Identification of a Src Tyrosine Kinase/SIAH2 E3 Ubiquitin Ligase Pathway That Regulates C/EBPδ Expression and Contributes to Transformation of Breast Tumor Cells

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The transcription factor CCAAT/enhancer-binding protein delta (C/EBPδ, CEBPD) is a tumor suppressor that is downregulated during breast cancer progression but may also promote metastasis. Here, we have investigated the mechanism(s) regulating C/EBPδ expression and its role in human breast cancer cells. We describe a novel pathway by which the tyrosine kinase Src downregulates C/EBPδ through the SIAH2 E3 ubiquitin ligase. Src phosphorylates SIAH2 in vitro and leads to tyrosine phosphorylation and activation of SIAH2 in breast tumor cell lines. SIAH2 interacts with C/EBPδ, but not C/EBPβ, and promotes its polyubiquitination and proteasomal degradation. Src/SIAH2-mediated inhibition of C/EBPδ expression supports elevated cyclin D1 levels, phosphorylation of retinoblastoma protein (Rb), motility, invasive properties, and survival of transformed cells. Pharmacological inhibition of Src family kinases by SKI-606 (bosutinib) induces C/EBPδ expression in an SIAH2-dependent manner, which is necessary for “therapeutic” responses to SKI-606 in vitro. Ectopic expression of degradation-resistant mutants of C/EBPδ, which do not interact with SIAH2 and/or cannot be polyubiquitinated, prevents full transformation of MCF-10A cells by activated Src (Src truncated at amino acid 531 [Src-531]) in vitro. These data reveal that C/EBPδ expression can be regulated at the protein level by oncogenic Src kinase signals through SIAH2, thus contributing to breast epithelial cell transformation.

The transcription factor CCAAT/enhancer-binding protein delta (C/EBPδ, CEBPD, or NFIL-6β) exhibits many tumor suppressor-like activities. In vitro, C/EBPδ inhibits the growth of tumor cell lines (20, 29, 45), promotes genomic stability of mouse embryonic fibroblasts (MEF) (28), and downregulates expression of cyclin D1 (3, 20, 47). Using a mouse model of mammary tumorigenesis, we have shown that, consistent with a tumor suppressor function, loss of C/EBPδ increased tumor incidence. However, distant metastases to the lung were reduced in the absence of C/EBPδ, suggesting additional tumor-promoting activity by C/EBPδ in this animal model (7). In meningiomas, C/EBPδ expression correlates with a low histological grade and progression-free patient survival (8), and CEBPD was in a 70-gene signature predicting longer survival of breast cancer patients (43). Indeed, C/EBPδ expression is downregulated in several types of cancers including cervix, liver, and breast (3, 38, 44, 48, 60).

Interestingly, the C/EBPδ gene promoter can be activated by the STAT3 transcription factor (13, 61, 72). However, STAT3 is frequently hyperactivated in cancer and is a well-characterized tumor-promoting factor (58). Thus, we were interested in understanding how activation of STAT3 signaling in breast cancer was compatible with downregulation of C/EBPδ in the same disease. Although the CEBPD gene was found to be methylated in a significant number of acute myelomonocytic leukemias, cervical and hepatocellular carcinomas, and a subset of breast tumors (3, 20, 38, 60), the sporadic pattern of methylation in breast tumors suggested that other mechanisms of repression exist. Therefore, we hypothesized that signaling pathways upstream of or parallel to STAT3 lead to inhibition of C/EBPδ expression in a manner that is dominant over activated STAT3. Because the c-myc proto-oncogene was shown to inhibit CEBPD promoter activity in a mouse mammary epithelial cell line (72) and because both STAT3 and c-myc can be activated by Src kinase signaling (1, 58), we investigated whether Src kinase signaling regulates C/EBPδ expression in breast epithelial cells.

Src and the related proteins Fyn and Yes form a subfamily of cytoplasmic tyrosine kinases that transmit signals from receptor tyrosine kinases, G-protein-coupled receptors, and integrins. Hence, these kinases are central mediators in multiple signaling pathways and regulate very diverse physiological processes (11, 66). Src family kinases are frequently overexpressed or highly activated in tumor tissues and are associated with progression of cancer (66). Abrupt activation of c-Src regulates many functions in tumor cells such as cell proliferation, cell-cell adhesion and motility, tumor cell migration, invasion, and metastasis (23, 53, 66). Therefore, inhibitors of Src family kinases such as dasatinib

and bosutinib (SKI-606) are being investigated and employed as therapeutic agents for cancer patients (12, 19, 36, 71).

To understand the role and regulation of C/EBP in breast cancer, we studied human breast epithelial cell lines and found that Src kinase activity downregulates C/EBP protein but not mRNA levels through a SIAH2 E3 ligase-dependent mechanism. Furthermore, our studies revealed that downregulation of C/EBP protein levels contributes to cell transformation by oncogenic Src kinase. These findings support a tumor suppressor activity of C/EBP in breast cancer.

MATERIALS AND METHODS

Cell culture and treatments. MCF-10A and MDA-MB-231 cells were cultured in Dulbecco’s modified Eagle’s medium–F-12 (HAM) (DMEM–F-12HAM; 1:1) medium supplemented with 10% fetal bovine serum (FBS), 10 μg/ml insulin, 100 ng/ml cholaer toxin, 0.5 μg/ml hydrocortisone, 20 ng/ml recombinant epidermal growth factor (EGF), 1 mM calcium chloride, 5 mM glutamine, and 0.5% penicillin-streptomycin. All other cells were grown in DMEM supplemented with 10% FBS, 5 mM glutamine, 0.5% penicillin-streptomycin, and MCF-7 with additional 5 mM sodium pyruvate. SKBR3 cells were grown in McCoy’s 5a medium with 10% FBS. Dimethyl sulfoxide (DMSO) was used in controls for treatments with proteasome inhibitors or SKI-606 (Selleck Chemicals). SKI-606 was used at 1 μM unless indicated otherwise. MG132 was added at 50 μM, 3 h before cell lysis. All cells were grown in a 5% CO₂ incubator at 37°C.

Transient RNA interference (RNAi). To generate cells with stable transfections, we used Mirrus. Appropriate vector-only transfections were used in all cases as negative controls. Lysates were prepared 24 h after transfection unless indicated otherwise.

Expression constructs. Chimeric C/EBP and C/EBP proteins were generated by introduction of BamHI sites at the appropriate locations within mouse C/EBP and rat C/EBP. Fragments were ligated as indicated in Fig. 5A. The point mutations of human C/EBP were introduced using a Quick Change Lightning Site-Directed Mutagenesis Kit (item 210518-5; Agilent Technologies) according to the manufacturer’s instructions. All constructs were verified by sequencing. All other expression constructs have been described before and were kind gifts from Amy Tang (Mayo Clinic Cancer Center) for C/EBP, Rosalyn Irby (Penn State College of Medicine) for Src-531 (Src truncated at amino acid 531), Ira Daar and Stanley Lipkowitz (NCI) for Src-527, and Allan Weissman (NCI) for MDM2 and NEDD4.

