Deletion of Na⁺/H⁺ exchanger regulatory factor 2 represses colon cancer progress by suppression of Stat3 and CD24

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Running title: NHERF2 is a positive regulator of colon cancer growth

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Abbreviations: Apc, adenomatous polyposis coli; CRC, colorectal cancer; ERM, ezrin, radixin and moesin; FITC, Fluorescein isothiocyanate; IFNα, interferon α; LPA, lysophosphatidic acid; LPA₂, lysophosphatidic acid receptor 2; MAPK, mitogen-activate protein kinase; MST, median survival time; NHE3, Na⁺/H⁺ exchanger 3; NHERF, Na⁺/H⁺ exchanger regulatory factor; PDZ, postsynaptic density 95, discs large, and zonula occludens-1; PI3K, phosphoinositide 3-kinase; Stat-3 signal transducer and activator of transcription 3
ABSTRACT

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 ApcMin/+ mice resulted in decreased tumor growth in ApcMin/+ mice and increased lifespan. Blocking NHERF2 interaction with a small peptide designed to bind the second PDZ domain of NHERF2 attenuated 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 genes largely differ from LPA₂-regulated genes. β-catenin and ERK1/2 activation was mitigated in ApcMin/+,Nherf2⁻/⁻ adenomas. Moreover, Stat3 phosphorylation and CD24 expression levels were suppressed in ApcMin/+,Nherf2⁻/⁻ 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.
INTRODUCTION

Transformation of normal cells into malignant derivatives entails accumulation of mutations in oncogenes or tumor-suppressor genes (40). Upon accumulation of oncogenic mutations, the expansion and progress of tumor relies on a multi-step process that increases tumor cell number through stimulation of cell division or inhibition of cell death. The progress and metastasis of cancer are driven by aberrant expression and activity of various cellular pathways such as the Wnt/β-catenin, mitogen-activate protein kinase (MAPK), phosphoinositide 3-kinase (PI3K)/Akt pathways and other (40). Scaffold proteins mediate protein-protein interaction to bring together key members of a pathway that drive cell division and growth.

The Na⁺/H⁺ exchanger regulatory factor (NHERF) family of proteins are scaffold proteins that contain two or four PDZ (postsynaptic density 95, discs large, and zonula occludens-1) domains. Among the NHERF proteins, NHERF1 and NHERF2 share most similarities with tandem PDZ domains and an ERM (ezrin, radixin and moesin) interacting motif in the carboxyl domain that enables anchoring to the actin cytoskeleton (17). NHERF1 and NHERF2 were initially identified as proteins that interact with Na⁺/H⁺ exchanger NHE3 to transduce PKA-mediated inhibition of Na⁺/H⁺ exchange (44). Currently, more than 30 proteins, including membrane receptors, protein kinases, transporters, and channels are known to interact with NHERF1/2 (1). One major function of NHERF1/2 is to recruit and spatially organize signaling proteins that either alters protein functions or downstream signaling pathways originating from receptor.

NHERF1 was shown to be an estrogen-inducible gene, but subsequent studies have shown that NHERF1 generally functions as a tumor suppressor. 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 (11, 25, 29). In comparison, the relevance of NHERF2 in tumorigenesis is less clear. We have shown
previously that NHERF2 interacts with serum- and glucocorticoid-inducible kinase 1 (SGK1) (42). SGK is closely related to Akt and breast cancer cells with elevated SGK1 possess Akt inhibitor-resistance (35), but whether the interaction between SGK1 and NHERF2 contributes to the inhibitor resistant phenotype is not known. NHERF2 also functions as a modulator of phospholipase C-β3 and MAPK activation by the lysophosphatidic acid receptor 2 (LPA₂), as such this interaction allows colon cancer cells to escape programmed cell death (32, 43). A recent study has shown that NHERF2 forms a complex with the steroid receptor coactivator to enhance estrogen receptor α (ERα) transactivation in breast cancer cells and overexpression of NHERF2 increases the tumorigenic potential of MCF7 xenografts (24). On the other hand, NHERF2 interacts with metabotropic glutamate receptor mGluR5 to prolong mGluR5-mediated calcium mobilization and potentiate 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), LPA₂ (shLPA₂), or CD24 (shCD24) were from Sigma (St. Louis, MO). pLKO.1-puro with non-target 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 was seeded at 3.0 ×10^4 cells/well in a complete media containing 10 % FBS. Cells were counted on day 2, 3, and 4 using a hemocytometer.

