Colorectal Tumors Require NUAK1 for Protection from Oxidative Stress

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Exploiting oxidative stress has recently emerged as a plausible strategy for treatment of human cancer, and antioxidant defenses are implicated in resistance to chemotherapy and radiotherapy. Targeted suppression of antioxidant defenses could thus broadly improve therapeutic outcomes. Here, we identify the AMPK-related kinase NUAK1 as a key component of the antioxidant stress response pathway and reveal a specific requirement for this role of NUAK1 in colorectal cancer. We show that NUAK1 is activated by oxidative stress and that this activation is required to facilitate nuclear import of the antioxidant master regulator NRF2. Activation of NUAK1 coordinates PP1β inhibition with AKT activation in order to suppress GSK3β-dependent inhibition of NRF2 nuclear import. Deletion of NUAK1 suppresses formation of colorectal tumors, whereas acute depletion of NUAK1 induces regression of preexisting autochthonous tumors. Importantly, elevated expression of NUAK1 in human colorectal cancer is associated with more aggressive disease and reduced overall survival.

SIGNIFICANCE: This work identifies NUAK1 as a key faciliator of the adaptive antioxidant response that is associated with aggressive disease and worse outcome in human colorectal cancer. Our data suggest that transient NUAK1 inhibition may provide a safe and effective means for treatment of human colorectal cancer via disruption of intrinsic antioxidant defenses. Cancer Discov; 8(5); 632-47. ©2018 AACR.

INTRODUCTION

The relentless drive to proliferate exposes tumor cells to considerable metabolic stress. Proliferating tumor cells increase nutrient consumption in order to balance the competing demands of macromolecular synthesis, toward which a large proportion of nutrient metabolites are diverted, with the energetic cost of sustaining viability, measured in ATP (1). Increased metabolic activity elevates production of reactive oxygen species (ROS), altering signal transduction and, at very high levels, inflicting damage upon lipids, proteins, and nucleic acids (2). In the context of a growing solid tumor with ineffective vascularity, tumor cells are commonly deprived of their preferred nutrients and exposed to hypoxia, which also increases ROS production, adding cell-extrinsic sources of further metabolic stress. In order to survive such stress, tumor cells must adapt flexibly and continuously by modulating their rates of macromolecular synthesis, cell growth, and proliferation, in order to maintain ATP homeostasis and counteract ROS. Failure to do so leads to ATP collapse, toxic levels of ROS, and loss of viability. As such, targeted suppression of adaptive measures used by tumor cells to counteract metabolic stress may yield therapeutic benefit in cancer treatment.

NUAK1 (aka ARK5) is one of 12 kinases related by sequence homology to the catalytic α subunits of AMPK (3). Collectively, these kinases play various roles in regulating cell adhesion and polarity, cellular and organistical metabolism, and in the cellular response to various forms of stress, including oxidative, osmotic, and energetic stress (4, 5). NUAK1 is a common target of several miRNAs that are frequently suppressed in cancer, suggesting a potential role for NUAK1 in tumorigenesis (6–8). Accordingly, we previously reported that NUAK1 is required to sustain viability of cancer cells when MYC is overexpressed (9).

In contrast with the widely studied AMPK, the molecular targets and downstream pathways governed by NUAK1 are poorly defined. To date, the best-characterized substrate of NUAK1 is the PPIβ subunit MYPT1 (encoded by PP1R12A). During cell detachment, phosphorylation of MYPT1 by NUAK1 inhibits PPIβ phosphatase activity toward myosin light chain. Inhibition of NUAK1 thus increases PPIβ activity, delaying cell detachment and suppressing cell migration (10). Other work points to a role for NUAK1 in metabolic regulation. Muscle-specific deletion of Ndufs1 protects mice from high-fat diet–induced diabetes, attributable to increased glucose uptake and increased conversion of glucose to glycogen by NUAK1-deficient skeletal muscle (11). An earlier study showed that NUAK1 protects cancer cells from nutrient deprivation–induced apoptosis (12). In the context of MYC overexpression, we showed that NUAK1 is required to maintain ATP homeostasis, in part by facilitating AMPK-dependent inhibition of TORC1-driven macromolecular synthesis (9, 13). Failure to engage this checkpoint results in cell death under conditions of metabolic stress (14–16).

Our previous work thus suggested that NUAK1 may present a good target for therapy, specifically in the context of MYC-driven cancers. Human colorectal cancer is uniformly characterized by deregulated expression of MYC, and mouse models have shown that expression of endogenous Myc is

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required for intestinal polyp formation upon loss of Apc, the most common tumor-initiating event in human colorectal cancer (17, 18). We asked therefore if NUAK1 is required to support tumor cell viability in colorectal cancer. Here, we show that NUAK1 is overexpressed in human colorectal cancer and that high NUAK1 expression correlates with reduced overall survival. Using genetically engineered mouse models of colorectal cancer driven by sporadic loss of Apc, we show that NUAK1 is required for both initiation and maintenance of autochthonous colorectal tumors. NUAK1 facilitates nuclear translocation of the antioxidant master regulator NRF2 by counteracting negative regulation of NRF2 by GSK3β. Depletion or inhibition of NUAK1 thus renders human colorectal cancer cells and murine colorectal tumors vulnerable to oxidative stress–induced cell death. Our data reveal NUAK1 as a candidate therapeutic target in human colorectal cancer.

