Deletion of Na\(^{+}/\)H\(^{+}\) exchanger regulatory factor 2 represses colon cancer progress by suppression of Stat3 and CD24

Michihiro Yoshida, Luqing Zhao, Gevorg Grigoryan, Hyunsuk Shim, Peijian He, and C. Chris Yun

1Division of Digestive Diseases, Department of Medicine, Emory University, Atlanta, Georgia; 2Department of Gastroenterology and Metabolism, Nagoya City University Graduate School of Medical Sciences, Nagoya, Japan; 3Division of Gastroenterology, Department of Medicine, Beijing Hospital of Traditional Chinese Medicine Affiliated to Capital Medical University, Beijing, China; 4Department of Computer Science, Dartmouth College, Hanover, New Hampshire; 5Winship Cancer Institute, Emory University, Atlanta, Georgia; and 6Department of Radiology and Imaging Sciences, Emory University, Atlanta, Georgia

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Yoshida M, Zhao L, Grigoryan G, Shim H, He P, Yun CC. Deletion of Na\(^{+}/\)H\(^{+}\) exchanger regulatory factor 2 represses colon cancer progress by suppression of Stat3 and CD24. Am J Physiol Gastrointest Liver Physiol 310: G586–G598, 2016. First published February 11, 2016; doi:10.1152/ajpgi.00419.2015.—The Na\(^{+}/\)H\(^{+}\) exchanger regulatory factor (NHERF) family of proteins is scaffolds that orchestrate interaction of receptors and cellular proteins. Previous studies have shown that NHERF1 functions as a tumor suppressor. The goal of this study is to determine whether the loss of NHERF2 alters colorectal cancer (CRC) progress. We found that NHERF2 expression is elevated in advanced-stage CRC. Knockdown of NHERF2 decreased cancer cell proliferation in vitro and in a mouse xenograft tumor model. In addition, deletion of NHERF2 in Apc\(^{Mino}\)/H11001 mice resulted in decreased tumor growth in Apc\(^{Mino}\)/H11001 mice and increased lifespan. Blocking NHERF2 interaction with a small peptide designed to bind the second PDZ domain of NHERF2 attenuated colorectal cancer cell proliferation in vitro and in a mouse xenograft tumor model. In addition, deletion of NHERF2 in Apc\(^{Mino}\)/H11001 mice resulted in decreased tumor growth in Apc\(^{Mino}\)/H11001 mice and increased lifespan. Blocking NHERF2 interaction with a small peptide designed to bind the second PDZ domain of NHERF2 attenuated colorectal cancer cell proliferation. Although NHERF2 is known to facilitate the effects of lysophosphatidic acid receptor 2 (LPA2), transcriptome analysis of xenograft tumors revealed that NHERF2-dependent transcriptionally up-regulated genes largely differ from LPA2-regulated genes. Activation of β-catenin and ERK1/2 was mitigated in Apc\(^{Mino}\)/Nherf2−/−/H11001 adenomas. Moreover, Stat3 phosphorylation and CD24 expression levels were suppressed in Apc\(^{Mino}\)/Nherf2−/−/H11001 adenomas. Consistently, NHERF2 knockdown attenuated Stat3 activation and CD24 expression in colon cancer cells. Interestingly, CD24 was important in the maintenance of Stat3 phosphorylation, whereas NHERF2-dependent increase in CD24 expression was blocked by inhibition of Stat3, suggesting that NHERF2 regulates Stat3 phosphorylation through a positive feedback mechanism between Stat3 and CD24. In summary, this study identifies NHERF2 as a novel oncogenic protein and a potential target for cancer treatment. NHERF2 potentiates the oncogenic effects in part by regulation of Stat3 and CD24.

colorectal cancer; NHERF2; Stat3; CD24

to prolong mGluR5-mediated calcium mobilization and potenti ate neuronal cell death, and exogenous NHERF2 negatively regulates endothelial cell proliferation (2, 30).

In this study, we explored the role of NHERF2 in colorectal cancer (CRC). We show that NHERF2 expression levels are elevated in human CRC tissues and the loss of NHERF2 markedly decreases CRC cell proliferation, suggesting an oncogenic role of NHERF2.

MATERIALS AND METHODS

Cell culture and plasmids. The human colon cancer HCT116, SW480, and HT-29 cells were obtained from the ATCC. HCT116 was cultured in McCoy’s 5A medium, and SW480 and HT-29 cells in RPMI 1640 medium, all supplemented with 10% FBS as previously described (18). pLKO.1 plasmids harboring short hairpin RNA (shRNA) targeting NHERF2 (shNHERF2), LPA3 (shLPA3), or CD24 (shCD24) were from Sigma (St. Louis, MO). pLKO.1-puro with nontarget shRNA (shCon) was used to generate control lentivirus. Stable knockdown was achieved by transduction with lentivirus and selection with puromycin as described previously (22).

Cell proliferation assay. Cells were seeded at 3.0 × 104 cells/well in a complete media containing 10% FBS. Cells were counted on days 2, 3, and 4 with a hemocytometer.