In vivo tumor growth

Five to six weeks 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 ×10^6 cells in 0.2 ml PBS) stably transduced with shNHERF2, shLPA2, or shCon. Tumor size was measured twice a week using calipers, and volume was calculated as length x width^2 x 0.5. On day 21, all mice were sacrificed, 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 Apc\textsuperscript{Min/+};Nherf2\textsuperscript{-/-} mice

Nherf2\textsuperscript{-/-} mice in C57BL/6 background were previously described (22). Apc\textsuperscript{Min/+} mice were purchased from Jackson Laboratory (Bar Harbor, ME). Apc\textsuperscript{Min/+} males were mated with Nherf2\textsuperscript{-/-} females to obtain male Apc\textsuperscript{Min/+};Nherf2\textsuperscript{-/-} mice, which were subsequently mated with Nherf2\textsuperscript{-/-} females to generate Apc\textsuperscript{Min/+}, Apc\textsuperscript{Min/+};Nherf2\textsuperscript{-/-}, and Apc\textsuperscript{Min/+};Nherf2\textsuperscript{-/-} mice. Littermate females were used in all studies. Mice were sacrificed at 9 and 14 week of age (n=6 mice per group). The entire small 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-AhxGGSGSTRF; Ahx: aminohexanoic acid) that interferes with the 2nd PDZ domain of NHERF2 was described previously (48). A scrambled 8-mer peptide (sc-FD2, FITC-Ahx-GGFTSGSR) was used as a control. All peptides linked to FITC at the N-terminus with unmodified C-terminus were synthesized by GenScript (Piscataway, NJ). HCT116 cells were seeded at 1.5 × 10^6 cells per well in McCoy’s 5A containing 5 % FBS. On day 1 and 2, peptides at 10 μg/ml, 50 μg/ml, or 100 μg/ml were added to the cells with BioPorter Protein Delivery Reagent (Genlantis, San Diego, CA) according to the manufacture’s instruction. Cells were counted on day 3 using a hemocytometer.

Immunohistochemistry (IHC)

Immunohistochemical analysis was performed as previously describe 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% H_2O_2/methanol solution, tissue sections were blocked with goat serum, and incubated with primary antibody overnight at 4°C. After 3 washes in PBS, sections were incubated with biotinylated secondary antibody and then with avidin-biotin horseradish peroxidase solution (Vector Laboratories, Burlingame, CA). Finally, tissue sections were incubated with 0.01 % H_2O_2 and 0.05 % 3,3’-diaminobenzidine tetrachloride. Nuclear counterstaining was accomplished using 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 antibodies (#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 vision at a magnification of x400 were scanned per tumor in xenograft tumors, and approximately 15 tumors were scanned per ApcMin/+ mouse.

**Human colon cancer tissue microarray (TMA)**

Human colon cancer TMA slides were purchased from OriGene (Rockville, MD). Immunohistochemical staining was performed as described above using 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.