RESULTS

NUAK1 Overexpression Is Associated with Worse Outcome in Human Colorectal Cancer

We used RNA-Scope in situ hybridization to examine NUAK1 expression in a 660-sample tissue microarray of human colorectal cancer (19). NUAK1 is weakly expressed in normal human colonic epithelium, but increased expression is significantly enriched in aggressive (Dukes’ stage B and C) colorectal cancer (Fig. 1A; Supplementary Fig. S1A and S1B). In situ examination of The Cancer Genome Atlas (TCGA) colorectal adenocarcinoma cohort similarly showed significantly elevated NUAK1 expression in advanced (T stage 3 and 4) versus early (T stage 1 and 2) disease, and in patients with lymph node metastasis versus none (Fig. 1B). Meta-analysis of 17 independent cohorts comprising 947 human colorectal cancer samples, via SurvExpress (20), also revealed significantly higher NUAK1 expression in the high-risk versus low-risk group, and elevated NUAK1 expression was associated with significantly reduced overall survival and a hazard ratio of 1.49 (Fig. 1C and D). A similar reduction of overall survival was borne out by individual analysis of two large cohorts in colorectal adenocarcinoma similarly showed significantly higher NUAK1 expression in the high-risk versus low-risk group, and elevated NUAK1 expression was associated with significantly reduced overall survival and a hazard ratio of 1.49 (Fig. 1C and D). A similar reduction of overall survival was borne out by individual analysis of two large cohorts in colorectal cancer (Fig. 1E and F). This cytotoxic effect was also observed using the dual NUAK inhibitor WZ4003, suggesting it reflects on-target activity of the inhibitors. Notably, WZ4003 gave greater suppression of phospho-MYPT1 than MYPT1 consistent with dual inhibition of NUAK1 and NUAK2, and showed somewhat greater potency, driving significant cell death at 5 μmol/L in SW480 cells (Supplementary Fig. S1M and S1N). Inhibition of NUAK1 is thus sufficient to drive apoptosis in colorectal cancer cells, and death does not require complete suppression of MYPT1 phosphorylation. In contrast with the colorectal cancer lines, wild-type mouse embryonic fibroblasts (MEF) and U2OS cells were comparatively resistant to both inhibitors, especially to the NUAK1-selective HTH-01-015 (Fig. 1G; Supplementary Fig. S1O and S1P), consistent with previous data showing that U2OS cells are refractory to NUAK1 depletion (9). Notably, both inhibitors completely suppressed MYPT1 phosphorylation in U2OS cells, indicating that NUAK1 accounts for the vast majority of MYPT1 phosphorylation in this cell type. As we showed previously in MEFs (5), overexpression of MYC strongly sensitized U2OS cells to HTH-01-015–induced apoptosis (Fig. 1H). Conversely, depletion of endogenous MYC rescued colorectal cancer cells from HTH-01-015–induced apoptosis, and rescue was proportional to the degree of MYC depletion, consistent with an ectopic requirement for NUAK1 in cells with deregulated MYC (Fig. 1I).

Nuak1 Is Required for Formation of Colonic Polyps in Mice

In order to investigate the in vivo requirement for NUAK1 in colorectal cancer, we bred mice bearing a floxed Nuak1 allele (Nuak1F/F; ref. 11) onto a tamoxifen (Tam)-inducible mouse model of sporadic intestinal cancer: Villin-CreERT2; ApcF/F; Isl-KrasG12D (VAK). In this model, transient Tam-dependent activation of CreERT2 in the intestines of adult mice drives widespread deletion of one copy of Apc simultaneous with expression of oncogenic KRASG12D. However, tumor formation requires stochastic loss of the second copy of Apc, consistent with an ectopic requirement for NUAK1 in cells with deregulated MYC (Fig. 1I).

Using a single injection of Tam to transiently activate CreERT2, we deleted Nuak1 in the intestines of adult Villin-CreERT2; ApcF/F; Isl-KrasG12D, Nuak1F/F, F/F (VAK) mice and aged mice until symptomatic in order to compare the intestinal tumor burden with that of symptomatic VAK mice. Deletion of Nuak1 profoundly suppressed both the number and size of individual tumors in the colon of VAK mice, compared with VAK controls (Fig. 2A–D). In contrast, we observed no significant difference in either the number or size of tumors that arose in the SI, and VAK mice required sacrifice concurrently with VAK mice (Supplementary Fig. S2B–S2D). However, qPCR analysis of Nuak1 expression in individual polyps harvested from the SI of VAK mice revealed enrichment of Nuak1 mRNA when compared with disease-free adjacent tissue (Supplementary Fig. S1E), indicating a failure of transient CreER activation to efficiently delete Nuak1 in the tumor-initiating population of the SI. The absence of a Nuak1F/F phenotype in SI tumors thus appears to reflect
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**Figure 1.** NUAK1 overexpression correlates with tumor progression, lymph node infiltrates, and reduced overall survival (OS) in human colorectal cancer.**

**A.** Summary of NUAK1 mRNA detection in a human colorectal cancer TMA (N = 660), sorted by Duke’s grade A–C. N, normal colon. Asterisks indicate significance (one-way ANOVA and post hoc Tukey test). **B.** Box and whisker plots of median-centered NUAK1 mRNA expression from the TCGA colorectal adenocarcinoma cohort accessed via Oncomine: left, early (T1, 2) versus late (T3, 4) stage colorectal cancer; right, tumors with (N1, 2) or without (N0) lymph node metastasis. **C.** Overall survival of human patients with colorectal cancer (N = 947) separated by high versus low NUAK1 expression. Log-rank P value, hazard ratio (HR), and 95% confidence interval (CI) shown. **D.** Box and whisker plots of NUAK1 mRNA levels in human colorectal cancer separated by risk group as per T test P value shown. **E.** Data were mined and graphs adapted from Metabase SurvExpress. **F.** Apoptosis induced in human colorectal cancer cell lines 48 hours after treatment with the indicated concentrations of HTH-01-015. Red bars, Annexin V/propidium iodide (AV/PI) double-positive cells; black bars, Annexin V-positive cells. Mean and SEM of 3 independent experiments shown; asterisks, significance [ANOVA and post hoc Tukey test, relative to vehicle-treated controls (-)]. **G–I.** Immunoblot of cell lines from human colorectal cancer cell lines showing reduction in MYPT1 Ser445 phosphorylation upon inhibition of NUAK1 (8 hours). The asterisk indicates a nonspecific band. **G.** Apoptosis induced in U2OS cells 48 hours after treatment with 10 μmol/L HTH-01-015 or WZ4003. Mean and SEM of 3 independent experiments shown; asterisks, significance [ANOVA and post hoc Tukey test, relative to vehicle-treated controls (vc)]. **H.** Immunoblot (bottom) shows suppression of phospho-MYPT1 Ser445 upon NUAK1 inhibition. **I.** Apoptosis induced in U2OS-MycER cells upon NUAK1 inhibition in the presence (+OHT) or absence (-OHT) of 4-hydroxy-Tam-dependent activation of overexpressed MycER. Mean and SEM of 3 independent experiments shown; asterisks, significance [two-way ANOVA and post hoc Tukey test]. Immunoblot shows nuclear stabilization of MycER by 4-OHT. I, Rescue of colorectal cancer cells from HTH-01-015-induced apoptosis upon deletion of MYC. Mean and SEM of a representative experiment (N ≥ 2) in each cell line shown; asterisks, significance (ANOVA and post hoc Tukey test). Immunoblots show depletion of MYC in the same cell lines (bottom). *P < 0.05; **P < 0.01; ***P < 0.001.
Figure 2. Deletion of NUAK1 suppresses colorectal tumor formation. A, Number of large intestine (LI) tumors per mouse in VAK (N = 12) and VAKN (N = 16) mice, harvested at endpoint. Black bar, mean tumor number; red bars, SEM. B, Total tumor burden (area) per mouse of the indicated genotypes. Mean and SEM shown. C, Size of individual tumors in mice of the indicated genotypes. Box plots depict the median (red bar) and interquartile range of individual tumor area; whiskers reflect maximum observed tumor size. N = 192 (VAK) and 119 (VAKN). A–C, P values from Mann–Whitney test shown. D, Representative hematoxylin and eosin (H&E)–stained images of tumors from VAK (top) and VAKN (bottom) mice. Panels i–iii: scale bars, 500 μm. iv: zoom of the inset from iii; scale bar, 200 μm; T, tumor; N, normal tissue. E, Number of spheroids arising from freshly isolated VAHomKN small and large intestine, normalized to VAHomK controls seeded on the same day. Mean and SEM from VAHomK (N = 4) and VAHomKN (N = 6) mice shown. ***, significance (unpaired t test). F, Representative images of spheroids from E. Scale bar, 500 μm. G, Detection of NUAK1 mRNA in colonic spheroids from VAHomKN mice relative to NUAK1 transcript levels in VAK spheroids. Mean of 4 VAHomK and 6 VAHomKN mice shown. Error bar, SEM. H, Numbers of VAHomK SI-derived spheroids after treatment with NUAK1 inhibitors HTH-01-015 (HTH) or WZ4003 (WZ), normalized to vehicle-treated control (vc). Mean and SEM of 3 independent experiments shown; asterisks, significance (one-way ANOVA and post hoc Tukey test, relative to vc controls). I, Numbers of VAHomK large intestine–derived spheroids treated and graphed as per H. *, P < 0.05; **, P < 0.01; ***, P < 0.001.
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NUAK1 Activity Is Required for Ex Vivo Spheroid Formation