In vivo tumor growth. Five- to 6-wk-old athymic female nude mice (n = 5, Harlan, Indianapolis, IN) were injected subcutaneously on both sides in the flank region with HCT116 cells (2.0 × 106 cells in 0.2 ml PBS) stably transfected with shNHERF2, shLPA3, or shCon. Tumor size was measured twice a week with calipers, and volume was calculated as length × width2 × 0.5. On day 21, all mice were euthanized, and tumor xenografts were quickly removed and either frozen in liquid nitrogen or fixed in formalin. Experiments with animals were carried out under approval by the Institutional Animal Care and Use Committee of Emory University and in accordance of the NIH Guide for the Care and Use of Laboratory Animals.

Generation of ApcMin+/−;Nherf2−/+ mice. Nherf2−/+ mice in C57BL/6 background were previously described (22). ApcMin+ mice were purchased from Jackson Laboratory (Bar Harbor, ME). ApcMin+/+, Nherf2−/+ females were used to generate ApcMin−/−, ApcMin+/−, and ApcMin+−/− females, which were subsequently mated with Nherf2−/+ females to generate ApcMin−/−, ApcMin+/−, ApcMin+−/−, and ApcMin−/+ mice. Littermate females were used in all studies. Mice were euthanized at 9 and 14 wk of age (n = 6 mice per group). The entire intestine was removed, and polyp numbers and size were measured under a dissecting microscope in a blinded manner.

NHERF2 blocking peptide. Design and generation of the peptide FD2 linked to fluorescein isothiocyanate (FITC-AhxGGGSGSTRF, where Ahx is aminohexanoic acid) that interferes with the 2nd PDZ domain of NHERF2 was described previously (48). A scrambled 8-mer peptide (sc-FD2, FITC-Ahx-GGFTGSGR) was used as a control. All peptides linked to FITC at the NH2-terminus with unmodified COOH-terminus were synthesized by GenScript (Piscataway, NJ). HCT116 cells were seeded at 1.5 × 105 cells per well in McCoy’s 5A containing 5% FBS. On days 1 and 2, peptides at 10, 50, or 100 µg/ml were added to the cells with BioPORTER Protein Delivery Reagent (Genlantis, San Diego, CA) according to the manufacturer’s instruction. Cells were counted on day 3 with a hemocytometer.

Immunohistochemistry. Immunohistochemical (IHC) analysis was performed as previously described with modifications (20, 21). Mouse intestine sections were flushed with cold PBS and were fixed overnight in 10% buffered formalin (Fisher). Intestinal tissues were paraffin embedded and sectioned at 4 µm. Tissue sections were deparaffinized and dehydrated in a graded series of xylene and ethanol. Antigen retrieval was achieved by heating the samples in 10 mM citrate buffer, pH 6.0 with 0.05% Tween 20, in a pressure cooker for 10 min at 125°C. After inhibition of endogenous peroxidase activity by immersion in 3% H2O2/methanol solution, tissue sections were blocked with goat serum and incubated with primary antibody overnight at 4°C. After three washes with PBS, sections were incubated with trinitiated secondary antibody and then with avidin-biotin horseradish peroxidase solution (Vector Laboratories, Burlingame, CA). Finally, tissue sections were incubated with 0.01% H2O2 and 0.05% 3,3′-diaminobenzidine tetrachloride. Nuclear counterstaining was accomplished with Mayer’s hematoxylin.

Ki67 and cleaved caspase-3 staining. Rabbit polyclonal anti-Ki67 antibody (NCL-Ki67p, 1:1,000 dilution, Leica, Newcastle, UK) and rabbit monoclonal anti-cleaved caspase-3 antibody (no. 9664, 1:2,000, Cell Signaling, Danvers, MA) were used for staining as described above. Proliferating and apoptotic cells were quantified by counting Ki67-positive and cleaved caspase-3-positive cells, respectively. Ten fields of view at a magnification of ×400 were scanned per tumor in xenograft tumors, and ~15 tumors were scanned per ApcMin+−/− mouse.

Human colon cancer TMA. Human colon cancer tissue microarray (TMA) slides were purchased from OriGene (Rockville, MD). Immunohistochemical staining was performed as described above with rabbit anti-NHERF2 polyclonal antibody (HPA001672, 1,250 dilution, Sigma). The histological scoring of NHERF2 expression was performed as previously described (16, 23). Briefly, the extent of NHERF2 positive was graded as follows: 0, none; 1, 1–25%; 2, 26–50%; 3, 51–75%; and 4, 76–100%. The intensity of cytoplasm and nucleus immunostaining was classified as 0 (negative), 1 (weak), 2 (moderate), and 3 (strong). All specimens were examined by two investigators who were blinded to all clinical information.

qRT-PCR. Total RNA was isolated from cells or tumor tissues by using an RNaseasy Mini Kit (Qiagen), and cDNA was synthesized by use of the SuperScript III First Strand Synthesis kit (Invitrogen). Quantitative RT-PCR (qRT-PCR) was performed as described (18). The amounts of CD24 mRNA were normalized to β-actin. The following primer pairs were used: CD24, human: 5′-CTCTTACACCGACGATTATTC-3′ and 5′-AGAGTGAGACCCAGGAGGAC-3′; CD24, mouse: 5′-GTTGCACTTTGGCTCGGTA-3′ and 5′-CCCCTCTGTGAGTACGTTA-3′; β-actin, human: 5′-CGTGGCGTACATTAAGGAGA-3′ and 5′-AGAGAAGAGGCTGAGGAG-3′; β-actin, mouse: 5′-GGCTGTATCTCCTTCTCAGTC-3′ and 5′-CCAGTTGGAACAACTGCAATGT-3′.