Generation of cells with stable transfection. To generate cells with stable depletion of endogenous C/EBP, MDA-MB-231T cells (46), which carry an integrated green fluorescence protein (GFP) and luciferase reporter (to be described elsewhere), were transfected with C/EBP silencing short hairpin RNA (shRNA) constructs or control constructs (7). After drug selection, clones were analyzed for loss of SKI-606-induced C/EBP expression while maintaining SKI-606-sensitive Src kinase activity. Data in this study are representative of two independent isolates of control and silenced clones. To generate MCF-10A cells with ectopic C/EBP expression, cells were transfected with expression constructs for hemagglutinin (HA)-tagged wild-type C/EBP (WT-C/EBP) or C/EBP with a K120A mutation (K120A-C/EBP) or with a control vector. After drug selection, pools of resistant clones were characterized and used for the subsequent studies.

Transient RNA interference (RNAi). On-Target plus SMARTpool SIAH2 small interfering RNA (siRNA) against SIAH2 (L-006561-00) and C/EBP siRNA (L-010453-00) and Accell Red nontargeting siRNA (catalog number D-001960-01-05) were purchased from Thermo Scientific, Lafayette, CO. MCF-10A, MDA-MB-468, and MDA-MB-231T cells were transfected with 100 nM On-Target plus SMARTpool siRNA at 2 × 10⁶/100 μl in Nucleofector solution V (Amaxa Biosystems, Germany) according to the manufacturer’s instructions. The pMaxGFP expression construct (200 ng; Amza Biosystems, Germany) was included to estimate transfection efficiency. The efficiency of siRNA on protein expression was assessed 2 days after nucleofection unless otherwise indicated. Scramble control oligonucleotides were used in all experiments for the negative control.

Real-time PCR. Total RNA was extracted by TRizol (Invitrogen), DNase I treated, and purified by RNAasy (Qiagen). Two micrograms of RNA was used to make the cDNA with Superscript Reverse Transcriptase III (Invitrogen) according to the manufacturer’s instructions. PCR was conducted with TaqMan gene expression primer/probe sets using a 7500 Fast Real Time PCR instrument (Applied Biosystems). Analysis was performed using the MxPro software (Stratagene). All reactions were performed in duplicates. The relative expression level of C/EBP (Hs00270931_s1; Applied Biosystems) was measured using the relative quantitation ∆∆Ct (where Ct is threshold cycle) method and normalized to β-actin (Hs99999903_m1; Applied Biosystems). Data are from three independent samples (means ± standard errors of the means [SEM]) and were normalized to β-actin.

Luciferase reporter assay. The C/EBP promoter (−2000 to +42) was isolated by PCR from a human bacterial artificial chromosome (BAC) using the following primers: hd-2000forward, AAAGCTAGCCATGTCGCGTAATGTCACACTCTATGC; hd+42reverse, ATAAAGCTTGGCGGCGTCTGCGTGGCGCGGCCGGTCCTTGC. The fragment was cloned into pGL4.12 by Nhel and HindIII. Cells were transfected with 200 ng of either the C/EBP reporter or a cyclin D1 vector reporter (−1745CD1LUC [5]), together with 200 ng of either vector or SrcY529F (Src with a Y529F mutation) expression constructs and with 200 ng of pRSV β-galactosidase (β-Gal) expression constructs as an internal control. The cells were lysed in reporter lysis buffer (Promega) and analyzed for luciferase activity per the manufacturer’s instructions. β-Galactosidase activity was assayed with O-nitrophenyl-β-D-galactopyranoside as a substrate as described previously (47) and used to normalize for the variations in transfection efficiencies. Data are from three independent experiments performed in duplicate.

Western blotting and antibodies. All Western analyses are with whole-cell lysates unless indicated otherwise. Cells were lysed in SDS sample buffer or in 1× reporter lysis buffer (catalog number E397A; Promega) with protease inhibitors: 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 μl/ml each of phosphatase inhibitor 1 (P2850; Sigma), phosphatase inhibitor II (P5726; Sigma), and protease inhibitor cocktail (P8340; Sigma). Proteins were resolved on SDS-PAGE gels, and blotted onto Protean nitrocellulose membranes (Schleicher & Schuell). Membranes were blocked with bovine serum albumin (BSA) in 10 mM Tris–HCl (pH 7.5), 150 mM NaCl, and 0.5% Tween 20 (TBS-T). Immunostaining was performed in TBS-T and visualized with Lumiglo Reserve Chemiluminescence reagent (KPL Inc.) according to the manufacturer’s instructions. Antibodies were from Cell Signaling (catalog number number RB-010-P0), Santa Cruz Biotechnology, Inc. (β-actin, sc-1616; C/EBP, sc-125; SIAH1, sc-5005; SIAH2, sc-5507; CEBPΔ, Sc-7962; pRb, sc-12901; CEBPΔ, sc-965; Pten, sc-133197), Abcam (retinoblastoma protein [Rb], Ab6075); BD Biosciences Pharmingen (C/EBP, BD69320, or BD69319 mouse monoclonal antibodies against the amino terminal 140 amino acids of human C/EBP), Rockland Immunologicals, Inc. (C/EBP, 600-401-A61; tubulin, 600-401-880), Millipore (phosphotyrosine, 05-1050), Pierce (anti-mouse and anti-rabbit horseradish peroxidase [HRP]-conjugated IgG, 1858413 and 1858415), Cell Signaling (Src, 2110; p-Src, 2101S; p53, 2524), Bethyl Laboratories, Inc. (Src, 2110; p-Src, 2101S; p53, 2524), and Antibodies, Inc. (cyclin D1, catalog number RB-010-P0); Santa Cruz Biotechnology, Inc. (β-actin, sc-1616; C/EBP, sc-7962; SIAH1, Sc-5005; SIAH2, sc-5507; CEBPΔ, Sc-7962; pRb, sc-12901; CEBPΔ, sc-965; Pten, sc-133197), Abcam (retinoblastoma protein [Rb], Ab6075); BD Biosciences Pharmingen (C/EBP, BD69320, or BD69319 mouse monoclonal antibodies against the amino terminal 140 amino acids of human C/EBP), Rockland Immunologicals, Inc. (C/EBP, 600-401-A61; tubulin, 600-401-880), Millipore (phosphotyrosine, 05-1050), Pierce (anti-mouse and anti-rabbit horseradish peroxidase [HRP]-conjugated IgG, 1858413 and 1858415), Cell Signaling (Src, 2110; p-Src, 2101S; p53, 2524), Bethyl Laboratories, Inc. (Flag, F3165), Thermo Scientific (anti-goat HRP-conjugated IgG, 31400), and 9E10 ascites fluid (anti-myc). The pan-C/EBP rabbit antibody used to analyze the chimeric proteins recognizes a conserved sequence in the C/EBP family basic region (67). Tubulin was used as loading control.