Homozygous deletion of Apc (A\(^{\text{Hm}}\)) in the gut rapidly gives rise to a Myc-dependent “crypt progenitor” phenotype, characterized by an extension of the transit-amplifying population into the normally quiescent villi of the SI (17). This phenotype was unimpaired by deletion of Nuak1 in VAK\(^{\text{Hm}}\)N intestines (Supplementary Fig. S3D). VASK transformed gut epithelium gives rise to spheroids when cultured in 3-D ex vivo, reflecting the tumor-initiating capacity of the transformed tissue (24). Primary VASK\(^{\text{Hm}}\)N colonic epithelium showed reduced spheroid-generating capacity, compared with VASK epithelium (Fig. 2E and F). Nuak1 expression was clearly detectable in the few VASK\(^{\text{Hm}}\)N spheroids that grew, suggesting that they likely arose from cells that escaped Cre-mediated Nuak1 deletion (Fig. 2G). Interestingly, a similar reduction in spheroid-generating capacity was also observed in primary epithelium isolated from the SI (Fig. 2E and F). Accordantly, pharmacologic inhibition of Nuak1 with either HTH-01-015 or WZ4003 profoundly suppressed formation of spheroids by VASK\(^{\text{Hm}}\)K gut epithelium from both small and large intestines (Fig. 2H and I), whereas wild-type organoids were refractory to treatment over the same time frame (Supplementary Fig. S3E).

NUAK1 Regulates the NRF2-Dependent Oxidative Stress Response

Reasoning that key physiologic roles of NUAK1 would be conserved across cell types, we exploited the fact that U2OS cells are refractory to NUAK1 suppression (in the absence of MYC overexpression) and express very little NUAK2 and performed an unbiased transcriptomic analysis after RNAi-mediated depletion of NUAK1. Metacore GeneGO pathway analysis revealed regulation of cholesterol synthesis, cell adhesion, and glutathione metabolism among the top-most pathways modulated upon NUAK1 depletion (Fig. 3A). The role of NUAK1 in regulating cell adhesion via phosphorylation of MYPT1 was described previously (10), whereas the modulation of glutathione metabolism suggested a novel role for NUAK1 in the antioxidant defense pathway. Strikingly, our transcriptomic analysis revealed a coordinated reduction in expression of a host of genes that are regulated by the antioxidant transcription factor NRF2 (encoded by NFE2L2; ref. 25), including the catalytic and regulatory.

**Table 1:** Nuak1 mRNA (% of control) and SREBP transcriptional control of cholesterol & FA synthesis (P-value and FDR).

**Figure 3:** NUAK1 promotes NRF2-dependent gene expression. A, Top 10 pathways modulated in U2OS cells after depletion of NUAK1 by shRNA, identified by Metacore GeneGO analysis of RNA-seq data. FDR, false discovery rate. B, RNA-seq read counts of selected NRF2 targets from A. Mean and SEM of 3 biological replicates shown; asterisks, significance (one-tailed t test). C, Reduction of NRF2 target gene expression upon inhibition of NUAK1 by shRNA. D, Comparison of selected NRF2 target gene expression upon depletion of NRF2 versus depletion of NUAK1 in colorectal cancer cell lines, HCT116 and SW480. N = 4. Mean and SEM shown; asterisks, significance (one-tailed t test). (continued on next page)
subunits of the glutamate-cysteine ligase; ROS scavengers thioredoxin, peroxiredoxin, and MGST; and glutathione reductase (Fig. 3B; Supplementary Fig. S4A). Acute inhibition of NUAK1 in U2OS cells with HTH-01-015 recapitulated these results (Fig. 3C), as did CRE-mediated deletion of Nuak1 in primary MEFs (Supplementary Fig. S4B and S4C), confirming the conservation of this effect across cell types and species. RNA sequencing (RNA-seq) analysis of SW480 colorectal cancer cells upon depletion of NUAK1 revealed a strong overlap with genes modulated upon depletion of NRF2 (NFE2L2), including several antioxidant pathway genes and the miR17-92 cluster, which was recently shown to negatively regulate LKB1 upstream of NUAK1 (26), suggestive of feedback regulation (Fig. 3D and E). Similar results were obtained in HCT116 cells (Fig. 3D). Pathway analysis showed broadly similar transcriptional effects of depletion of either NUAK1 or NRF2, whereas analysis of downregulated genes revealed significant enrichment for pathways “Oxidative stress” and “NRF2 regulation of oxidative stress” in both instances (Supplementary Tables S1 and S2).
The reduced expression of NRF2 target genes suggested that NUAK1-deficient cells would be hypersensitive to oxidative stress. Accordingly, we detected elevated levels of cytosolic H$_2$O$_2$ in U2OS cells, multiple colorectal cancer cell lines, and VAK colonic spheroids, after acute treatment with HTH-01-015 (Fig. 3F–I), whereas depletion of NUAK1 sensitized U2OS cells and multiple colorectal cancer cell lines to H$_2$O$_2$-induced cell death, consistent with the inhibitor and RNAi each reducing antioxidant buffering capacity, albeit to different degrees (Fig. 3J). Similar sensitization to H$_2$O$_2$-induced cell death was also observed upon CRE-mediated deletion of NUAK1 in MEFs, providing genetic confirmation of the specificity of this effect (Supplementary Fig. S4D and S4E). Colorectal cancer lines with lower levels of NUAK1 were inherently more sensitive to ROS-induced cell death, even in the absence of NUAK1 depletion, compared with cells expressing higher levels of NUAK1, whereas the relatively modest sensitization of SW480 cells compared with U2OS cells may reflect differences in the efficiency of NUAK1 depletion or indeed the relative expression of NUAK2. Notably, depletion of NUAK1 in some colorectal cancer lines resulted in increased NUAK2 expression (Supplementary Fig. S1L). Importantly, provision of exogenous antioxidants significantly rescued human colorectal cancer cells (Fig. 3K; Supplementary Fig. S4F) and VAK spheroids (Fig. 3L; Supplementary Fig. S4G) from NUAK1 inhibitor–induced apoptosis, indicating that ROS contributes substantially to cell death in both settings. The remaining levels of cell death measured in the colorectal cancer cell lines likely reflect exhaustion of the exogenous antioxidant, evidenced by intermediate levels of ROS in cells treated for 8 hours with NUAK1 inhibitor in the presence of Trolox (Supplementary Fig. S4H and S4I).