RNA sequencing analyses of xenografts. RNA sequencing (RNA-Seq) and data analysis were performed at the Emory Integrated Genomics Core. Briefly, total RNA from xenograft tumors (n = 3/ group) was extracted by use of the Qiagen miRNasey Mini Kit (Valencia, CA). Double-stranded cDNA libraries were prepared according to the NEBNext RNA-Seq protocol and sequenced on an Illumina 2000 HiSeq using v3 chemistry according to the Illumina Sequencing User’s Guide (San Diego, CA); 50 million 100-bp paired-end reads were generated per library. Base calling and FASTQ were done with Illumina’s HiSeq Control Software version 1.5.15.1 (RTA v1.13.18 and bclfastq v1.8.3) (6). The RNA-Seq data have been deposited to the Gene Expression Omnibus with the data identifier GSE67931.

Western blot and antibodies. Western blot was performed as previously described (21). Blots were visualized with a Li-COR Odyssey Imager. Rabbit polyclonal anti-NHERF2 and anti-NHERF1 antibodies were described previously (45). Additional rabbit polyclonal anti-NHERF2 (HPA001672, 1,250 dilution for IHC) and mouse monoclonal anti-β-actin (A1978, 1:5,000 for Western blot) antibodies were obtained from Sigma. Rabbit monoclonal anti- phospho-ERK1/2 (p-ERK1/2) (no. 4370, 1:2,000 for Western blot and 1:400 for IHC); mouse monoclonal anti-ERK1/2 (no. 4696, 1:2,000 for Western blot), rabbit monoclonal anti-phospho-Stat3 (no. 9145, 1:2,000 for Western blot and 1:400 for IHC), and rabbit monoclonal anti-ERK1/2 (p-ERK1/2) (no. 4370, 1:2,000 for Western blot and 1:400 for IHC).
anti-Stat3 (no. 4904, 1:2,000 for Western blot) antibodies were purchased from Cell Signaling.

Statistical analysis. Statistical significance was assessed by two-tailed unpaired Student’s t-test, Welch’s t-test, Mann-Whitney’s U-test, or mxn/H92732 test. Overall survival curves were generated by the Kaplan-Meier methods and differences were evaluated with the log-rank test. Values are expressed as means ± SE. A P value of <0.05 was considered statistically significant.

RESULTS

NHERF2 expression is upregulated in advanced human CRC. We showed previously that NHERF2 expression was elevated in CRC, but we could not find changes in NHERF2 expression levels in different stages of CRC (18). To determine whether NHERF2 expression varies during CRC progress, we analyzed NHERF2 expression in a colon cancer TMA from another source. The TMA comprises a cohort of 41 colon cancer specimens of different clinical stages. We detected NHERF2 expression in 36 of 41 specimens (88%). NHERF2 was detected in the nuclei and cytoplasm of epithelial cells within the tumors where more than 80% of the total area was NHERF2 positive (Fig. 1A). To determine whether NHERF2 expression varies during CRC progress, we analyzed NHERF2 expression in a colon cancer TMA from another source. The TMA comprises a cohort of 41 colon cancer specimens of different clinical stages. We detected NHERF2 expression in 36 of 41 specimens (88%). NHERF2 was detected in the nuclei and cytoplasm of epithelial cells within the tumors where more than 80% of the total area was NHERF2 positive (Fig. 1A).

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Table 1. Patient characteristics

<table>
<thead>
<tr>
<th>NHERF2 Expression</th>
<th>High, n = 36</th>
<th>Low, n = 5</th>
<th>P</th>
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<tr>
<td>Sex</td>
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<td></td>
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</tr>
<tr>
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<td>20</td>
<td>2</td>
<td>0.51</td>
</tr>
<tr>
<td>female</td>
<td>16</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Age, yr</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>median (range)</td>
<td>70 (31–90)</td>
<td>80 (67–89)</td>
<td>0.19</td>
</tr>
<tr>
<td>Primary tumor site</td>
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<td></td>
<td></td>
</tr>
<tr>
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<td>2</td>
<td>0.4</td>
</tr>
<tr>
<td>transverse colon</td>
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</tr>
<tr>
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<tr>
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<td>0</td>
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</tr>
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<td></td>
</tr>
<tr>
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<td>32</td>
<td>4</td>
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</tr>
<tr>
<td>diffuse</td>
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<td>1</td>
<td></td>
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<td>Stage of primary tumor</td>
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<tr>
<td>pT1, T2</td>
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<td>0.002*</td>
</tr>
<tr>
<td>pT3, T4</td>
<td>30</td>
<td>1</td>
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</tr>
</tbody>
</table>

High, high NHERF2 expression; Low, low NHERF2 expression. *Significant difference.
and HT-29 cell lines with stable knockdown of NHERF2 by transduction with lentiviral shNHERF2 or shCon. Both SW480 and HT29 cells have mutated Apc gene, whereas HCT116 cells are heterozygous for β-catenin, harboring one WT allele and one mutant allele (26). As a result, all the three cell lines have activation of the Wnt-β-catenin pathway. In all cell lines, knockdown efficiency of ≥80% was achieved without affecting NHERF1 expression (Fig. 2A). Importantly, shNHERF2-transduced cells proliferated at lower rates than control cells, demonstrating that NHERF2 positively regulates cancer cell proliferation (Fig. 2B). Therefore, HCT116 cells were used primarily in the following experiments.