Coimmunoprecipitation assay. Cells were lysed in 30 mM Tris, pH 8.0, 75 mM NaCl, 10% glycerol, 1% Triton X-100, 1 mM PMSF, and protease and phosphatase inhibitors. One milligram of protein lysate was mixed with 5 μg of antibody and rotated overnight at 4°C. An equivalent aliquot of lysate used for specific antibodies as indicated was used for immunoprecipitation (IP) with IgG as a negative control. Protein A/G beads were blocked with 1% BSA and 1 mg/ml single-stranded DNA.
(ssDNA) overnight. Forty microliters of preblocked beads was added to the lystate and antibody mixture for 4 h at 4°C before centrifugation at 3,000 rpm (Eppendorf) for 30 s, followed by five washes with lysis buffer. Forty microliters of preblocked sample buffer (Bio-Rad) was then added in each IP mixture and denatured at 100°C for another 5 min. Ten microliters of each IP product was loaded. For immunoprecipitation under denatured conditions, cells were lysed in denaturing lysis buffer (50 mM Tris, pH 7.5, 70 mM β-mercaptoethanol) and boiled for 10 min. Four volumes of cold cell lysis buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na3VO4, 1 μg/ml leupeptin) was added to the previous lysate before the IP reaction.

**In vitro phosphorylation analysis.** SIAH2 was immunoprecipitated with anti-Flag from radioimmunoprecipitation assay (RIPA) buffer extracts of HEK293T cells transfected with Flag-SIAH2 and incubated with 1 ng of purified active Src (catalog number 14-746; Millipore) in kinase assay buffer as described previously (42). Reactions were resolved on SDS-PAGE gels and transferred to nitrocellulose. Following autoradiography the SIAH2 bands were digested from the membrane with trypsin (and chymotrypsin as indicated in Fig. 3K) and analyzed by reverse-phase high-performance liquid chromatography (HPLC), phosphoamino acid analysis (PAA), and Edman degradation (42).

**Cell proliferation assay.** Cells were seeded in 96-well plates at 3,000 to 5,000 cells/well and treated the next day with SKI-606 as indicated in the figures. Each experiment was done with triplicate wells. Cells were incubated with 10% Alamar blue (BioSource) for 3 h, and fluorescence was quantified with a NovoStares (BMG) plate reader.

**Soft-agar assay.** Cells (1 × 105) were suspended in medium (DMEM–F-12HAM at 1:1, containing appropriate components as described above) containing 0.3% agarose. The cell mixture was then seeded on a layer of 0.5% bottom agar in a six-well plate and allowed to grow for 2 weeks. The culture medium was changed once a week. After 2 weeks colonies were stained with Giemsa, and representative views from triplicate experiments were photographed. Colonies were counted in three independent fields at a magnification of ×10 for each of three independent experiments.

**Cell motility assay.** Cells (approximately 3 × 105 cells/well) were seeded in culture inserts (Ibidi) and allowed to adhere for 8 to 10 h in medium containing 0.5% FBS. Culture inserts were removed using sterile tweezers, and the wall was filled with fresh medium containing 0.5% FBS.

**Invasion assay.** Twenty-four-well plates with precoated growth factor-reduced Matrigel (Matrigel-GFR) chambers (BD Biosciences) were prepared according to the manufacturer’s instructions. The lower well of the chamber was filled with DMEM supplemented with 10% FBS. Cells were plated in the upper chamber suspended in medium containing 1% FBS at a density of 105 cells/ml. Following incubation for 24 h at 37°C, cells were removed from the filter top with a cotton swab, and the filter was placed in the chamber in DMEM supplemented with 10% FBS and cultured for 72 h. Culture inserts were removed using sterile tweezers, and the wall was filled with fresh medium containing 0.5% FBS.

**Matrigel assay.** MCF-10A cells were grown on Matrigel as described previously (16). Acini were fixed using 2% paraformaldehyde for 20 min and permeabilized with 0.5% Triton X-100 for 10 min at 4°C. Cells were rinsed with PBS-glycine solution and blocked with primary blocking solution containing 130 mM NaCl, 7 mM Na2HPO4, 3.5 mM NaH2PO4, 7.7 mM NaNO3, 0.1% bovine serum albumin, 0.2% Triton X-100, and 0.05% Tween 20 plus 10% goat serum for 1 h. The acini were next blocked with secondary blocking solution [primary blocking solution plus 20 μg/ml goat anti-mouse F(ab)2 fragment] for 40 min. Acini were then incubated with anti-laminin 5 (ab14509; Abcam), anti-active caspase-3 (ab2302; Abcam), and anti-Ki67 (Mab4190; Millipore) in the secondary blocking solution overnight at room temperature. After acini were rinsed with IF buffer (130 mM NaCl, 7 mM Na2HPO4, 3.5 mM NaH2PO4, 7.7 mM NaNO3, 0.1% bovine serum albumin, 0.2% Triton X-100, 0.05% Tween 20) at room temperature, they were incubated with secondary antibody (Alexa Fluor 488-conjugated goat anti-rabbit antibody [A11034; Invitrogen] or Alexa Fluor 594-conjugated goat anti-mouse antibody [A11032; Invitrogen]) for 50 min at room temperature. After acini were washed with IF buffer, they were incubated with PBS containing 4’,6’-diamidino-2-phenylindole (DAPI; 0.5 ng/ml) for 15 min. Acini were washed with PBS for 5 min and mounted with Vectashield (Vector laboratories, Inc., Burlingame, CA). Images were captured using a Zeiss Laser Scanning LSM 510.

**Quantitative analyses.** Quantified data are from three independent biological replicates expressed as means ± SEM, unless indicated otherwise. Data were analyzed by t test or unequal variance t test with a Satterthwaite correction (see Fig. 6E and F and 7E).

**RESULTS**

Src kinase downregulates C/EBPδ protein expression. We have previously shown that breast cancer cell lines express significantly less C/EBPδ protein than untransformed breast epithelial cell lines (47). Many breast cancer cell lines are known to contain activated Src kinase (63), and, as expected, we found that C/EBPδ expression inversely correlated with the phosphorylation of Src family kinases at tyrosine 419 (Fig. 1A), which is indicative of activity (62). The tumor cell lines MCF-7, MDA-MB-231, SKBR3, BT474, and MDA-MB-468 harbor activated endogenous Src kinases, whereas the untransformed MCF-10A and MCF-12A cells do not. These cell lines therefore reflect that Src activity is induced in breast tumors (52). On the other hand, MCF-10A and MCF-12A cells express significantly higher levels of C/EBPδ mRNA (44; also data not shown) and protein (Fig. 1A) (47) than breast tumor cell lines. Indeed, in transient cotransfection experiments, constitutively active Src downregulated a C/EBPδ promoter-reporter construct, while the cyclin D1 promoter was induced as expected (Fig. 1B).