**NUAK1 Promotes Nuclear Translocation of NRF2 by Antagonizing GSK3β**

NRF2 was recently described to contain an AMPK-substrate consensus phosphomotif (27) that could potentially be targeted for phosphorylation by NUAK1. We used immunoprecipitation (IP) of FLAG-tagged NRF2 followed by immunoblotting with a pan–phospho-AMPK-substrate antibody to assess the influence of NUAK1 inhibition on NRF2 phosphorylation levels but detected no difference (Supplementary Fig. S5A). Similarly, purified NUAK1 showed no activity toward a corresponding NRF2 peptide in vitro (Supplementary Fig. S5B). Thus, NRF2 does not appear to be a direct target of NUAK1 kinase activity.

We noticed that acute inhibition of NUAK1 resulted in decreased total NRF2 levels (Fig. 4A). NRF2 is regulated by KEAP1, which sequesters NRF2 in the cytoplasm while targeting it continuously for ubiquitin-dependent degradation (28, 29). We asked if KEAP1 is required for regulation of NRF2 by NUAK1. As expected, RNAi-mediated depletion of KEAP1 increased basal levels of NRF2, yet concomitant inhibition of NUAK1 continued to reduce total NRF2 protein levels (Fig. 4B). Accordingly, cyclohexamide time-course analysis showed that NUAK1 depletion reduces total NRF2 levels but does not affect the rate of NRF2 degradation, per se (Supplementary Fig. S5C). KEAP1 contains a number of cysteine residues that are subject to oxidation and, in the presence of ROS, oxidized KEAP1 releases NRF2, allowing it to translocate to the nucleus and activate transcription (30). We therefore examined NUAK1-depleted or HTH-01-015-treated U2OS cells for nuclear accumulation of NRF2 after acute treatment with H$_2$O$_2$ and found that loss of NUAK1 activity strongly suppressed ROS-induced nuclear accumulation of NRF2 (Fig. 4C). Accordingly, ROS-induced translocation of NRF2 targets was also suppressed upon depletion of NUAK1 (Fig. 4D). Analysis of multiple colorectal cancer cell lines likewise revealed that depletion of NUAK1 suppresses ROS-driven NRF2 nuclear accumulation, indicating that this role of NUAK1 is conserved in colorectal cancer (Fig. 4E; Supplementary Fig. S5D). Additionally, this role of NUAK1 is at least partially shared with NUAK2, as depletion of NUAK2 in SW620 cells similarly suppressed peroxide-induced nuclear accumulation of NRF2 (Supplementary Fig. S5E).

We used unbiased, SILAC-based phosphoproteomics to identify candidate mediators of NRF2 regulation upon acute inhibition of NUAK1 in U2OS cells (see schematic, Supplementary Fig. S5F). Ser$^{309}$ of MYPT1 was the only site resident within a recognizable AMPK-related kinase consensus motif that was consistently reduced upon NUAK1 inhibition. This analysis also revealed reduced inhibitory phosphorylation of GSK3β at Ser$^\beta$ and a corresponding increase in phosphorylation of multiple GSK3β targets (Fig. 5A; Supplementary Table S3). GSK3β is known to suppress nuclear accumulation of NRF2: In the presence of oxidative stress, activation of AKT inhibits GSK3β via Ser$^\beta$ phosphorylation, allowing nuclear accumulation of NRF2 (31). We therefore examined the influence of NUAK1 depletion on ROS-driven signal transduction via AKT and GSK3β. Treatment of U2OS cells with H$_2$O$_2$ rapidly activated AKT, leading to increased GSK3β$^{309}$ phosphorylation. Upon depletion of NUAK1, activation of AKT by H$_2$O$_2$ was unimpaired; however, the inhibitory phosphorylation of GSK3β was strongly reduced, suggesting that NUAK1 may limit dephosphorylation of GSK3β$^{309}$ (Fig. 5B). Similar results were observed upon H$_2$O$_2$ treatment of NUAK1-depleted SW480 cells (Fig. 5C), whereas treatment with NUAK1 inhibitor suppressed GSK3β$^{309}$ phosphorylation in SW480 cells and VAK large intestine (LI) spheroids (Fig. 5D and E). Notably, phosphorylation of MYPT1 by NUAK1 inhibits PP1β activity (10), and PP1β was previously shown to dephosphorylate GSK3β (32, 33). Strikingly, H$_2$O$_2$ led to a clear increase in NUAK1-dependent MYPT1 phosphorylation (Fig. 5B), suggesting that ROS coordinately activates AKT and inactivates PP1β (via NUAK1) in order to suppress GSK3β activity. Significantly, inhibition of GSK3β stabilized total NRF2 levels and rescued nuclear accumulation of NRF2 in NUAK1-deficient SW480 cells (Fig. 5F; Supplementary Fig. S5G). Interestingly, depletion of PTEN similarly rescued nuclear NRF2, suggesting that the requirement for NUAK1 in this pathway can be overcome by strongly deregulated AKT signaling (Supplementary Fig. S5H).