To confirm the role of NHERF2 on cancer cell proliferation, we implanted HCT116 cells transduced with shNHERF2 or shCon into the flanks of nude mice. During the course of 21 days, the growth of shNHERF2 xenograft tumor was consistently slower compared with shCon xenografts (n = 10 per group). The average size of shNHERF2 xenograft tumors was significantly smaller compared with control (tumor size in mm³: 603.3 ± 113.5 vs. 2,013 ± 214.1, P < 0.001; tumor weight in g: 0.73 ± 0.11 vs. 1.55 ± 0.15, P < 0.001) (Fig. 2, C–D). Immunostaining tumor sections for Ki67 revealed NHERF2 depletion decreased the number of Ki67-positive cells almost by half (Fig. 2E; Ki67 index: 41.7 ± 1.83 vs. 69.6 ± 1.58, P < 0.001), consistent with the results of in vitro proliferation assay. However, there was no significant difference in the number of apoptotic cells (Fig. 2F). Together, these results suggest that NHERF2 stimulates colon cancer cell proliferation.

Blocking the second PDZ domain of NHERF2 with a small peptide decreases cancer cell proliferation in vitro. The primary mode of NHERF2-dependent functions is protein-protein interaction via its PDZ domains. To explore the idea of NHERF2 as a new molecular target for cancer therapy, we determined the effect of interrupting PDZ interaction on cell proliferation. We have recently reported designing of peptides...
based on a computational design framework that models peptide flexibility in binding of PDZ domain, and the specific peptide FD2 showed a selective preference to bind the second PDZ domain of NHERF2 (48). To deliver peptides into HCT116 cells, we used BioPORTER protein delivery reagent. Because FD2 was linked to FITC, we gauged peptide uptake by increased fluorescence levels in cells. Peptide alone had a marginal change in fluorescence levels within the cells, whereas BioPORTER significantly increased peptide uptake (Fig. 3A). To determine whether FD2 alters cancer cell proliferation, the peptides were added to the culture media daily. BioPORTER alone or FD2 without BioPORTER did not significantly alter cell proliferation (Fig. 3B). Similarly, control scFD2 with BioPORTER had no effect. On the other hand, FD2 complexed with BioPORTER decreased proliferation in a dose-dependent manner ($10^{-6}$, $10^{-5}$, $10^{-4}$, and $10^{-3}$ μg/ml, 0.68 ± 0.04; 100 μg/ml, 0.66 ± 0.05, $P < 0.01$). These results demonstrate that interfering PDZ interaction of NHERF2 decreases colon cancer cell proliferation.

The absence of NHERF2 reduces tumor growth in Apc$^{Min/+}$ mice. To further ascertain the role of NHERF2 in intestinal tumorigenesis, we used the Apc$^{Min/+}$ model to determine the effect of loss of NHERF2 function. We compared the number and size of adenomas that developed in Apc$^{Min/+}$, Apc$^{Min/+}$; Nherf2$^{-/-}$, and Apc$^{Min/+}$;Nherf2$^{-/-}$ mice at 9 and 14 wk of age. Surprisingly, there was no significant difference in the number of tumors among three strains, with Apc$^{Min/+}$, Apc$^{Min/+}$; Nherf2$^{+/+}$, and Apc$^{Min/+}$;Nherf2$^{-/-}$ mice having an average of 31.2 ± 3.8, 30.8 ± 2.9, and 32.7 ± 2.9 tumors per mouse in the small intestine at 9 wk (Fig. 4A). Similarly, the number of colonic adenomas was not altered by the absence of NHERF2. Although tumor numbers increased in all strains at 14 wk, again no variation among three strains was observed. On the other hand, there was a significant shift in the tumor size in the small intestine in the absence of NHERF2. Apc$^{Min/+}$; Nherf2$^{-/-}$ mice developed smaller tumors compared with Apc$^{Min/+}$ mice at 9 wk of age (mean size in mm, 1.01 ± 0.02 for Apc$^{Min/+}$;Nherf2$^{-/-}$ vs. 1.28 ± 0.04 for Apc$^{Min/+}$, $P < 0.01$) (Fig. 4B). At 14 wk, the difference in mean tumor size between Apc$^{Min/+}$;Nherf2$^{-/-}$ and Apc$^{Min/+}$ mice was greater with 1.19 ± 0.05 mm for Apc$^{Min/+}$;Nherf2$^{-/-}$ mice compared with 2.00 ± 0.10 mm for Apc$^{Min/+}$ mice ($P < 0.001$). Although there was no significant change in colonic tumor size at 9 wk, tumors in Apc$^{Min/+}$;Nherf2$^{-/-}$ mice at 14 wk were significant smaller compared with those in Apc$^{Min/+}$ mice (mean size in mm, 1.70 ± 0.21 for Apc$^{Min/+}$;Nherf2$^{-/-}$ vs. 2.54 ± 0.19 for Apc$^{Min/+}$, $P < 0.05$). No difference between Apc$^{Min/+}$ and Apc$^{Min/+}$;Nherf2$^{-/-}$ mice was observed. Figure 4C depicts a shift in tumor sizes from $\approx 1$ to $\approx 1$ mm from 9 to 14 wk of age in both Apc$^{Min/+}$ and Apc$^{Min/+}$;Nherf2$^{-/-}$ mice, whereas half