To assess a causal relationship between Src activation and the reduced expression of endogenous C/EBPδ, we treated MDA-MB-231 (Fig. 1C) and MDA-MB-468 (Fig. 1D) cells with the Src family kinase inhibitor SKI-606 (22). Loss of Src phosphorylation was accompanied by induction of C/EBPδ protein expression in these tumor cell lines. In our hands the kinetics of Src inhibition by SKI-606 was slower than previously reported (32, 63), which could be due to differences in phosphatase activities toward Src between cell lines or in bioavailability of SKI-606 due to, e.g., efflux pumps. Surprisingly, there was no effect of SKI-606 on C/EBPδ mRNA levels in either cell line (Fig. 1E). Next, we tested the effect of Src activation on C/EBPδ expression in untransformed cells. Overexpression of a constitutively activated Src truncated at amino acid 531 (Src-531) (30) in MCF-10A cells resulted in the downregulation of endogenous C/EBPδ protein levels, whereas expression of the related protein C/EBPδ was not affected (Fig. 1F). Again, there was no significant effect of Src activity on C/EBPδ mRNA levels (Fig. 1G). Src kinase with an activating Y527F mutation (33) also downregulated C/EBPδ, while a kinase-dead K295M mutant (33) did not (Fig. 1H).
FIG 1 Src kinase inhibits C/EBPδ protein expression in breast epithelial cells. (A) Western analysis of C/EBPδ protein levels in comparison to an antibody to the autophosphorylation site of Src family proteins (Y419 in human Src [pSrcY419]) in human mammary epithelial cell lines (Normal, nontumorigenic; Cancer, tumorigenic). (B) Luciferase reporter gene activity in MCF-10A cells transfected with the indicated promoter-reporter constructs (***, *P < 0.01; ****, *P < 0.0001). (C) Western analysis of the indicated proteins in MDA-MB-231 cells treated with SKI-606 for various times. (D) MDA-MB-468 cells were treated for 24 h with SKI-606 and analyzed as described for panel C. Data are from the same blot with intermediate lanes deleted. (E) Quantitative PCR analysis of C/EBPδ mRNA levels in MDA-MB-231 and MDA-MB-468 cells treated for 24 h with SKI-606. Normalized data are relative to untreated controls. (F) Western analysis of the indicated proteins in MCF-10A cells transfected with a Src-531 expression construct. (G) Quantitative PCR analysis of C/EBPδ expression in MCF-10A cells from the experiment shown in panel F. (H) Western analysis of MCF-10A cells transfected with expression constructs for Src-531, SrcY529F (chicken-mouse chimeras), SrcY529L (chicken-human chimera), K295M inactivated Src (kinase dead), or vector control. Endogenous Src is not seen because the exposure was optimized for overexpressed protein.

Together, these data show that Src kinase activity can downregulate C/EBPδ expression by a mechanism that acts primarily at the protein level.

Src activity leads to proteasomal degradation of C/EBPδ. Next, we assessed the effect of activated Src on C/EBPδ protein stability. Inhibition of protein synthesis by cycloheximide in MCF-10A cells revealed that C/EBPδ was less stable when expressed in the presence of Src-531 (Fig. 2A). C/EBPδ overexpression was used for this experiment in order to have detectable protein levels at the beginning of the time course with Src-531. Many proteins are turned over by proteasome-dependent degradation (18). When MCF-10A cells were treated with the proteasome inhibitor lactacystin, endogenous C/EBPδ expression could be rescued in the presence of Src-531 (Fig. 2B, upper panel). In the absence of Src-531, proteasome inhibition had no significant effect on C/EBPδ levels in MCF-10A cells. On the other hand, inhibition of the proteasome (by MG132) was sufficient to induce C/EBPδ expression in MDA-MB-468 cells (Fig. 2B, lower panel). Because almost all proteasome substrates are targeted for degradation by polyubiquitination, we next examined if C/EBPδ was polyubiquitinated. C/EBPδ has a predicted molecular mass of 28 kDa but runs at about 36 kDa on SDS-PAGE gels. Immunoprecipitation of C/EBPδ from MCF-10A cells expressing HA-tagged ubiquitin demonstrated that Src-531 led to high-molecular-weight (MW), polyubiquitinated species of C/EBPδ (Fig. 2C). In summary, these data show that in the presence of active Src kinase, C/EBPδ is an unstable protein due to proteasomal degradation.

SIAH2 is necessary and sufficient for Src kinase-mediated degradation of C/EBPδ. Polyubiquitination of proteins is usually achieved by substrate-specific E3 ubiquitin ligases. SIAH2 is an E3 ligase, which is activated by the Ras signaling pathway and Src kinase (56, 70). When we overexpressed SIAH2 in MCF-10A cells, it alone downregulated endogenous C/EBPδ (Fig. 3A). To address specificity for SIAH2, we also expressed MDM2 or NEDD4, which did not alter C/EBPδ protein levels, while levels of known substrates, p53 and PTEN, respectively, decreased (Fig. 3A). Short-term treatment with MG132 rescued C/EBPδ expression in the presence of SIAH2, consistent with a role of SIAH2 in targeting C/EBPδ for proteasomal degradation (Fig. 3B). Comparison of several human breast epithelial cell lines revealed that SIAH2 was more highly expressed in the breast tumor cell lines, especially MDA-MB-468 and MDA-MB-231, than in untransformed MCF-10A cells (Fig. 3C). In contrast, expression of the related SIAH1 protein was more similar across these cell lines. To test the causal role of endogenous SIAH in the degradation of C/EBPδ, we inactivated the protein by two different approaches in breast cancer cells. C/EBPδ protein expression was induced by expression of a dominant negative (DN) SIAH2 protein (4) in MDA-MD-231T or MDA-MD-468 cells (Fig. 3D). Furthermore, depletion of SIAH2 by RNAi silencing in MDA-MB-231T cells also increased C/EBPδ protein expression (Fig. 3E). Importantly, Y419 phosphorylation of endogenous Src was not significantly affected by inactivation of SIAH2, ruling out such an indirect mechanism for C/EBPδ stabilization by SIAH2 inhibition (Fig. 3D and E). Comparison of SIAH2 inhibition by siRNA and overexpression of DN-SIAH2 resulted in similar levels of C/EBPδ accumulation in MDA-MB-231T cells (Fig. 3F). Collectively, these data show that SIAH2 is necessary for constitutive C/EBPδ degradation in breast tumor cells and is sufficient to downregulate C/EBPδ in MCF-10A cells. Although other E3 ligases may also target C/EBPδ, the above results led us to focus our subsequent analyses on the role of SIAH2 in regulation of C/EBPδ expression.