**Regulation of NUAK1 by ROS and NRF2**

We asked if NUAK1 is an integral part of the oxidative stress response pathway. Depletion of NRF2 with two independent siRNAs consistently reduced NUAK1 protein levels (Fig. 6A). Examination of the NUAK1 promoter revealed a near-consensus antioxidant response element (ARE) located approximately 1.2 kb upstream of the NUAK1 transcription start site, and NRF2 chromatin IPs showed specific binding...
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Acetamide labeling of FLAG-NUAK1 IPs from cells treated for tated NUAK1 (Fig. 6G). Consistently, MS analysis of iodo-
in increased dimedone labeling of FLAG-immunoprecipi-
tagged NUAK1 to measure cysteine oxidation after H2O2 first used dimedone labeling (35) of cells expressing FLAG-
modify NUAK1 (Fig. 6F). To investigate this hypothesis, we
of PTEN by ROS (34), suggesting that ROS may directly
AKT phosphorylation, known to result from direct inactivation
these changes occur within the same time frame as increased
AKT phosphorylation, known to result from direct inactivation
of PTEN by ROS (34), suggesting that ROS may directly
modify NUAK1 (Fig. 6F). To investigate this hypothesis, we
first used dimedone labeling (35) of cells expressing FLAG-
tagged NUAK1 to measure cysteine oxidation after H2O2
Treatments. Treatment with increasing doses of H2O2 resulted
H2O2, with and without prior depletion of NUAK1 by two distinct siRNAs (left and center), or upon NUAK1 inhibition by HTH-01-015 (10 µmol/L). All blots
(A–C) are representative of at least 3 independent experiments. D, Expression analysis of NRF2 target genes GCLC and GCLM shows suppression of
H2O2-induced mRNA levels upon depletion of NUAK1. Mean and SEM of 3 independent experiments, normalized to preperoxide treatment, are shown.
Asterisks, significance (two-way ANOVA and post hoc Tukey test). E, Suppression of ROS-induced NRF2 nuclear translocation in multiple human colorec-
tal cancer cell lines upon depletion of NUAK1. *, P < 0.05; **, P < 0.01; ***, P < 0.001.

Figure 4. NUAK1 promotes nuclear accumulation of NRF2. A, NRF2 immunoblot of U2OS whole-cell extracts harvested after 4 or 8 hours of NUAK1 inhibition. Reduced phospo-MYPT1 confirms NUAK1 inhibition. Bottom, densitometry of the NRF2 blot shown. B, NRF2 immunoblot of KEAP1-depleted U2OS cells upon NUAK1 inhibition for 8 hours. Bottom, densitometry of the NRF2 blot shown. Right, confirmation of KEAP1 depletion with two independ-
tent siRNAs. C, Immunoblots of NRF2 protein levels in nuclear extracts from U2OS cells after acute (30 minutes) treatment of cells with 500 µmol/L
H2O2, with and without prior depletion of NUAK1 by two distinct siRNAs. D, Immunoblots of KEAP1-depleted U2OS cells upon NUAK1 inhibition for 8 hours. Bottom, densitometry of the NRF2 blot shown. Right, confirmation of KEAP1 depletion with two independ-
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tent siRNAs.

of NRF2 to the putative NUAK1 ARE, albeit at much lower efficiency than to the canonical NRF2 target HMBOX1 (Fig. 6B and C). Treatment of U2OS cells with H2O2 modestly increased NUAK1 mRNA but had much greater influence on NUAK1 protein, suggesting that posttranslational regulation may have greater functional impact (Fig. 6D and E). Time-course analysis revealed that H2O2 treatment rapidly increased phosphorylation of NUAK1 at Thr211, and consequent MYPT1S445 phosphorylation, downstream.

5 minutes with H2O2 similarly revealed increased oxidation of multiple NUAK1 cysteines, as compared with untreated controls (Fig. 6H). Collectively, our data suggest a model wherein ROS-dependent activation of NUAK1 coordinates inhibition of PPIβ with activation of AKT in order to counteract suppression of nuclear NRF2 by GSK3β (Fig. 6I).

Modeling the Therapeutic Potential of Nuak1 Suppression In Vivo

The above data collectively suggest that NUAK1 may be an excellent target for therapeutic intervention in colorectal cancer. However, the relatively poor potency of the NUAK1 inhibitors used above precludes their use in vivo. We therefore used a doxycycline (dox)-inducible RNAi approach to assess the impact of acute Nuak1 suppression on preexisting tumors. We used Villin-CreER72 to limit expression of rtTA3 to the mouse intestine. Upon activation with dox, rtTA3 was then used to drive expression of either of two shRNAs, targeting Nuak1 mRNA from nucleotide 612 or 1533, respectively,
Figure 5. NUAK1 inhibits negative regulation of NRF2 by GSK3β. A, Summary of phosphoproteomic changes induced in U2OS cells upon treatment with 10 μmol/L HTH-01-015 for 1 hour. Left, comparison of “forward” (x-axis) with “reverse” (y-axis) SILAC-labeled cells. Phosphorylation sites in the bottom left quadrant thus show consistent reduction in levels, whereas those in the top right quadrant show consistently higher phosphorylation levels detected by mass spectrometry. The previously validated NUAK1 substrate MYPT1 was used to set a threshold for acceptance/rejection of modulated phosphopeptides. Right, zoom of the inset from left, with known (red) and predicted (orange) GSK3β substrates highlighted. B, Immunoblots of lysates from NUAK1-depleted or control U2OS cells after treatment with H2O2 (500 μmol/L) showing effects on AKT, GSK3β, and MYPT1 phosphorylation. Bottom, the ratio of Ser9-phospho-/total GSK3β, measured by ImageJ analysis of the presented immunoblots. C, Immunoblots of NUAK1-depleted or control SW480 cytosolic fractions after treatment with H2O2 (30 minutes). Bottom, the ratio of Ser9-phospho-/total GSK3β, measured by ImageJ analysis of the presented immunoblots. D, Immunoblots show reduced Ser9 phosphorylation of GSK3β in the presence of NUAK1 inhibitors. Bottom, the ratio of Ser9-phospho-/total GSK3β, measured by ImageJ analysis of the presented immunoblots. E, Immunoblots show suppression of nuclear NRF2 and Ser9 phosphorylation of GSK3β upon inhibition of NUAK1 (5 μmol/L HTH-01-015 for 16 hours) in VAK large intestine–derived spheroids. Note that the presence of Matrigel likely blunts the impact of treatment with exogenous H2O2 (2 mmol/L for 1 hour). Bottom, quantification of nuclear NRF2 levels by ImageJ analysis. F, Pretreatment of NUAK1-depleted SW480 cells with GSK3β inhibitors BIO-acetoxime [a; 1 μmol/L for 6 hours] or CHIR99021 [b; 3 μmol/L for 6 hours] restores ROS-induced NRF2 nuclear translocation. All images are representative of at least 3 independent experiments.
Figure 6. Regulation of NUAK1 by NRF2 and ROS. A, Immunoblots show reduced NUAK1 protein and reduced MYPT1 S445 phosphorylation in U2OS cells upon depletion of NRF2 using 2 distinct siRNAs. B, Alignment of a putative antioxidant response element (ARE) in the NUAK1 promoter with the NRF2-binding consensus sequence. C, Chromatin IP of NRF2-bound DNA probed with primer pairs flanking (F1/R1; F2/R2) or distal to (F3/R3) the putative ARE in the NUAK1 promoter (see diagram). Right, NRF2 binding to the canonical target gene HMOX1 from the same analysis. Mean and SEM of technical replicates from 1 of 2 independent experiments shown. D, QPCR measurement of NUAK1 mRNA in U2OS cells treated with or without H2O2 (100 μmol/L) for 4 hours. Mean and SEM of 3 experiments. *, Significance (paired t test). E, Immunoblot of NUAK1 protein levels after treatment of U2OS cells (1 hour) with the indicated concentrations of H2O2. F, Immunoblots of T211 NUAK1 and S445 MYPT1 phosphorylation upon acute treatment of U2OS (left) or SW480 (right) with H2O2 for the indicated times. G, Oxidation of NUAK1 protein detected by dimedone labeling of U2OS cells expressing FLAG-tagged NUAK1 and treated for 5 minutes with 500 mmol/L H2O2. H, Identification of oxidized cysteines in FLAG-tagged NUAK1 by MS analysis of iodoacetamide labeling of U2OS-FLAG-NUAK1 cells treated with/without H2O2 for 5 minutes. Lysates were labeled with heavy (13C) or light (12C) iodoacetamide, followed by IP of FLAG-NUAK1. Plot shows analysis of reciprocally labeled samples from 2 independent experiments. Mean and SD indicated. I, Model integrating NUAK1 suppression of PP1β-dependent dephosphorylation of GSK3β as an integral step in nuclear mobilization of NRF2 in response to oxidative stress.
stringently selected to specifically deplete NUAK1 as previously described (see Supplementary Methods). Supplementary Fig. S6A shows depletion of NUAK1 in MEFs upon dox-dependent expression of Nuak1 shRNA.