![Image](http://ajpgi.physiology.org/)

**Fig. 3.** Blocking NHERF2 PDZ interaction attenuates cell proliferation. A: uptake of FD2 peptide by HCT116 cells was determined. Cells were loaded with FD2 alone, FD2+BioPORTER, or BioPORTER alone for 4 h. FD2 was conjugated with FITC, and uptake of FD2 by the cells was assessed by fluorescence microscopy. F-actin and nuclear (DAPI) staining show the location of cells. B: proliferation of HCT116 cells in the presence of a peptide that blocks NHERF2 interaction was determined. Cells were seeded in a complete medium, and the PDZ blocking peptide, FD2, or control scrambled peptide, scFD2, was added to the medium next day. Media and peptides at a concentration indicated were replaced daily and cell numbers were counted on day 3. BioPORTER was used as the carrier of the peptide as described in the text. The rate of cell proliferation under each condition is presented relative to the rate in the absence of BioPORTER or peptide. Data represent means ± SE from 4 independent experiments in triplicate. *$P < 0.01$ vs. control without BioPORTER peptide.
of the tumors in ApcMin+/Nherf2−/− mice still remained less than 1 mm in size and ~5% were greater than 2 mm at 14 wk. Because the absence of NHERF2 decreased tumor growth in ApcMin/− mice, we determined whether NHERF2 affects overall survival of ApcMin/− mice. Figure 4D shows that ApcMin+/−; Nherf2−/− mice had a longer lifespan than ApcMin+/− mice [median survival time (MST) in days: 152 vs. 131, P < 0.05]. MST of ApcMin+/−; Nherf2+/− mice was not different from that of ApcMin+/− mice (132 vs. 131). These results suggest that the absence of NHERF2 decreases tumor growth and improves the survival of ApcMin+/− mice. However, the lack of difference in tumor numbers indicated that NHERF2 does not influence the tumor initiation.

NHERF2-dependent gene regulation is largely independent of LPA. We have shown previously that NHERF2 modulates LPA2 functions. However, the effects of tumor growth without altering tumor numbers by NHERF2 differ from the LPA2 study, in which both tumor growth and numbers in ApcMin+/− mice were decreased (20, 21). To ascertain that the functions of NHERF2 are not primarily dependent on LPA2, we compared xenograft tumor growth of HCT116 transduced with shLPA2 (32, 43). The rate of shLPA2 tumor growth was markedly slower than shCon, consistent with a previous study (41). To investigate further whether the NHERF2-dependent effects are a subset of LPA2-mediated oncogenic effects, we performed genome-wide RNA-Seq of HCT116/shNHERF2, HCT116/shLPA2, and HCT116/shCon tumors. If NHERF2-mediated effects are primarily dependent on LPA2-mediated signaling, we would expect a significant overlap between the transcriptome profiles of shNHERF2 and shLPA2 tumors. A comparison of transcript sequences of shNHERF2 and shCon tumors revealed that 106 genes (P < 0.01, fold change > 1.5) were upregulated, whereas 70 (P < 0.01, fold change > 1.5) were downregulated in shNHERF2 tumor compared with control (Fig. 5 and Supplementary Tables S1 and S2). Similarly, a total of 109 genes were upregulated, while 137 genes were downregulated in shLPA2 tumor compared with control (Fig. 5 and Supplementary Tables S3 and S4). Importantly, a little over 10 genes were increased or decreased equally in both shNHERF2 and shLPA2 tumors. These results appear to suggest that, although the growth patterns of shNHERF2 and shLPA2 xenograft tumors are similar, the genetic programming by NHERF2 and LPA2 only partially overlaps, suggesting that the molecular basis of NHERF2-dependent cancer growth probably differs from that of LPA2.
Reduced proliferation is associated with decreased β-catenin and ERK activation. The transcriptome analysis of shNHERF2 xenograft tumor identified a number of Wnt pathway-associated genes, including Sox4, Id2, TACSTD2, ZC-CHCH12, and IGFBP6 (Supplementary Tables S1 and S2). Hence, we determined whether the loss of NHERF2 affected Wnt pathway activation in ApcMin mice. We did not find a difference in β-catenin expression between ApcMin/+ and ApcMin/+;Nherf2−/− mice (Fig. 6A), but IHC analysis shows that the loss of NHERF2 decreased the nuclear expression of β-catenin (Fig. 6B), indicating that NHERF2 modulates β-catenin activation. Previous studies have shown that NHERF2 is associated with activation of ERK in Caco-2 and Rat1 embryonic fibroblasts (14, 43). In addition, the Wnt pathway interacts with the ERK pathway to potentiate carcinogenesis (5, 13). Hence, we determined whether ERK activation was altered by NHERF2 in ApcMin/+ mice. There was a pronounced decrease in the expression level of phospho-ERK1/2 in ApcMin/+;Nherf2−/− compared with ApcMin/+ mice without a change in ERK1/2 abundance (Fig. 6, A and B). In line with the decreased Wnt and ERK signaling, the number of proliferating cells identified by Ki67 staining was significantly reduced in ApcMin/+;Nherf2−/− compared with ApcMin/+ mice (Ki67 index, %: 32.2 ± 1.94 for ApcMin/+;Nherf2−/− vs. 54.5 ± 3.43 for ApcMin/+; P < 0.001) (Fig. 6C).