**Quantitative RT-PCR (qRT-PCR)**

Total RNA was isolated from cells or tumor tissues using RNasey Mini Kit (Qiagen), and cDNA was synthesized using the SuperScript III First Strand Synthesis kit (Invitrogen). 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'-CTCCTACCCACGCAGATTATTC-3' and 5'-AGAGTGAGACCACGAAGAGAC-3'; CD24, mouse: 5'-GTTGCACCGTTTCCCGGTAA-3' and 5'-CCCCTCTGGTGGTAGCGTTA-3'; β-actin, human: 5'-CGTGCGTGACATTAAGGAGA-3' and 5'-AGGAAGGAAGGCTGGAAGAG-3'; β-actin, mouse: 5'-GGCTGTATTCCCCTCCATCG-3' and 5'-CCAGTTGGTAACAATGCCATGT-3'.
RNA sequencing (RNA-seq) analyses of xenografts

RNA-seq and data analysis were performed at the Emory Integrated Genomics Core. Briefly, total RNA from xenograft tumors \( (n = 3/\text{group}) \) was extracted using the Qiagen miRNeasy 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 100bp paired-end reads were generated per library. Base calling and FASTQ was done using Illumina’s HiSeq Control Software version 1.5.15.1 (RTA v1.13.18 and bcftools v1.8.3) (6). Sequenced reads were mapped to the human reference genome UCSC version mm9 with TopHat version 2.0.5, and differentially expressed genes were assessed using Cuffdiff, a part of the Cufflinks version 2.1.1 package (39). The RNAseq 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 using a Li-COR Odyssey Imager. Rabbit anti-NHERF2 polyclonal and anti-NHERF1 polyclonal antibodies were described previously (45). Additional rabbit anti-NHERF2 polyclonal antibody (HPA001672, 1:250 dilution for IHC) and mouse monoclonal anti-β-actin (A1978, 1:5,000 for WB) were obtained from Sigma. Rabbit monoclonal anti-phospho-ERK1/2 (p-ERK1/2) (#4370, 1:2,000 for WB and 1:400 for IHC), mouse monoclonal anti-ERK1/2 (#4696, 1:2,000 for WB), rabbit monoclonal anti-phospho-Stat3 (#9145, 1:2,000 for WB and 1:400 for IHC), and rabbit monoclonal anti-Stat3 (#4904, 1:2,000 for WB) were purchased from Cell signaling (Danvers, MA).
**Statistical analysis**

Statistical significance was assessed by two-tailed unpaired Student’s *t* test, Welch’s *t* test, Mann-Whitney’s U test, or mxi chi square test. Overall survival curves were generated using the Kaplan-Meier methods and differences were evaluated with the log-rank test. Values are expressed as mean ± standard error of mean (SEM). A *P* value of < 0.05 was considered statistically significant.

**RESULTS**

**NHERF2 expression is up-regulated 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 out 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 (Figure 1A). To determine whether there is correlation between NHERF2 expression levels and progression of CRC, CRC tissues were allocated into two groups: (1) high-NHERF2 group, which had stronger immunostaining intensity with a score of 2 or 3; and (2) low-NHERF2 group with staining scores of ≤ 1. Thirty six tumors had scores of 2-3 (high-NHERF2), while five belonged to the low-NHERF2 group. The characteristics of the patients in high- and low-NHERF2 groups are shown in Table 1. No significant difference was noted between these two groups in their genders, ages, primary tumor sites, and histological types, despite the difference in the group size. However, there was a significant difference in tumor stage between the two groups. Thirty of high-NHERF2 expressing
tumors were in pT3 or pT4 stage (83.3 %), while six were in pT1 or pT2 stage tumors (Figure 1B). In contrast, low-NHERF2 group consisted of one in pT3/T4 stage and four in pT1/pT2 stages. Collectively, these results suggest that NHERF2 expression is elevated in CRC and high NHERF2 expression is more prevalent in advanced ($P = 0.002$) than early CRC.