Tumors were initiated in heterozygous floxed Apc (VA) mice by Tam-dependent activation of CreER72, and tumor development in the colon was accelerated by treatment with dextran sulfate sodium salt (DSS). DSS-treated VA mice developed colonic polyps within 70 days of CreER72 activation with >90% penetrance (36), and this time after induction was chosen to commence dox-dependent induction of either shRNA. Mice were maintained on dox for 1 week and then harvested for analysis (for a schematic, see Supplementary Fig. S6B). DSS-treated VA mice lacking either Nuak1 shRNA or rtTA alleles were similarly administered dox, to control for effects of the antibiotic. Depletion of NUAK1 for just 1 week strongly reduced the number of tumors per mouse and moreover suppressed the size of the remaining tumors found upon examination (Fig. 7A). Similar results were obtained with both Nuak1 shRNA alleles, strongly suggesting that the observed effects reflect the “on-target” depletion of NUAK1. Of the tumors that persisted in Nuak1 shRNA-expressing mice, all expressed readily detectable levels of Nuak1 mRNA, as measured by ISH (Supplementary Fig. S6C), indicating that some tumors escape shRNA-mediated NUAK1 depletion. PEARL imaging of intestines of mice injected overnight with Li-COR ROSstar reagent revealed elevated ROS levels in colonic tumors in situ after just 2 days of NUAK1 depletion (Supplementary Fig. S6D), whereas IHC analysis showed increased oxidative damage (8-oxo-dG), increased apoptosis (TUNEL), and reduced proliferation [bromodeoxyuridine (BrdUrd)] in NUAK1-depleted tumors within the same time frame (Fig. 7B and C). Consistent with our in vitro data, transcriptomic analysis of NUAK1-depleted tumors revealed significantly reduced expression of a host of NRF2 target genes within 2 days of NUAK1 depletion (Supplementary Fig. S6E). Importantly, exogenous provision of the antioxidant N-acetyl-cysteine (NAC) in drinking water reversed the tumor-suppressive effect of NUAK1 depletion (Fig. 7D; Supplementary Fig. S6F), but had no effect on NUAK1-replete tumors (Supplementary Fig. S6G). We conclude from these results that impairment of cellular antioxidant defenses is the underlying mechanism of the tumoricidal effect of NUAK1 suppression in the gut.

**DISCUSSION**

Here, we demonstrate that the AMPK-related kinase NUAK1 plays a key role in protecting colorectal tumors from oxidative stress. Using a combination of genetic and pharmacologic approaches, we show that NUAK1 is required for both formation and maintenance of colorectal tumors after loss of APC; that suppression of NUAK1 reduces viability of transformed intestinal spheroids and of human colorectal cell lines; and that protecting cells from toxic levels of ROS, via facilitation of NRF2-dependent antioxidant gene expression, is a key tumor-promoting activity of NUAK1. We show that NUAK1 kinase activity is rapidly increased by ROS following cysteine oxidation and, moreover, that NUAK1 is transcriptionally regulated by NRF2, placing NUAK1 squarely within the oxidative stress response pathway. Noting that NUAK1 expression is normally highest in highly oxidative tissues (11), it thus appears that protecting cells from oxidative stress is a major physiologic role of NUAK1 that has been co-opted by tumor cells to support their survival in the typically harsh tumor microenvironment. AMPK also participates in antioxidant defense, albeit indirectly, by conserving NADPH levels via inhibition of lipid biosynthesis (37), and a recent paper has shown a genetic requirement for this activity in MYC-overexpressing melanoma (38). Although AMPK may under certain circumstances directly phosphorylate NRF2 (27), in our system, the observed level of NRF2 phosphorylation is extremely low and is not modulated by NUAK1 inhibition. As such, AMPK does not presently appear to contribute to regulation of NRF2 by NUAK1.