Next, we examined if Src could regulate SIAH2 expression. Indeed, ectopic activated Src-531 kinase induced the expression of
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**FIG 2** Src kinase mediates proteasome-dependent C/EBPβ degradation. (A) MCF-10A cells were transfected with expression constructs for HA-tagged C/EBPβ and Src-531 as indicated, and 24 h later cycloheximide (100 μM) was added for the indicated times. Data are from the same blot with the intermediate lane deleted. (B) Western analysis of MCF-10A cells with or without added for the indicated times. Data are from the same blot with the intermediate lane deleted. (B) Western analysis of MCF-10A cells with or without Src-531 and lactacystin (1 μM, 6 h) treatment or from MDA-MB-468 cells treated with MG132 (50 μM, 3 h). (C) MCF-10A cells were transfected with expression vectors for HA-tagged ubiquitin (Ub) and Src-531 and 24 h later treated with MG132. Denatured lysates were immunoprecipitated with anti-HA antibody and immunoblotted for HA tag and for C/EBPβ as an IP control. Inputs were analyzed as indicated. Numbers on the right indicate the position of MW markers.

SIAH2 in MCF-10A cells (Fig. 3G) and, as shown before, down-regulated C/EBPβ expression. Coexpression of DN-SIAH2 attenuated downregulation of C/EBPβ by Src-531 (Fig. 3H). Collectively, these data show that Src-531 inhibits C/EBPβ expression in MCF-10A cells at least in part by inducing SIAH2 expression.

Whereas Src-531 induced SIAH2 in MCF-10A cells, the Src kinase family inhibitor SKI-606 had no effect on SIAH2 expression levels in the breast tumor cell lines MDA-MB-231, MCF-7, and MDA-MB-468 (Fig. 3I). Thus, the higher levels of SIAH2 expression in tumor cell lines (Fig. 3C) appear independent of Src family kinase activity. MCF-7 cells contained less SIAH2 than MDA-MB-468 and MDA-MB-231 cells (Fig. 3C and I), which is accompanied by detectable basal levels of C/EBPβ, albeit much lower levels than those in MCF-10A and MCF-12A cells (Fig. 1). However, Src may also regulate the activity of SIAH2. The highly homologous protein SIAH1 can be phosphorylated on at least three tyrosines. Mutation of all three tyrosines or the pharmacological Src family tyrosine kinase inhibitor PP2 blocks SIAH1-mediated ubiquitination of its target phospholipase Cε (PLCe) (70). Therefore, we next assessed the phosphorylation status of SIAH2. Indeed, SIAH2 was phosphorylated on tyrosine in the breast tumor cell lines, which was reduced upon SKI-606 treatment (Fig. 3I). In response to SKI-606, C/EBPβ expression was induced in all three cell lines shown here. In *vitro* kinase assays showed that Src can indeed phosphorylate SIAH2 directly (Fig. 3J). Phosphopeptide and phosphoamino acid analysis revealed that Y86, Y140, and Y263 were targeted by Src *in vitro* (Fig. 3K). The previously identified tyrosine phosphorylation sites of SIAH1 (70) correspond to amino acids (aa) 86, 239, and 263 of SIAH2. Thus, our analysis confirmed two sites and identified one new one. These data suggest that Src kinases contribute to SIAH2 activation in breast tumor cell lines by promoting its phosphorylation even though SIAH2 expression levels were not affected directly.

To assess the functional role of sites of tyrosine phosphorylation on SIAH2 activity toward C/EBPβ, we individually mutated all six tyrosine residues (aa 86, 140, 163, 166, 239, and 263) to phenylalanine. Transfection of SIAH2 variants into MCF-10A cells revealed that the sites of *in vitro* phosphorylation (Y86, Y140, and Y263) were indeed important for SIAH2 activity (Fig. 3L) because the mutants did not downregulate C/EBPβ to the same extent as wild-type SIAH2. The tyrosine residues Y86 and Y140 are both essential, conceivably because they reside with the critical ring finger and Zn²⁺ finger domains, respectively. The Y263F mutation is located within the C-terminal portion of the protein, which may be involved in folding of the substrate binding domain, and has a more modest effect on SIAH2 activity toward C/EBPβ. Expression in MDA-MB-231 cells showed that the same three mutants led to an increase in endogenous C/EBPβ expression, suggesting a dominant negative effect of the SIAH2 mutants (Fig. 3M). Collectively, these data indicate that tyrosines 86, 140, and 263 contribute to destabilization of C/EBPβ and are targets of phosphorylation in the presence of Src activity.

**SIAH2 interacts with C/EBPβ, leading to its polyubiquitination.** To assess if SIAH2 was directly interacting with C/EBPβ, coimmunoprecipitation was performed in MDA-MB-468 cells. In fact, endogenous C/EBPβ and SIAH2 were found in complex and at increased levels when the cells were treated with MG132, which stabilizes C/EBPβ (Fig. 4A). Next, we examined polyubiquitination of C/EBPβ after immunoprecipitation of ectopic HA-ubiquitin from the tumor cell lines MDA-MB-468 and MDA-MB-231T. As shown in Fig. 4B, high-MW forms of C/EBPβ were detected in MG132-treated cells unless a dominant negative SIAH2 was cotransfected. These data demonstrate that C/EBPβ is polyubiquitinated in an SIAH2-dependent manner in these breast tumor cell lines.

Polyubiquitination occurs commonly on lysine residues, which can also be sites of sumoylation (17). The K120 residue of C/EBPβ has been identified as a site of sumoylation (65) and was therefore a candidate site for ubiquitination. Indeed, a K120 mutation of C/EBPβ prevented both Src-531- and SIAH2-mediated downregulation of C/EBPβ in MCF-10A cells, while a nearby E122A mutation had no effect (Fig. 4C). Although the K120A mutation protected C/EBPβ from degradation, both the wild-type and K120A forms of C/EBPβ were able to interact with ectopic SIAH2 in MCF-10A cells (Fig. 4D, left and right panels, respectively). However, the K120A mutation prevented C/EBPβ polyubiquitination. WT-C/EBPβ alone was relatively stable in MCF-10A cells (Fig. 2A and B) and not significantly polyubiquitinated even in the presence of MG132 (Fig. 4E). Ectopic SIAH2 caused
polyubiquitination of WT-C/EBPα, which accumulated in the presence of MG132. In contrast, no association of ubiquitin with the K120A mutant was detected in the presence of SIAH2 and MG132 (Fig. 4E). These data demonstrate that the K120A residue of C/EBPα is necessary for the polyubiquitination and degradation of C/EBPα in response to Src/SIAH2 signaling even though it is not required for the interaction with SIAH2.