**Depletion of NHERF2 decreases cancer cell proliferation**

To investigate whether NHERF2 regulates colon cancer growth, we generated human colon cancer HCT116, SW480 and HT-29 cell lines with stable knockdown of NHERF2 by transduction with lentiviral shNHERF2 or shCon. In all cell lines, knockdown efficiency of $\geq 80 \%$ was achieved without affecting NHERF1 expression (Figure 2A). Importantly, shNHERF2 transduced cells proliferated at reduced rates than control cells, demonstrating that NHERF2 positively regulates cancer cell proliferation (Figure 2B). Both SW480 and HT29 cells have mutated Apc gene, while HCT116 cells are heterozygous for β-catenin, harboring one WT allele and one mutant allele (26). As a result, all the three cell lines activation of the Wnt-beta-catenin pathway, and we have shown previously that silencing of NHERF2 had a similar effect on proliferation of these 3 cell lines all of which have activated Wnt signaling (18, 46).

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 mm$^3$; 603.3±113.5 vs. 2,013±214.1, $P < 0.001$; tumor weight in grams; 0.73 ± 0.11 vs. 1.55 ± 0.15, $P < 0.001$) (Figure 2C-D). Immunostaining tumor sections for Ki67 revealed NHERF2 depletion decreased the number of Ki67-positive cells almost by half (Figure 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 (Figure 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
(Figure 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 (Figure 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
μg/ml, 0.75 ± 0.03; 50 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
weeks 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 weeks (Figure 4A). Similarly, the number of colonic adenomas was not altered by the absence of NHERF2. Although tumor numbers increased in all strains at 14 weeks, again no variation among three strains was observed. On the other hand, there was a significant shift in the tumor size in the absence of NHERF2. $Apc^{Min/+;Nherf2^{-/-}}$ mice developed smaller tumors compared with $Apc^{Min/+}$ mice at 9 weeks of age (mean size in mm, 1.01 ± 0.02 for $Apc^{Min/+;Nherf2^{-/-}}$ vs. 1.28 ± 0.04 for $Apc^{Min/+}$, $P < 0.01$) (Figure 4B). At 14 weeks, 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 weeks, tumors in $Apc^{Min/+;Nherf2^{-/-}}$ mice at 14 weeks 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 ≤ 1 mm to ≥ 1 mm from 9 weeks to 14 weeks of age in both $Apc^{Min/+}$ and $Apc^{Min/+;Nherf2^{-/-}}$ mice, whereas half of the tumors in $Apc^{Min/+;Nherf2^{-/-}}$ mice still remained less than 1 mm in size and about 5% were greater than 2 mm at 14 weeks. Because the absence of NHERF2 decreased tumor growth in $Apc^{Min/+}$ mice, we determined whether NHERF2 affects overall survival of $Apc^{Min/+}$ mice. Figure 4D shows that $Apc^{Min/+;Nherf2^{-/-}}$ mice had a longer lifespan than $Apc^{Min/+}$ mice (median survival time (MST) in days: 152 vs. 131, $P < 0.05$). MST of $Apc^{Min/+;Nherf2^{-/-}}$ mouse was not different from that of $Apc^{Min/+}$ mice (132 vs. 131). These results suggest that the absence of NHERF2 decreases tumor growth and improves the survival of $Apc^{Min/+}$ 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 Apc\textsuperscript{Min/+} 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 sequencing 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 \(\geq 1.5\)) were up-regulated, while 70 (\(P < 0.01\), fold change \(\leq 1.5\)) were down-regulated in shNHERF2 tumor compared with control (Figure 5 and Supplementary Table S1-2). Similarly, a total of 109 genes were up-regulated, while 137 genes were down-regulated in shLPA2 tumor compared with control (Figure 5 and Supplementary Table S3-4). 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, ZCCHCH12, and IGFBP6 (Supplementary Table S1-2). Hence, we determined whether the loss of NHERF2 affected Wnt