Decreased CD24 expression and Stat3 activation in the absence of NHERF2. We have shown above that depletion of NHERF2 led to reprogramming of a large number of genes. Among the downregulated genes, CD24, a glycosylated protein that is linked to the membrane via a glycosyl-phosphatidyinositol anchor, has been shown to be frequently overexpressed in a variety of human carcinomas (33). It has been shown that deletion of CD24 attenuates colon cancer development in rodent models (28). Silencing of NHERF2 reduced CD24 mRNA levels in HCT116 cells (Fig. 7A). The same results obtained in SW480 and HT29 cells (not shown). Additionally, we observed decreased CD24 expression in ApcMin/+;Nherf2−/− mice (Fig. 7, B and C). To assess the role of CD24, we knocked down CD24 and determined proliferation of HCT116 cells. Knockdown of CD24 did not alter NHERF2 expression (Fig. 7D), but it decreased the rate of HCT116 cell proliferation (Fig. 7E). Simultaneous depletion of NHERF2 and CD24 had a greater effect than knockdown of CD24 or NHERF2 alone. The additive effect of CD24 and NHERF2 was not surprising in light of other effects of NHERF2, such as ERK1/2 and β-catenin, which are likely be independent of CD24.

The activity of signal transducer and activator of transcription 3 (Stat3) is closely linked to progression and metastasis of various cancers, including gastrointestinal cancer (31). It has been shown that CD24 knockdown results in decreased Stat3 expression and activity in human cancer cells (4). Hence, we investigated whether NHERF2 may regulate Stat3 activation through a change in CD24 expression. Stat3 activation requires phosphorylation of Stat3 on Tyr-705, which moves Stat3 into the nucleus to change gene expression of the cell. We confirmed that CD24 knockdown in HCT116 cells decreased basal phospho-Stat3 levels without altering Stat3 expression, providing potential linkage between CD24 and Stat3 (Fig. 8A). Consistent with knockdown of CD24 in HCT116 cells, the level of phospho-Stat3 activation determined by immunoblotting and immunohistochemical analysis was markedly lower in ApcMin/+;Nherf2−/− mice compared with ApcMin/+ mice (Fig. 8, B and C).

CD24 has been suggested as a potential cancer stem cell marker (8, 12), but the regulatory pathways that modulate CD24 expression are not well known. On the contrary, the JAK-Stat3 pathway is robustly regulated by IL-6, which links inflammation and cancer (10). Hence, we next evaluated whether IL-6 regulates Stat3 and CD24 through a NHERF2-dependent mechanism. IL-6 acutely increased phospho-Stat3 level in HCT116 cells without altering Stat3 expression (Fig. 8D). In comparison, knockdown of NHERF2 significantly attenuated baseline and IL-6-induced Stat3 activation. Similar results were obtained in SW480 and HT29 cells (not shown). To determine whether the effect of NHERF2 knockdown on Stat3 phosphorylation is specific to IL-6, we assessed interferon α (IFNα)-induced activation of Stat3. Similarly, knockdown of NHERF2 attenuated Stat3 activation by IFNα in HCT116 cells (Fig. 8E), implying that NHERF2 plays a broader role as a modulator of Stat3 activation. It has been shown recently that LPA induces Stat3 phosphorylation in ovarian cancer OVACAR-3 cells (34), but we could not observe LPA-dependent effect on phospho-Stat3 in any of the colon cancer cell lines used in this study.