In order to identify the SIAH2 binding region of C/EBPα, we performed coimmunoprecipitation experiments with different chimeric constructs of C/EBPα and C/EBPβ (Fig. 5A) because C/EBPβ is not a target of SIAH2 (Fig. 1F and 4A). Upon cotransfection with SIAH2 into MCF-10A cells, only proteins that contained amino acids (aa) 93 to 181 of C/EBPα were detected in immunoprecipitates of SIAH2. An internal deletion of aa 153 to 199 (64) did not impair the interaction with SIAH2, which further defined the interaction domain to aa 93 to 153 (data not shown). Within this domain, the conserved amino acids A137 and V139 align with the core of the consensus of SIAH substrate recognition motifs (Fig. 5B), which further supports that this region is a bona fide SIAH2 interaction domain (25, 26). Moreover, this sequence is not conserved in C/EBPβ, which did not undergo SIAH2-mediated degradation. Therefore, we introduced two individual point mutations into C/EBPα which change its sequence to that of C/EBPβ (Fig. 5B). The conservative V139A mutation did not impair the interaction of SIAH2 with C/EBPα. Changing A137 to a tyrosine, as in the corresponding sequence of C/EBPβ, completely abolished interaction with SIAH2 (Fig. 5B). Accordingly, A137Y-C/EBPα was protected from SIAH2-mediated downregulation in MCF-10A cells (Fig. 5C). Taken together, these results demonstrate that SIAH2 directly in-

FIG 3 SIAH2 is necessary and sufficient for C/EBPα downregulation. (A) Western analysis of MCF-10A cells transfected with expression constructs for the indicated proteins. (B) MCF-10A cells were transfected with SIAH2, treated with MG132, and analyzed as described in panel A. (C) Western analysis of the indicated cell lines for SIAH1 and SIAH2 expression. (D) Western analysis of MDA-MB-231T (top) and MDA-MB-468 (bottom) cells transfected with the indicated SIAH proteins, which were detected with anti-Flag antibodies. (E) Western analysis of MDA-MB-231T cells after nucleofection of siRNA against SIAH2. (F) Western analysis of MDA-MB-231T cells transfected with a DN-SIAH2 expression construct or nucleofected with siRNA against SIAH2. Anti-Flag was used to detect specifically DN-SIAH2 which has a higher MW than endogenous SIAH2. (G) Western analysis of MCF-10A cells cotransfected with Src-531. (H) Western analysis of MCF-10A cells cotransfected with Src-531 and/or DN-SIAH2. (I) Western analysis of the indicated cell lines treated with SKI-606 for 24 h. The same lysates were immunoprecipitated with anti-SIAH2 and immunoblotted with anti-phospho-tyrosine. (J) In vitro phosphorylation assay conducted by incubating affinity purified Flag-SIAH2 with (+) or without (−) purified Src-530 in the presence of [32P]ATP. Samples were resolved by SDS-PAGE, transferred to nitrocellulose, and subjected to autoradiography (left panel). Labeled SIAH2 was digested from the membrane and subjected to phosphoamino acid analysis (PAA; right panel). Expression of the input SIAH2 by Western analysis is also shown. (K) Results from HPLC analysis of SIAH2 with or without individual tyrosine-to-phenylalanine mutations (see panel L) after in vitro phosphorylation assays as in panel J, compared to unphosphorylated WT SIAH2, Edman degradation, and phosphoamino acid analysis. T, trypsin; T+C, trypsin followed by chymotrypsin. (L) Western analysis of MCF-10A cells transfected with expression constructs for the indicated SIAH2 proteins. (M) Western analysis of MDA-MB-231 cells transfected as described for panel L.
interacts with C/EBPβ, which is necessary but not sufficient for its polyubiquitination and degradation.

Src/SIAH2-induced degradation of C/EBPβ is necessary for cyclin D1 protein expression in breast tumor cell lines, cell motility, and invasion. We have previously shown that ectopic C/EBPβ promotes degradation of cyclin D1 in breast tumor cell lines (47). To address the physiological relevance of Src-mediated degradation of endogenous C/EBPβ, we assessed cyclin D1 expression in response to SKI-606. In agreement with previous reports (15, 69), inhibition of Src family kinase activity in the MDA-MB-468 and MDA-MB-231T breast tumor cell lines resulted in reduced cyclin D1 expression (Fig. 6A and B). However, C/EBPβ silencing by either transient (MDA-MB-468) or stable (MDA-MB-231T) shRNA-mediated depletion attenuated inhibition of cyclin D1 expression by SKI-606 (Fig. 6A and B). As expected, MDA-MB-231T cell motility in a wound closure assay was inhibited by SKI-606 (Fig. 6E). However, when C/EBPβ was silenced, SKI-606 was much less potent, and cells migrated even in the presence of SKI-606, i.e., in the absence of endogenous Src family kinase activity.

To further address the role of SIAH2 in the regulation of cell motility, we silenced SIAH2 in MDA-MB-231 cells and observed that, along with induced C/EBPβ expression, cyclin D1 levels as well as Rb1 phosphorylation declined (Fig. 6H). In addition, RNAi-mediated depletion of SIAH2 alone was sufficient to significantly reduce cell motility of MDA-MB-231T cells in the wound closure assay (Fig. 6H). These data corroborate the role of SIAH2 in silencing the C/EBPβ tumor suppressor pathway in this cancer cell line.

SKI-606 at 1 μM inhibits cell motility and invasion of breast tumor cell lines in vitro. Inhibition of cell growth appears to depend on cell lines and culture conditions (24, 32, 63). In our hands, 1 μM SKI-606 did not inhibit proliferation of MDA-MB-468 cells (Fig. 6C) or MDA-MB-231T cells with or without stable silencing of C/EBPβ (Fig. 6D). As expected, MDA-MB-231T cell motility in a wound closure assay was inhibited by SKI-606 (Fig. 6E). However, when C/EBPβ was silenced, SKI-606 was much less potent, and cells migrated even in the presence of SKI-606, i.e., in the absence of endogenous Src family kinase activity.
Because cyclin D1 was also shown to promote cell motility (41, 73), we overexpressed wild-type cyclin D1 and a degradation-resistant cyclin D1 with a mutation of RK to EE (47). As seen in Fig. 6I, ectopic cyclin D1 was sufficient to promote cell motility (Fig. 6J) and phosphorylation of Rb1 (Fig. 6K) in MCF-10A cells. Ectopic wild-type cyclin D1 was expressed at lower levels than the degradation-resistant mutant (Fig. 6K) but at levels sufficient for biological effects, most likely because endogenous factors for its degradation (47) are limiting. Consistently, we also observed lower levels of total Rb1 protein when cyclin D1 was overexpressed in MCF-10A cells. Collectively, these data show that C/EBPδ-dependent downregulation of cyclin D1 is an important event in the inhibition of cell motility in untransformed MCF-10A cells.