pathway activation in \(Apc^{Min}\) mice. We did not find a difference in \(\beta\)-catenin expression between \(Apc^{Min/+}\) and \(Apc^{Min/+};Nherf2^{-/-}\) mice (Figure 6A), but IHC analysis shows that the loss of NHERF2 decreased the nuclear expression of \(\beta\)-catenin (Figure 6B), indicating that NHERF2 modulates \(\beta\)-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 \(Apc^{Min/+}\) mice. There was a pronounced decrease in the expression level of phospho-ERK1/2 in \(Apc^{Min/+};Nherf2^{-/-}\) compared with \(Apc^{Min/+}\) mice without a change in ERK1/2 abundance (Figure 6A-B). In line with the decreased Wnt and ERK signaling, the number of proliferating cells identified by Ki67 staining was significantly reduced in \(Apc^{Min/+};Nherf2^{-/-}\) compared with \(Apc^{Min/+}\) mice (Ki67 index, %; 32.2 ± 1.94 for \(Apc^{Min/+};Nherf2^{-/-}\) vs. 54.5 ± 3.43 for \(Apc^{Min/+}\), \(P < 0.001\)) (Figure 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 down-regulated 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 (Figure 7A). The same results obtained in SW480 and HT29 cells (not shown). Additionally, we observed decreased CD24 expression in \(Apc^{Min/+};Nherf2^{-/-}\) mice (Figure 7B-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 (Figure 7D), but it decreased the rate of HCT116 cell proliferation (Figure 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 in the context of NHERF2 (Figure 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 (Figure 8B-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 (Figure 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 (Figure 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 9A-B shows that IL-6 had a small but statistically significant increase in CD24 mRNA expression, which was ablated by NHERF2 knockdown. Comparison of data in Figure 8D and Figure 9A indicated that the kinetics of Stat3 activation was greater compared with CD24 regulation (<1/2 h for Stat3 versus 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 (Figure 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 and Stat3 activation by IL-6 was also decreased by CD24 knockdown. (Figure 9D). However, when the decreased basal phospho-Stat3 level was taken into the account, the relative changes in Stat3 phosphorylation with or without CD24 knockdown (3.6x in shCon vs 4.1x 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 (Figure 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 ER-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 current 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 principal, 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-encode gene SRY in the nucleus of pre-Sartoli 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 anti-apoptotic 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/+ intestinal tumor model. This observation differs from the loss of LPA2 in Apc Min/+ mice, where both tumor multiplicity and growth was observed (20). Second, the transcriptome analysis of xenograft tumors identified a small number of genes co-regulated by LPA2 and NHERF2. Since LPA2 interacts with NHERF2, finding genes concurrently altered in shNHERF2 and shLPA2 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 ApcMin/+ mice. The gene expression analysis showed that several Wnt pathway associated genes were down-regulated 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 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 \(Apc^{Min/+}\) mice \((28, 33)\). It has been suggested that CD24 expression is regulated by COX2 activating through \(\beta\)-catenin \((28)\), but the knowledge about the molecular basis of CD24 oncogenic effects is limited. The current study reveals that NHERF2 regulates CD24 transcript abundance. Altered CD24 expression in NHERF2-deficient \(Apc^{Min/+}\) 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 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 \(Apc^{Min/+}\) 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 \(Apc^{Min/+}\) 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 as 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 current 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.