Although Stat3 is regulated by IL-6 and IFNα in a NHERF2-dependent manner, whether CD24 is similarly regulated is not known. We next determined whether CD24 expression is regulated using IL-6 as a prototypic agonist. Figure 9, A and B shows that IL-6 had an small but statistically significant increase in CD24 mRNA expression, which was ablated by NHERF2 knockdown. Comparison of data in Fig. 8D and Fig. 9A indicated that the kinetics of Stat3 activation was greater compared with CD24 regulation (<1/2 h for Stat3 vs. 3 h for CD24). Hence, we next tested whether Stat3 is involved in the regulation of CD24 transcripts by IL-6. Inhibition of Stat3 by the Stat3 inhibitor STA-21 completely blocked the transcriptional activation of CD24 by IL-6, indicating that IL-6 regulates CD24 expression through activation of Stat3 (Fig. 9C). Silencing of NHERF2 in the presence of STA-21 further lowered the expression levels of CD24 mRNA. Cells with CD24 knocked down were treated with IL-6 to evaluate the role of CD24 on Stat3 activation. Knockdown of CD24 significantly decreased Stat3 phosphorylation under basal conditions.
Fig. 6. Decreased activation of ERK1/2 and β-catenin in ApcMin/+;Nherf2−/− mice. A: immunoblot compares expression levels of β-catenin, p-ERK1/2, ERK1/2, NHERF2, and NHERF1 in ApcMin/+ and ApcMin/+;Nherf2−/− mice. B: intestinal sections from 14-wk-old ApcMin/+ and ApcMin/+;Nherf2−/− mice were immunolabeled with anti-β-catenin or anti-p-ERK1/2 antibody. Representative images of immunohistochemical staining of β-catenin (top) and p-ERK1/2 (bottom) (magnification: ×200) in tumor and normal-looking epithelium are shown. Middle: magnified view (×400) of boxed area. C: proliferating cells in tumor and normal looking epithelium were identified by Ki67 staining (magnification: ×200). Approximately 15 tumors were scanned per mouse, and for each tumor one or more fields of vision were counted depending on the size of the tumor. The numbers shown are the number of Ki67+ cells per field of vision; n = 3. **P < 0.01.
and Stat3 activation by IL-6 was also decreased by CD24 knockdown (Fig. 9D). However, when the decreased basal phospho-Stat3 level was taken into account, the relative changes in Stat3 phosphorylation with or without CD24 knockdown (3.6 ± H1003/H11003 in shCon vs. 4.1 ± H1003/H195 in shCD24) in response to IL-6 was not significant. Together, these results show that NHERF2 potentiates Stat3 activation in part by direct effect on Stat3 phosphorylation and transcriptional regulation of CD24, which in turn alters basal Stat3 activity (Fig. 9E).

**DISCUSSION**

NHERF1 and NHERF2 are structurally related proteins, but it is becoming increasingly evident that NHERF1 and NHERF2 are not redundant proteins. Although NHERF1 has been reported to be overexpressed in estrogen receptor-positive breast carcinomas, evidence shows that NHERF1 generally functions as a tumor suppressor (36). NHERF1 inhibits platelet-derived growth factor signaling by interacting with the tumor suppressor PTEN to counterbalance PI3K/Akt oncogenic signaling, and silencing NHERF1 expression stimulates breast cancer cell growth and epithelial-mesenchymal transition of human intestinal epithelial Caco-2 cells, indicating that NHERF1 functions like a tumor suppressor (11, 29). In this study, we have demonstrated that depletion of NHERF2 decreased colon cancer cell growth in vitro and in xenograft implants. These findings were corroborated by the decreased tumor growth and increased life span of ApcMin/+ mice lacking NHERF2. We showed previously that NHERF2 expression is elevated in human CRC tissues (18). In the present study, we found that increased NHERF2 expression correlated with advanced stages of primary tumor, suggesting that the effects of NHERF2 may incur at a late step. This view is, in principle, supported by our finding that NHERF2 ablation decreased tumor growth without a significant change in tumor number in ApcMin/+ mice. NHERF2 modulates colon cancer cell proliferation via multiple mechanisms, including inactivation of Erk1/2 and Stat3, and decreased CD24 expression. Transcriptome analysis of NHERF2-depleted xenografts showed that a number of genes are regulated by NHERF2, but the precise mechanism of NHERF2-dependent gene regulation remains unknown. In this regard, it is noteworthy that a recent report has shown that NHERF2 is a coactivator of ERα in MCF7 breast cancer cells and it possesses intrinsic transcriptional activity (24). Additionally, it has been shown previously that NHERF2 interacts with the Y chromosome-encoded gene SRY in the nucleus of pre-Sertoli cells in the testis (38).
The effect of NHERF2 depletion on colon cancer growth raises a question whether NHERF2 can be a potential target for therapeutic intervention. In this study, we tested the effect of FD2 peptide on HCT116 cells to examine this possibility. FD2 has been modeled to target the second PDZ domain of NHERF2, and it does not bind the fifth PDZ domain of MAGI-3, which also interacts with LPA2 (18, 48). Although the scope of peptide delivery was limited, our study provides a proof of concept that interruption of NHERF2 interaction attenuates cancer cell proliferation, indicating that NHERF2 could be a candidate target for therapeutic intervention. Future studies to optimize peptide delivery and enhance peptide stability are needed to fully appreciate the efficacy of FD2 in blocking NHERF2 interaction and tumor suppression.

LPA2-mediated effects are modulated in part by NHERF2 such that loss of NHERF2 decreases IL-8 secretion and mitigates antipapoptotic effect of LPA (32, 43). Consistently, depletion of either NHERF2 or LPA2 decreased cancer cell proliferation. However, at least three lines of evidence show that the effects of NHERF2 are largely independent of LPA2. First, NHERF2 loss decreased tumor growth without a significant effect on tumor multiplicity in the ApcMin/H11001 intestinal tumor model. This observation differs from the loss of LPA2 in ApcMin/H11001 mice, where both tumor multiplicity and growth was observed (20). Second, the transcriptome analysis of xenograft tumors identified a small number of genes coregulated by LPA2 and NHERF2. Since LPA2 interacts with NHERF2, finding genes concurrently altered in shNHERF2 and shLPA2 would provide further evidence of their independent roles.