Next, we tested the effect of C/EBPδ on more aggressive tumor cell properties such as migration through Matrigel and growth in soft agar. Similar to the results from the wound-healing assay (Fig. 6E), knockdown of C/EBPδ significantly reduced the ability of SKI-606 to prevent cell migration in a Matrigel transwell invasion assay (Fig. 6F). In agreement with previous reports (63) SKI-606 also impaired the colony formation of MDA-MB-231T cells in soft agar (Fig. 6G). Although SKI-606 still inhibited colony formation even when C/EBPδ expression was silenced, significantly more colonies could still form (Fig. 6G). Taken together, these data show that induction of C/EBPδ expression augments the tumor cell response to SKI-606.

C/EBPδ inhibits Src-mediated transformation of MCF-10A cells. Although cyclin D1 alone is not an oncogene, it can contribute significantly to cell transformation (41). For these reasons we assessed the role of C/EBPδ downregulation on oncogenic Src signaling in MCF-10A breast epithelial cells. Indeed, Src-531 induced expression of cyclin D1 and, as expected, downregulated ectopic WT-C/EBPδ (Fig. 7A). In contrast, the degradation-resistant K120A-C/EBPδ (Fig. 4C) or A137Y-C/EBPδ (Fig. 5C) prevented cyclin D1 induction and attenuated Rb phosphorylation despite the continued presence of Src-531 activity (Fig. 7A). As shown before (Fig. 5C), expression of SIAH2 alone in MCF-10A cells also downregulated wild-type but not mutant C/EBPδ. Consistent with our previous report (47), downregulation of C/EBPδ also resulted in elevated levels of cyclin D1 protein. However, phosphorylation of Rb was less pronounced in response to SIAH2 overexpression than Src-531 (Fig. 7A).

Expression of oncogenic Src induces invasive properties of MCF-10A cells when grown in Matrigel (10). Typically, untransformed MCF-10A cells form hollow, spherical acini when plated in laminin 5 showing that K120A-C/EBPδ as well as A137Y-C/EBPδ restored basal deposition of basement membrane components by Src-531-transfected MCF-10A cells (Fig. 7C). Src-531 caused a greater than 2-fold increase in the size of acini. However, there was no size difference between acini from WT- and K120A-transfected cells with or without Src (data not shown). Similarly, the number of acini was comparable between WT- and K120A-C/EBPδ-

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**FIG 5** Identification of the SIAH2 interaction domain in C/EBPδ. (A) Six different C/EBPδ-C/EBP zipper. (B) Schematic of the sequence of human C/EBPδ and C/EBPα aligned with the consensus for SIAH2 interaction domains and two point mutations in C/EBPδ (underlined amino acids match the consensus motif). Immunoprecipitates with anti-Flag antibody and input were analyzed with antibodies specific for C/EBPδ and C/EBPα and with anti-Flag to detect SIAH2. Cells had been treated with MG132 to stabilize C/EBPδ.

**FIG 6** Expression by Src and SIAH2. (A) Five different C/EBPδ-C/EBP zipper. These chimeric proteins, as shown in the schematic and the full-length proteins, were cotransfected with Flag-SIAH2 in MCF-10A cells. Immunoprecipitates with anti-Flag antibody and input were analyzed with a pan-C/EBP and anti-Flag antibody for SIAH2. Cells had been treated with MG132 to stabilize C/EBPδ. TA, transactivation domain; BR, basic region; LZ, leucine zipper. (B) Schematic of the sequence of human C/EBPδ and C/EBPα aligned with the consensus for SIAH2 interaction domains and two point mutations in C/EBPδ (underlined amino acids match the consensus motif). Immunoprecipitates with anti-Flag antibody and input were analyzed with antibodies specific for C/EBPδ and C/EBPα and with anti-Flag to detect SIAH2. Cells had been treated with MG132 to stabilize C/EBPδ. (C) Western analysis of MCF-10A cells after transfection with the indicated C/EBPδ expression constructs and Flag-SIAH2.
transfected MCF-10A cells with and without Src-531 (data not shown). However, instead of individual cells invading the Matrigel, the acini were distorted and more hollow (Fig. 7B and C).

To further characterize the effect of C/EBPβ on acinus formation of Src-531-expressing cells, we stained for the proliferation marker Ki67 and for active caspase-3 as an indicator of cell death. In the absence of Src-531, 10-day-old acini were negative for Ki67 (data not shown). In the presence of Src-531, Ki67 was detected in all acini whether cotransfected with WT-C/EBPβ or mutant C/EBPβ (Fig. 7D). After 14 days in culture, Ki67 staining persisted in WT-C/EBPβ acini but was lost in the presence of mutant C/EBPβ (data not shown). On the other hand, caspase-3 activity

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was detected in only K120A-C/EBP\(\beta\)- or A137Y-C/EBP\(\beta\)-expressing acini but not with WT-C/EBP\(\beta\) after both 10 days (Fig. 7D) and 14 days of culture (data not shown). Thus, forced C/EBP\(\beta\) expression promotes anoikis of Src-531-expressing MCF-10A cells in Matrigel while having a more modest effect on cell proliferation. The latter may explain why there is no dramatic effect on the size of acini. These results are in agreement with a previously characterized proapoptotic activity of C/EBP\(\beta\) in vivo (61).

In contrast to acinar growth on Matrigel, MCF-10A cells cannot form colonies in soft agar unless transformed by oncogenes, which permits anchorage-independent growth and is one of the features of transformed cells (21, 31). When MCF-10A cells were transfected with Src-531, they were able to form colonies in soft agar. When stable K120A-C/EBP\(\beta\) or A137Y-C/EBP\(\beta\) was coexpressed with Src-531 instead of WT-C/EBP\(\beta\), significantly fewer colonies formed that were also smaller than controls (Fig. 7E). Thus, C/EBP\(\beta\) that escapes downregulation by Src by either loss of SIAH2 interaction (A137Y) or resistance to polyubiquitination (K120A) interferes with full transformation of MCF-10A cells by Src. Collectively, these findings provide evidence that inhibition of C/EBP\(\beta\) expression is an important step for the induction of cyclin D1 expression and for complete transformation of breast tumor cell lines by Src signaling.