Acknowledgements
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REFERENCES


11. Hayashi Y, Molina JR, Hamilton SR, and Georgescu MM. NHERF1/EBP50 is a new


and Kraus S. CD24 knockout prevents colorectal cancer in chemically induced colon
carcinogenesis and in APC(Min)/CD24 double knockout transgenic mice. Int J Cancer 135:
1048-1059, 2014.

exchanger regulatory factor 1 (NHERF1). Breast cancer research : BCR 8: R63, 2006.

30. Paquet M, Asay MJ, Fam SR, Inuzuka H, Castleberry AM, Oller H, Smith Y, Yun CC,
Traynelis SF, and Hall RA. The PDZ scaffold NHERF-2 interacts with mGluR5 and regulates

31. Quintas-Cardama A and Verstovsek S. Molecular pathways: Jak/STAT pathway:

32. Rusovici R, Ghaleb A, Shim H, Yang VW, and Yun CC. Lysophosphatidic acid prevents
apoptosis of Caco-2 colon cancer cells via activation of mitogen-activated protein kinase and

33. Sagiv E, Memeo L, Karin A, Kazanov D, Jacob-Hirsch J, Mansukhani M, Rechavi G,
Hibshoosh H, and Arber N. CD24 is a new oncogene, early at the multistep process of

34. Seo JH, Jeong KJ, Oh WJ, Sul HJ, Sohn JS, Kim YK, Cho do Y, Kang JK, Park CG, and
Lee HY. Lysophosphatidic acid induces STAT3 phosphorylation and ovarian cancer cell motility:

35. Sommer EM, Dry H, Cross D, Guichard S, Davies BR, and Alessi DR. Elevated SGK1

D, Roy JE, Cohen WA, Ramesh V, and Louis DN. NHE-RF, a merlin-interacting protein, is
primarily expressed in luminal epithelia, proliferative endometrium, and estrogen receptor-

37. Theisen CS, Wahl JK, 3rd, Johnson KR, and Wheelock MJ. NHERF links the N-


46. Zhang H, Bialkowska A, Rusovici R, Chanchevalap S, Shim H, Katz JP, Yang VW, and Yun CC. Lysophosphatidic acid facilitates proliferation of colon cancer cells via induction of


FIGURE LEGENDS

Figure 1: Expression of NHERF2 in human colon cancer TMA

(A) Table shows the characteristics of tumors in TMA. (B) Representative images of NHERF2 expression in colonic cancer and normal intestinal tissues are shown. Insets show a magnified view of the boxed area. Magnification: x200; inset, x400. (C) The relative NHERF2 expression levels in colon cancer TMA was determined as described in Methods and Materials. Numbers of tumors in different stages are shown. High, high NHERF2 expression; Low, low NHERF2 expression.

Figure 2: NHERF2 knockdown reduces cell proliferation

(A) HCT116, SW480, and HT-29 cells were transduced with lentiviral shNHERF2 (N) or non-targeting scrambled shCon (C). Equal amounts of lysates were immunoblotted for NHERF2, NHERF1, and β-actin (loading control). (B) Cells transduced with shNHERF2 or shCon were cultured in complete media with 10% FBS. Number of cells was counted daily. Representative results from four independent experiments are shown. Data represent mean ± SEM. * P < 0.01, and ** P < 0.001, shNHERF2 vs shCon. (C) HCT116 cells transduced with shNHERF2 or shCon were implanted in nude mice and tumors were measured as described in Materials and
Methods. n=10 per group. Data represent mean ± SEM. * P < 0.01, and ** P < 0.001, shNHERF2 vs shCon. (D) Xenograft tumor weight on day 21 after implantation are shown. n=10 for each group. ** P < 0.001. (E) Proliferating cells in xenograft tumor sections were identified by Ki67 staining. 20 fields of vision were counter per tumor. n = 4. Magnification: x100. Right panel shows Ki67 proliferation index. ** P < 0.001. (F) Apoptotic cells in xenograft tumors were identified by cleaved Caspase-3 staining. Ten fields of vision were counted per each tumor. n = 4. Magnification: x100. Right panel shows cleaved Caspase-3 index.

Figure 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 lone for 4h. FD2 was conjugated with FITC, and uptake of FD2 by the cells was assessed by fluorescence microscopy. F-actin and nuclear (DAPI) 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 media, and the PDZ blocking peptide, FD2, or control scrambled peptide, scFD2, was added to the media 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 mean ± SEM from four independent experiments in triplicates. * P < 0.01 vs control without BioPorter/peptide.