**Fig. 8.** NHERF2 modulates Stat3 phosphorylation. A: effect of CD24 depletion on Stat3 was determined in HCT116 cells. The levels of phospho-Stat3 (p-Stat3) and Stat3 were normalized to Stat3 and β-actin, respectively. *P < 0.05 compared with shCon; n = 3. B: phospho-Stat3 and total Stat3 expression in tumors of ApcMin/+ and ApcMin/+; Nherf2-/- mice was determined. Relative levels Stat3 phosphorylation normalized to total Stat3 expression (means ± SE) are shown. *P < 0.05. C: representative images of phospho-Stat3 in ApcMin/+ and ApcMin/+; Nherf2-/- mice are shown. Magnification: ×200. D: HCT116 cells transduced with shRNA as indicated were treated with IL-6 (25 ng/ml) for the indicated time duration. Expression of phospho-Stat3 and total Stat3 was determined. Right: quantification of phospho-Stat3 expression normalized to total Stat3 levels. *P < 0.05; n = 3. E: HCT116 cells were treated with IFN and phospho- and total Stat3 levels were determined. *P < 0.05; n = 3.
tumors is not surprising, but the fact that the majority of the differentially regulated genes fall on one group of tumor indicates that LPA2-mediated effects constitute only a small part of NHERF2-dependent transcriptional programming. Third, whereas NHERF2 depletion clearly modulated Stat3 phosphorylation, LPA did not alter Stat3 phosphorylation, demonstrating that Stat3 regulation is independent of LPA2.

It is interesting that NHERF2 alters β-catenin in ApcaMin+/+ mice. The gene expression analysis showed that several Wnt pathway-associated genes were downregulated by depletion of NHERF2. In addition, it was shown that NHERF2 complexes with β-catenin (37). NHERF2 interacts with β-catenin at the cell junction and at the membrane ruffle (37). Furthermore, evidence shows that NHERF2 interacts with transcription factors in the nucleus (15, 38). Hence, we speculate that NHERF2 may interact with β-catenin in the nucleus, stabilizing β-catenin.

CD24 expression is linked to tumor initiation and progression and CD24 deletion reduces tumor burden in ApcaMin+/+ mice (28, 33). It has been suggested that CD24 expression is regulated by COX2 activating through β-catenin (28), but the knowledge about the molecular basis of CD24 oncogenic effects is limited. The present study reveals that NHERF2 regulates CD24 transcript abundance. Altered CD24 expression in NHERF2-deficient ApcaMin+/+ tumors confirmed the effect of NHERF2 on CD24 expression. However, we could not determine the changes in CD24 protein expression in colon cancer cells. We tested three anti-CD24 antibodies, but these antibodies failed to yield consistent results. Ironically, the most of commercially available anti-CD24 antibodies are recommended for immunohistochemical or FACS analysis, implying that the epitope recognition by these antibodies under denaturing conditions of Western blotting is not optimum.

Similarly to CD24, persistent activation of Stat3 correlates with malignant transformation and with activation of Stat3 promotes proliferation and survival of cancer cells (7, 31). Intestinal epithelial cell (IEC)-specific deletion of Stat3 or pharmaceutical inhibition of Stat3 ameliorates gut inflammation and reduces colitis-associated cancer (3, 9). Similarly, IEC-specific ablation of Stat3 in ApcaMin+/+ mice decreases tumor multiplicity, although a compensatory change in Stat1 expression increases tumor invasiveness (27). We found that NHERF2 depletion reduced phospho-Stat3 levels in HCT116 cells and ApcaMin+/+ mice. In addition, acute stimulation of Stat3 phosphorylation was critical for the transcriptional regulation of CD24. CD24 knockdown lowered basal Stat3 activation in colon cancer cells, consistently with previous studies that CD24 expression correlates with Stat3 and Stat3 target gene expression in ovarian carcinoma, lung adenocarcinoma, or glioblastoma cell lines (4, 19). Hence, NHERF2 maintains elevated phospho-Stat3 levels through a positive feedback between Stat3 and CD24. How NHERF2 modulates Stat3 activation is not known. We could not find interaction of NHERF2 with JAK, Stat3, or receptor for activated C kinase 1 (RACK1), which recruits Stat3 to insulin-like growth factor receptor (47). IL-6 receptor and glycoprotein 130 are unlikely NHERF2-interacting proteins because they lack a PDZ binding motif. A future study is needed to elucidate the underpinning mechanism of NHERF2-dependent regulation of Stat3.

In summary, our present study demonstrates that NHERF2 stimulates colon cancer growth by intersecting at multiple signaling nodes. Our study points to the NHERF2-Stat3-CD24 cascade that may intensify oncogenic effect by further potentiating Stat3 and MAPK. The identification of oncogenic signaling by NHERF2 provides NHERF2 as an attractive therapeutic target to limit tumor growth.

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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

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