**DISCUSSION**

In this study we have identified a molecular mechanism by which C/EBP\(\beta\) protein is downregulated in breast tumor cells (Fig. 8). We show that the Src kinase, which is frequently activated in can-
cer, causes the tyrosine phosphorylation and activation of the SIAH2 E3 ubiquitin ligase, which in turn interacts with C/EBPδ, leading to polyubiquitination and proteasomal degradation of C/EBPδ. This pathway contributes to modulation of cell “transformation,” a term that is used here to comprise enhanced cell migration and invasion, growth in soft agar, loss of basement membrane deposition, and reduced anoikis in vitro. Downregulation of C/EBPδ protein was accompanied by upregulation of the pro-oncogenic protein cyclin D1 and concomitant phosphorylation of the tumor suppressor Rb1. Expression of C/EBPδ interfered with MCF-10A cell transformation by a constitutively activated Src-531 and SKI-606-induced C/EBPδ expression in breast tumor cells contributed to the drug’s effect on invasive properties such as cell motility, transwell migration, and growth in soft agar. Collectively, these data support a tumor suppressor-like function for C/EBPδ in human breast epithelial cells, which modulates the cellular response to Src kinase activity. Previously, we reported that C/EBPδ inhibits expression of the tumor suppressor FBXW7, which leads to activation of the AKT/mTOR/HIF-1 pathway specifically under hypoxia when C/EBPδ expression is induced (7). Both hypoxia and the mTOR/AKT pathway promote tumor progression (59). In fact, C/EBPδ null mice exhibit higher Erbb2 Neu-induced mammary tumor incidence but reduced lung metastasis, suggesting that C/EBPδ can act as both a tumor suppressor and tumor promoter (7). Given that these results were obtained with mice carrying a germ line mutation of C/EBPδ, both activities could be due to functions in tumor cells and/or cells of the tumor environment. The data reported in the present study support a tumor suppressor-like function of C/EBPδ within breast epithelial cells under normoxic conditions. As both Src and SIAH are activated under hypoxia (19, 27), we hypothesize that hypoxia provides additional signals that prevent SIAH-mediated C/EBPδ degradation and may also modulate the downstream effects of C/EBPδ.

We began this study with the hypothesis that Src regulates the C/EBPδ gene promoter. In transient cotransfection experiments activated Src, indeed, inhibited a C/EBPδ promoter-reporter construct. Thus, we do not rule out that Src can also inhibit C/EBPδ transcription, perhaps through activation of c-myc (72). However, we show that regulation of protein stability is an important mechanism controlling C/EBPδ expression levels in breast tumor cell lines, which acts subsequent to any regulation of promoter activity. A previous report had shown that C/EBPδ is degraded by the proteasome in vitro but suggested that this was ubiquitin independent (74). The same study reported a short half-life (120 min) for C/EBPδ in growth-arrested HC11 mouse mammary epithelial cells although C/EBPδ expression is in fact induced at G0 (74). In contrast, we found that C/EBPδ was rather stable in untransformed MCF-10A cells and that the SIAH2 E3 ligase and polyubiquitination were necessary for reduced C/EBPδ protein levels in human breast tumor cell lines. Among C/EBP family members, this is a unique pathway because the A137 residue of C/EBPδ, which was necessary for interaction with SIAH2, is replaced by tyrosine or phenylalanine in other C/EBP proteins. To our knowledge, among C/EBP proteins, ubiquitin-mediated degradation has been reported only for C/EBPβ via interaction with FBXW7 (9, 57), which—as mentioned above—is a C/EBPδ repressed gene (7).

SIAH ubiquitin ligases can require cofactors for interaction with and targeting of substrates (55, 68), and substrate interaction may depend on extracellular signals, such as EGF, as described for the substrate PLCζ (70). We found that the K120 residue of C/EBPδ was necessary for its polyubiquitination. Given that we have not demonstrated polyubiquitination at K120 directly, other explanations for the phenotype of the K120A mutation could be loss of phosphorylation or cofactor interaction necessary for ubiquitination at other lysine residues.

Previous studies showed that K120 was necessary for sumoylation of C/EBPδ and its interaction with HDAC1 and HDAC3 to repress gene expression (65). In our experiments, K120A-C/EBPδ was able to downregulate cyclin D1 expression and to partially prevent Src-531 transformation of MCF-10A cells. Therefore, we conclude that sumoylation at K120 is not necessary for these tumor suppressor-like activities. However, sumoylation or acetylation at K120 could be a physiological mechanism to prevent C/EBPδ degradation. Given that C/EBPδ expression is often only transiently induced by various stimuli, we speculate that protein (de)stability may play a significant role in other cellular systems such as the inflammatory response (51) or hypoxia (7).

Destabilization of C/EBPδ in breast epithelial cells was dependent on Src kinase signaling. While ectopic Src-531 in MCF-10A cells mimicked many of the activities attributed to SKI-606 inhibited kinase activities in tumor cell lines, it is very likely that other Src family kinases may also contribute to C/EBPδ degradation. This will probably depend on the ability of such other kinases to induce expression and/or activity of the SIAH2 ligase or other necessary cofactors. Also, it remains to be determined why SIAH2 overexpression was sufficient to downregulate C/EBPδ in MCF-10A cells even though these cells do not exhibit significant levels of endogenous phosphorylated Src kinases. Possibly, the transient lower levels of endogenous Src family kinase activity are sufficient for activation of SIAH2, or other kinases that alone do not induce expression of endogenous SIAH2 can contribute to its catalytic activation, such as p38 mitogen-activated protein kinase (MAPK) (34). We found that activated Src phosphorylated SIAH2 in vitro

![Diagram](https://example.com/diagram.png)
on three tyrosines, each of which contributes to SIAH2 activity in vivo. It remains to be determined if Src and other Src family kinases mediate the direct activation of SIAH2 in vivo and how the tyrosine phosphorylations functionally interact with SIAH2 activation by p38 MAPK (34).

In animal and xenograft models, SIAH2 was characterized as a tumor promoter (4, 27, 50, 56). A recent report using SIAH2-specific immunostaining showed that SIAH2 is most highly expressed in advanced, basal-like breast cancer due to gene amplification (14). Similarly, cytoplasmic Src family kinase activity is associated with more aggressive breast cancer and also shorter patient survival (19). In vitro data support the notion that this may be due to the promotion of invasive features by Src (54) and is in part mediated by elevated levels of cyclin D1 (41). We found that downregulation of C/EBPδ augmented Src-induced cyclin D1 protein expression in breast tumor cell lines. We previously reported that C/EBPδ promotes cyclin D1 degradation through upregulation of Cdc27/APP3 expression (47). We do not rule out that transcriptional mechanisms contributed to the cyclin D1 regulation observed in the present study. However, even when cyclin D1 mRNA levels are high, continued expression of C/EBPδ may promote cyclin D1 degradation (47). Cyclin D1 is overexpressed in different cancers, including breast, and many studies have associated cyclin D1 with malignant cell features and tumor progression (35) including cell motility (41). In some reports, cyclin D1 was associated with reduced cell migration and more favorable prognosis (39), which may depend on other cofactors such as splice variants and availability of p27KIP1 (40). Thus, the precise role of cyclin D1 downregulation in inhibition of cell transformation by C/EBPδ signaling remains to be determined.

Lastly, SKI-606 (bosutinib) shows very promising activity in clinical and preclinical trials (12). Our results raise the possibility that C/EBPδ and its downstream mediators may be critical for therapeutic response and that further investigations into this pathway could lead to the identification of predictive biomarkers.

In conclusion, this study revealed a novel signaling pathway, which links Src kinase(s), the SIAH2 E3 ubiquitin ligase, and the C/EBPδ transcription factor and shows that this pathway contributes to transformed features of breast epithelial cell lines.

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