Figure 4: Loss of NHERF2 decreases tumor growth in ApcMin/+ mice

(A) Numbers and (B) sizes (Mean ± SEM) of adenomas in the small intestine (left) and colon (right) of ApcMin/+, ApcMin/+;Nherf2+/+, and ApcMin/+;Nherf2−/− mice of 9 (open bar) or 14 (hatched bar) weeks of age were determined. n = 6 per each genotype at each time point. * P < 0.05, ** P < 0.01, *** P < 0.001. (C) Distribution of tumors by their sizes at the age of 9 and 14 weeks
are shown. (D) Overall survival of \(Apc^{\text{Min}+}, Apc^{\text{Min}+};Nherf2^{+/},\) and \(Apc^{\text{Min}+};Nherf2^{-/}\) mice were determined. \(n = 12\) per strain. * \(P < 0.05, Apc^{\text{Min}+} \) vs. \(Apc^{\text{Min}+};Nherf2^{-/}\).

**Figure 5: Comparison of gene expression between shNHERF2 and shLPA_2 xenograft tumors.** Venn diagrams depict differentially expressed genes in the shNHERF2 or shLPA_2 xenograft tumors compared with shCon tumors. Genes were considered significant when their p-values were below 0.01 with more than 1.5 fold changes of expression compared with shCon.

**Figure 6: Decreased activation of ERK1/2 and \(\beta\)-catenin in \(Apc^{\text{Min}+};Nherf2^{-/}\) mice**

(A) Immunoblot compares expression levels of \(\beta\)-catenin, p-ERK1/2, ERK1/2, NHERF2 and NHERF1 in \(Apc^{\text{Min}+}\) and \(Apc^{\text{Min}+};Nherf2^{-/}\) mice. (B) Intestinal sections from 14 weeks old \(Apc^{\text{Min}+}\) and \(Apc^{\text{Min}+};Nherf2^{-/}\) mice were immunolabeled using anti-\(\beta\)-catenin or anti-p-ERK1/2 antibody. Representative images of immunochemical staining of \(\beta\)-catenin and p-ERK1/2 (magnification: x200) in tumor and normal looking epithelium are shown. Middle panels show a magnified view (x400) of boxed area. (C) Proliferating cells in tumor and normal looking epithelium were identified by Ki67 staining (magnification: x200). 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. \(n=3\). * \(P < 0.01\).

**Figure 7: Loss of NHERF2 attenuates CD24 levels**

(A) CD24 mRNA levels were determined by qRT-PCR in HCT116 cells stably transduced with lentiviral shCon or shNHERF2. * \(P < 0.05\). (B) CD24 expression in tumors of \(Apc^{\text{Min}+}\) and \(Apc^{\text{Min}+};Nherf2^{-/}\) mice was determined by qRT-PCR. * \(P < 0.05\). \(n=3\). (C) Representative images of CD24 in \(Apc^{\text{Min}+}\) and \(Apc^{\text{Min}+};Nherf2^{-/}\) mice are shown Magnification: x200. (D) CD24 mRNA (upper) and NHERF2 protein (bottom) levels were determined in HCT116 cells transduced with
shCon, shCD24, shNHERF2, or shCD24+shNHERF2. Data represent mean ± SEM. n=3; * P < 0.05 and ** P < 0.01 compared with shCon. (E) HCT116 cells transduced as indicated were cultured in complete media with 10 % FBS. Numbers of cells (mean ± SEM) were counted daily. Representative results from four independent experiments are shown. ** P < 0.01, shCD24 vs shCon; † P < 0.01, shNHERF2 vs shCon; ‡ P < 0.001, shCD24/shNHERF2 vs shCon.

Figure 8: NHERF2 modulates Stat3 phosphorylation

(A) The effect of CD24 depletion on Stat3 was determined in HCT116 cells. The levels of 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 (mean ± SEM) are shown. * P < 0.05. (C) Representative images of phospho-Stat3 in ApcMin/+ and ApcMin/+;Nherf2−/− mice are shown. Magnification: x200. (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 panel shows 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.

