphorylation affects cytoskeletal signaling as well (17). We have been studying the role of Trask in cytoskeletal signaling and have identified mechanistic links between Trask and components of the actin cytoskeleton, although this evidence is only preliminary at this point.

The fact that the phosphorylation of Trask is entirely dependent on src kinases may suggest its use as an in vivo marker of the activity of Src kinases. Its phosphorylation in PyMT-induced tumors and in many human cancers is consistent with this. However, it is unlikely that the level of Trask phosphorylation faithfully reflects the activity of Src kinases. In the tumor cell panel reported here, the level of Trask phosphorylation varies widely and does not parallel the much more uniform activity of Src kinases previously published by ourselves and others (37, 38). In addition, although epithelial cells undergoing mitosis or detachment show modest increases in Src kinase activity (39), the abundant phosphorylation of Trask seen in these circumstances greatly exceeds increases in Src kinase activities (26). In addition, Trask phosphorylation seems to be partly dependent on Trask expression, and its phosphorylation can be induced by its overexpression (17). Clearly, there are many variables other than the steady-state activity of Src kinases that regulate the Src-mediated phosphorylation of Trask.

In summary, Trask is a recently identified Src substrate that may be highly relevant to epithelial tumorigenesis. The evidence indicates that Trask is a principal marker of anchorage-independent signaling in epithelial cells and its Src-driven phosphorylation is tightly regulated in epithelial cells. The deregulation of Trask phosphorylation is a common and early event in epithelial tumorigenesis, and further mechanistic exploration of its signaling functions should be of high priority.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We thank the Immunohistochemical Core, the Tissue Core, and the Mouse Pathology Core facilities of the University of California at San Francisco Comprehensive Cancer Center for their services in this work, and in particular, the technical assistance of Loretta Chan of the immunohistochemical core.

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Phosphorylation of the src Epithelial Substrate Trask Is Tightly Regulated in Normal Epithelia but Widespread in Many Human Epithelial Cancers

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