Instead, we show that NUAK1 facilitates nuclear import of NRF2 by counteracting negative regulation of this process by GSK3β, and that direct inhibition of GSK3β restores NRF2 nuclear import in NUAK1-deficient cells. ROS-mediated inactivation of PTEN activates AKT, resulting in direct inhibitory phosphorylation of GSK3β on Ser9 (31, 39). This phosphorylation is opposed by PP1β, which reactivates GSK3β (33). We show that activation of AKT by ROS is unaffected by NUAK1 suppression; however, NUAK1 facilitates AKT-dependent regulation of GSK3β by inhibiting PP1β via phosphorylation of the PP1β regulatory subunit MYPT1. NUAK1 is thus required to coordinate inhibition of PP1β with AKT activation in response to ROS, thereby allowing GSK3β to be switched off long enough to permit NRF2 nuclear accumulation, providing fascinating new insight into temporal coordination of Redox signal transduction. This role of NUAK1 is likely to be shared with NUAK2, which similarly suppresses PP1β via MYPT1, and, indeed, we show that depletion of NUAK2 similarly reduces nuclear NRF2 in cells that highly express NUAK2. However, further work is needed to distinguish between specific effects of NUAK1 and NUAK2 on PP1β and beyond.

This mechanism of regulation suggests that the effects of NUAK1 suppression may be quite pleiotropic and, indeed, our phosphoproteomic analysis indicated modulation of multiple GSK3β targets in addition to NRF2. Moreover, transcriptional regulation by NRF2 reaches far beyond antioxidant gene expression, as previously noted (25). However, the central role of NRF2-dependent antioxidant gene expression in supporting tumor cell viability is attested to (i) by the hypersensitivity of NUAK1-depleted colorectal cancer tumor lines to oxidative stress-induced apoptosis and (ii) by the dramatic rescue of NUAK1-depleted colorectal tumors and inhibitor-treated spheroids upon provision of exogenous antioxidants. The more modest (but nonetheless significant) antioxidant rescue observed in HTH-01-015–treated colorectal cancer cells likely reflects the limits of trying to buffer against oxidative stress in standard cell culture (40). Although attempts to recapitulate the cytotoxic effects of NUAK1 inhibition in colorectal cancer cells using RNAi were unsuccessful, we believe that the effects of HTH-01-015 are specific for NUAK1 for several reasons: (i) this compound has been tested against more than 120 kinases and is extremely selective for NUAK1, although at higher concentrations it
Figure 7. Acute depletion of NUAK1 reverses colorectal tumors via increased ROS. A, Colonic tumor number per mouse (left), total tumor burden (center), and individual tumor size (right) in DSS-treated VA mice after 7 days of NUAK1 depletion in the gut using either of two dox-inducible shRNAs (1533, N = 10; or 612, N = 7), compared with dox-treated controls lacking either shRNA or the rtTA3 allele (−, N = 7). Graphs depict mean (blue lines) and SEM (red bars). Red asterisks indicate significance, relative to untreated controls (one-way ANOVA and post hoc Tukey test). An outlier mouse in the shNuak-612 cohort is circled (center), and all tumors present within the LI of that mouse are labeled in blue (right). The outlier and corresponding tumors were included in the statistical analysis. Total tumor burden is significantly reduced by shNuak1-612 if the outlier is omitted.

B, Representative IHC analysis of proliferation (BrdU), apoptosis (TUNEL), and oxidative damage (nuclear 8-oxo-deoxyguanine) with corresponding ISH analysis of NUAK1 mRNA (red dots) in selected tumors from control (top) and shNuak1-1533 mice treated for 2 days with dox. C, HALO automated quantification of BrdU, TUNEL, and 8-oxo-guanine IHC in individual tumors from shNuak1 expressing (N = 6) or control (N = 6) mice, as per B. Mean (blue bars) and SEM (red bars) indicated. Red asterisks indicate significance (Mann–Whitney test).

D, Tumor number, total tumor burden, and individual tumor size in DSS-treated VA mice after 7 days of NUAK1 depletion in the gut using shNuak1-1533, in mice given N-acetyl-cysteine (NAC; N = 8) compared with no exogenous antioxidant (nt). Note that the NAC-untreated data are the same as used in A. Red asterisks indicate significance (Mann–Whitney test). *, P < 0.05; **, P < 0.01; ***, P < 0.001.
does show some activity toward NUAK2 and possibly MARK3 (23); (ii) cytotoxicity was observed only at concentrations that yielded a clear reduction in MYPT1 phosphorylation, thus indicating greater suppression of either NUAK1 or a NUAK1-like activity; (iii) cytotoxicity was reproducible with the unrelated compound WZ4003; (iv) consistent with our previous demonstration of a synthetic lethal relationship between MYC and NUAK1 (9), sensitivity to HTH-01-015 was MYC dependent and colorectal cancer cell death was rescued by MYC depletion. It is thus unclear why cytotoxicity was not observed using RNAi in the cell-culture setting, except in instances of simultaneous peroxide challenge. It may be that very low levels of residual NUAK1 suffice to suppress cell death, consistent with our data in SW620 cells, which do express very low levels of NUAK1. Additionally, the asynchronous nature of RNAi may allow cultured cell populations time to quench H2O2 before the threshold for loss of viability is breached and, accordingly, depletion of NUAK1 resulted in upregulation of NUAK2 in multiple colorectal cancer lines, likely dampening the impact of NUAK1 depletion. Furthermore, HTH-01-015 has been shown to partially inhibit NUAK2 at the 10 μmol/L dose that exhibited cytotoxicity in colorectal cancer lines (23) and, although we cannot entirely exclude the possibility of an off-target effect of the inhibitor, the fact that colorectal cancer cytotoxicity at this dose was significantly rescued by both antioxidant provision and by depletion of c-MYC strongly supports our interpretation that the on-target effect of the inhibitor is responsible for induction of tumor cell death. The differential sensitivity of some cells (e.g., SW480) to the NUAK1 inhibitor versus peroxide challenge after NUAK1 depletion by RNAi may thus reflect expression of NUAK2 and/or the continued biochemical activity of residual levels of NUAK1 after RNAi-mediated depletion.

Our previous work linked the selective requirement of tumor cells for NUAK1 to MYC overexpression, and this link is borne out here by the rescue of NUAK1 inhibitor–induced death upon depletion of MYC from colorectal cancer cell lines. In the intestine, loss of Apc leads to β-catenin–dependent overexpression of endogenous MYC. Although deregulated MYC is alone insufficient for intestinal tumor formation (41), it is nonetheless required for β-catenin–driven polyposis and, significantly, is also required for the elevation of ROS levels observed in vivo upon loss of Apc (42).