Figure 9: IL-6 regulates CD24 expression

(A) The effect of IL-6 on CD24 mRNA levels was determined by qRT-PCR in HCT116 cells. CD24 mRNA levels were normalized to β-actin mRNA levels. n=4. * P < 0.05 compared with 0 h. (B) HCT116 cells transduced with shCon or shNHERF2 were treated with IL-6 for 3 h. CD24 mRNA expression levels were normalized to β-actin. ** P < 0.01. n = 3. (C) Cells transduced
with shCon or shNHERF2 were treated with IL-6 in the presence or absence of STA-21 (10µM). CD24 mRNA levels were determined by qRT-PCR and normalized to β-actin mRNA levels. **P < 0.01. n = 3.

(D) Cells were treated with IL-6 for 30 min and phospho-Stat3 and Stat3 levels were determined. Stat3 expression was normalized to β-actin expression. Phospho-Stat3 levels were normalized the Stat3/β-actin ratios. * P < 0.01, phospho-Stat3 levels compared with that of untreated shCon. # P < 0.01, phospho-Stat3 levels compared with that of untreated shCD24. (E) A putative model of NHERF2-dependent regulation of Stat3 and CD24.
Fig. 1

A

adenocarcinoma  adenocarcinoma  normal

B

![Graph showing number of cases](image)

- pT1, pT2: 6 High, 4 Low
- pT3, pT4: 30 High, 1 Low
**Fig. 2**

**A**

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**B**

**HCT116**

- **Cell number (x10^4)**
  - shCon: ●
  - shNHERF2: ■
  - Days: 0, 1, 2, 3, 4

**SW480**

- **Cell number (x10^4)**
  - shCon: ●
  - shNHERF2: ■
  - Days: 0, 1, 2, 3, 4

**C**

**HT-29**

- **Cell number (x10^4)**
  - shCon: ●
  - shNHERF2: ■
  - Days: 0, 1, 2, 3, 4

**D**

- **Tumor weight (g)**
  - shCon: ![Box Plot]
  - shNHERF2: ![Box Plot]

**E**

- **Ki67 (%)**
  - shCon: ![Image]
  - shNHERF2: ![Image]

- **Cleaved Caspase-3 (%)**
  - shCon: ![Image]
  - shNHERF2: ![Image]
A

Tumor number

Small Intestine

Colon

B

Small Intestine

Colon

Tumor size (mm)

9 weeks

14 weeks

- ***

- **

- *

C

Tumor number

≤1.0 mm

1.0-2.0 mm

2.0-3.0 mm

>3.0 mm

D

Survival rate

Apc<sub>Min/+</sub>

Apc<sub>Min/+; Nherf2<sup>+/−</sup></sub>

Apc<sub>Min/+; Nherf2<sup>−/−</sup></sub>

70

120

170

(Days)

Fig. 4
Up-regulated genes

shNHERF2  shLPA2
106  11  109

Down-regulated genes

shNHERF2  shLPA2
70  15  137

Fig. 5
Fig. 6
Fig. 7

A. Graph showing CD24 mRNA levels in shCon and shNHERF2 groups.

B. Graph showing CD24 mRNA levels in Apc<sup>Min/+</sup> and Apc<sup>Min/+;Nherf2⁻/⁻</sup> groups.

C. Immunohistochemistry images of CD24 expression in Apc<sup>Min/+</sup> and Apc<sup>Min/+;Nherf2⁻/⁻</sup> groups, comparing tumor and normal tissue.

D. Western blot for NHERF2 and β-actin in shCon, shCD24, shNHERF2, and shCD24/shNHERF2 groups.

E. Graph showing cell numbers over time with different treatments: shCon, shCD24, shNHERF2, and shCD24/shNHERF2. The graph includes statistical annotations (†, ‡, **).
Fig. 9

A - B and C show the expression of CD24 mRNA in response to different treatments.

D - Western blot analysis of p-Stat3, Stat3, and β-actin with corresponding molecular weights.

E - Schematic representation of the relationship between IL-6, INFα, and NHERF2 with CD24 mRNA expression.

* and ** indicate statistical significance.
Table 1. Patient characteristics

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