Colorectal tumors will thus have evolved in the face of continuous oxidative stress, and cells derived therefrom would likely be better buffered against oxidative stress than cells (e.g., U2OS) that lack MYC deregulation. Accordingly, we show that U2OS cells depleted of NUAK1 are exquisitely sensitive to a peroxide challenge and that this is phenocopied by MYC overexpression. Note that the absence of NUAK2 expression from U2OS cells likely increases their reliance upon NUAK1. NUAK1 thus functions in two major tumor-protective pathways, ATP homeostasis and the oxidative stress response, that are rapidly engaged to support viability upon MYC overexpression (43). As such, NUAK1 appears to be more intimately linked with the downstream metabolic consequences of MYC deregulation than with the absolute levels of MYC protein per se, and we recently linked MYC deregulation to calcium-dependent activation of NUAK1 in LKB1-deficient cells (13).

Exploiting the heightened sensitivity of tumor cells to ROS is emerging as a plausible strategy for cancer therapy (44, 45). Recently, intravenous injection of very high doses of dihydroxyacetone was shown to suppress colorectal tumor formation by saturating ROS scavengers, and subsequent work suggests that this strategy may indeed show clinical benefit (46, 47). With increasing evidence linking elevated NRF2 to aggressive disease (48, 49), disabling antioxidant defenses via transient inhibition of NUAK1 may offer a new strategy for improving therapeutic outcomes in cancer.

METHODS
Mouse Experiments and Analyses
All experiments involving mice were approved by the local ethics committee and conducted in accordance with UK Home Office license numbers 70/7950 and 70/8646. Mice were housed in a constant 12-hour light/dark cycle, fed, and watered ad libitum. Mice bearing dox-inducible shRNAs targeting Nuak1 are described in the Supplementary Materials section. All mice were maintained on mixed (FVBN × C57BL/6 × 129/SV) background, and littermate controls were used for all experiments. To induce allele recombination, transient activation of CreER(T2) in the intestine was performed on mice ages 6 to 12 weeks via single IP injection of 50 mg/kg Tam. For survival analysis, humane endpoints were defined as exhibition of two or more symptoms: >15% weight loss, pale feet, lethargy, and bloody stool. Where indicated, 1.75% DSS, m.w. 35–50 kDa (M.P. Biochemicals) was administered in drinking water for 5 days, commencing 4 days after allele induction, followed by distilled water for 1 week, then tap water. Doxycycline (Sigma; in H2O) was administered by oral gavage in 2 mg daily boluses, from day 64 to day 70 after induction. N-acetylcysteine (Sigma; 4% w/v solution) was administered in drinking water, starting 3 days before shRNA induction, and replaced every 3 to 4 days until sacrifice. All mice were sacrificed using a schedule 1 procedure. ROSstar 650 reagent (LI-COR) was injected IV the day before tissue harvesting and signal was detected by PEARL imaging.

Crypt Culture
Primary spheroid cultures of intestinal crypts were established as previously described (50) from the SI and colon of VAHomK and VAHomKN mice. Adult mice were induced as above, and tissues were harvested 4 days later. Intestines were flushed with ice-cold PBS and longitudinally, and villi were removed using a glass coverslip. Established crypt cultures were split 1 to 2 times per week by manual disruption followed by incubation in Cell Dissociation solution (Thermo) until a single-cell suspension was achieved. Cells were then centrifuged (Thermo) until a single-cell suspension was achieved. Cells were then counted and pelleted at 600 rpm for 2 minutes in a table-top centrifuge. Resuspended crypts were counted by hemocytometer, then seeded in Matrigel (BD Bioscience) with advanced DMEM/F12 media (Invitrogen), supplemented with 10 mmol/L HEPES; 2 mmol/L glutamine; 0.1% FBS; penicillin/streptomycin, N-2 and B-27 supplements (1x, Invitrogen). Alternatively, for quantification of primary spheroid formation, isolated crypts were further incubated in Cell Dissociation solution (Thermo) until a single-cell suspension was achieved. Cells were then counted and seeded at normalized density as above. Growth factors Noggin (100 ng/mL) and EGF (50 ng/mL; Peprotech) were added to primary cultures but removed from subsequent passages. Spheroids were counted manually 3 or 4 days after seeding. Wild-type organoid cultures were prepared similarly but additionally supplemented with R-Spondin (500 ng/mL; R&D Systems). Established crypt cultures were split 1 to 2 times per week by manual disruption followed by incubation in Cell Dissociation solution (Thermo) until a single-cell suspension was achieved. Cells were then
counted and reseeded at normalized density. NUAK1 inhibitors, HTH-01-015 (Apex Biotech) or WZ4003 (Medchem Express) in DMSO, were added to single-cell suspensions at a final concentration of 500 μmol/L for 16 hours prior to HTH-01-015 and replenished daily for 3 days. ROS detection was performed by confocal fluorescent microscopy using 5 μmol/L CellRox green (Thermo; 3 hours at 37°C) after overnight treatment of preformed spheroids with HTH-01-015.

**Cell Lines**

U2OS (2009), HCT116, SW620, and SW480 (all in 2013) cell lines were obtained from the ATCC and cultured in DMEM supplemented with penicillin/streptomycin and 10% FBS. Cells were expanded initially upon receipt and aliquoted into frozen stocks. Upon resuscitation, cells were passaged as required and discarded after no more than 3 months of continuous culture. Cell lines were periodically validated using the Promega Geneprint 10 authentication kit, most recently in August 2017. All cell lines in culture were tested every 3 months for Mycoplasma.

**Transcriptomic Analysis**

Whole-transcriptome analysis was performed by Illumina RNA-seq. The following datasets are available through ArrayExpress: U2OS ± NUAK1 shRNA, accession number E-MTAB-6244; SW480 ± siNRF2 or siNUAK1, accession number EMtab-6264; Apc/DSS-induced colonic tumors ± shNUAK1, accession number E-MTAB-6265. A full description of methodology is provided in the Supplementary Materials.

**Statistical Analysis**

All experiments were performed at least 3 times except where noted in the text. Raw data obtained from quantitative real-time PCR, FACS, and spheroid generation assays were copied into Excel (Microsotc) or Prism (Graphpad) spreadsheets. All mean and SEM values of biological replicates were calculated using the calculator function. Graphical representation of such data was also produced in Excel or in Prism. Box and spider plots were generated using Prism. Statistical significance for pairwise data was determined by Student’s paired or paired t test, as indicated. For multiple comparisons, ANOVA was used with a post hoc Tukey test. *P < 0.05; **P < 0.01; ***P < 0.001. For Kaplan–Meier plots, Mantel–Cox log-rank P values are presented; for tumor enumeration, Mann–Whitney tests were performed.

Additional methods are described in the Supplementary Materials.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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**REFERENCES**


NUAK1 Protects Colorectal Tumors from Oxidative Stress


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