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The interplay between epigenetic silencing, oncogenic KRas and HIF-1 regulatory pathways in control of *BNIP3* expression in human colorectal cancer cells

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ABSTRACT

Bcl-2/adenovirus E1B-19 kDa-interacting protein 3 (BNIP3) is an important mediator of cell survival and a member of the Bcl-2 family of proteins that regulate programmed cell death and autophagy. We have previously established a link between the expression of oncogenic HRas and up-regulation of BNIP3 and the control of autophagy in cancer cells. However, in view of varied expression of BNIP3 in different tumor types and emerging uncertainties as to the role of epigenetic silencing, oncogenic regulation and the role of BNIP3 in cancer are still poorly understood.

In the present study we describe profound effect of KRas on the expression of methylated *BNIP3* in colorectal cancer cells and explore the interplay between HIF-1, hypoxia pathway and oncogenic KRas in this context. We observed that *BNIP3* mRNA remains undetectable in aggressive DLD-1 cells harboring G13D mutant *KRAS* and HT-29 colorectal cancer cells unless the cells are exposed to demethylating agents such as 5-aza-2′-deoxycytidine. Following this treatment *BNIP3* expression remains uniquely dependent on the Ras activity. We found that hypoxia or pharmacological activation of HIF-1 alone contributes to, but is not sufficient for efficient induction of *BNIP3* mRNA transcription in cells lacking mutant KRas activity. The up-regulation of *BNIP3* by KRas in this setting is mediated by the MAPK pathway, and is attenuated by the respective inhibitors (PD98059, U0126). Thus, we demonstrate the novel mechanism where activity of Ras is essential for 5-aza-2′-deoxycytidine-mediated *BNIP3* expression. Moreover, we found that 5-aza-2′-deoxycytidine-mediated or enforced up-regulation of *BNIP3* in DLD-1 cells results in KRas-dependent resistance to 5-Fluorouracil.

1. Introduction

BNIP3 (Bcl-2/adenovirus E1B-19 kDa-interacting protein 3, formerly Nip3) is a member of Bcl-2 family of proteins. Despite of classification of BNIP3 as a BH3-only pro-apoptotic protein evidence suggests lack of or only a weak pro-apoptotic activity associated with BNIP3. Instead, BNIP3 emerged as one of the key inducers of autophagy, a process of controlled self-digestion that is critical for cellular adaptation to stress conditions [1,2].

Autophagy regulated by BNIP3 is often regarded as an important regulatory mechanism involved in tumor growth and responses to anticancer therapies, but the nature and determinants of this relationship remain largely obscure. The levels of *BNIP3* were found to be elevated in several cancers, including: breast, cervical, prostate, and lung [3,4]. In contrast, *BNIP3* expression is decreased or the gene is completely silenced in cases of colorectal [5] and pancreatic cancers [6]. Moreover, this is often correlated with altered sensitivity to anticancer drugs. For example, *BNIP3* silencing in colon cancer is associated with increased resistance to S-1 therapy combined with camptothecin 11 (CPT-11) [7] and 5-aza-2′-deoxycytidine (5-aza-dC) mediated up-regulation of *BNIP3* in HCT-15 cells restored sensitivity to CPT-11 [8]. On the other hand, BNIP3 expression in hepatoma cells provides the resistance to etoposide [9], temozolomide- or hypoxia-induced cell death of gliomas [10,11].

BNIP3 expression is regulated mainly at the level of gene transcription [12]. While HIF-1 is considered to be the principal transcription factor controlling *BNIP3* expression, the other transcription factors have also been implicated in this process, including NF-κB [13], and especially E2F1 [13,14].

It is then reasonable to consider the role of BNIP3 in the context of more specific transforming circumstances, such as those exemplified by the activation of the GTPase Ras (Ras) pathway. In this regard autophagy has already been implicated as a mechanism

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important to maintain tumorigenesis of certain Ras-dependent cancers [15], but *in vitro* studies have suggested the increase in cell survival related to Ras-mediated down-regulation of *BNIP3* and some of the key mediators of autophagy [16,17]. On the other hand, there is a recurrent interest in using demethylating agents, such as 5-aza-dC or 5-azacytidine in treatment of myelodysplastic syndrome, as well as solid tumors, including colorectal cancer (CRC) and other malignancies harboring oncogenic *KRAS* mutations [18,19]. Especially, 5-aza-dC have been presented as a chemosensitizer. that could increase sensitivity of myeolid leukemia cells to busulfan [20] and CRC cell lines to CPT-11- or SN-38-induced cell death [7,8]. In this context, the control of *BNIP3* level by intracellular and extracellular stimuli (hypoxia) may represent an important aspect of therapeutic efficacy of demethylating agents, and especially their combinations.

For these reasons, in the present study we set out to explore the link between naturally occurring activating mutations of the *KRAS* oncogene in CRC cells and the 5-aza-dC-mediated regulation of *BNIP3* expression under relevant microenvironmental conditions such as normoxia and hypoxia.

2. Materials and methods

2.1. Cell culture

DLD-1, DKs-8 and HT-29 CRC cell lines were cultured in Dulbecco Modified Eagle's Medium (DMEM; HyClone, Waltham, MA, USA) supplemented with 10% fetal bovine serum (Gibco, Carlsbad, CA, USA) and with Antibiotic and Antimycotic Solution (Sigma–Aldrich, St. Louis, MO, USA). Culture plates and flasks were purchased from Corning Inc. (Tewksbury, MA, USA). Cells were grown at 37 °C, 5% CO_2 and 95% humidity (NuAire, Plymouth, MN, USA).

2.2. Chemicals, reagents, antibodies, plasmids and siRNAs

Phosphate Buffered Saline (PBS), cobalt chloride ($CoCl_2$), trypsin, Tris–HCl, 5-aza-2′-deoxycytidine, 5-Fluorouracil were purchased from Sigma–Aldrich. Ethanol and sodium chloride were obtained from POCh (Gliwice, Poland). U0126, U0124 and PD98059 were purchased from Calbiochem (Merck, Darmstadt, Germany). Antibodies: mouse anti-Ras (1:200; Pierce, Rockford, IL USA), mouse anti-HIF-1-α (5 μg/ml; Abcam Inc., Cambridge, MA, USA), goat anti-actin and rabbit anti-histone H1 (both 2 μg/ml; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), anti-mouse, anti-rabbit and anti-goat secondary horseradish peroxidase (HRP)-conjugated antibodies (1:1000 or 1:2000; Dako, Glostrup, Denmark). RT-PCR primer/probe sets: *BNIP3*-Hs00969291_m1, *HIF1A*-Hs00153153_m1 and *E2F1*-Hs00153451_m1, endogenous control GAPDH-4333764F (Applied Biosystems, Carlsbad, CA, USA). Plasmids: pCMV-RasN17 (RasN17) (Clontech Labs, Inc. Mountain View, CA USA), pcDNA3-BNIP3 [21] and pcDNA3 (Invitrogen Corp., Carlsbad, CA, USA), HRE-luciferase reporter plasmid (generous gift from Navdeep Chandel; Addgene plasmid 26731, Cambridge, MA, USA) [22]. Plasmids were extracted using EZ-200 Spin Column Plasmids DNA Medi-Preps Kit (Bio Basic, ON, Canada). siRNA targeting *HIF1A*, *E2F1* and non-targeting Pool D were obtained from Dharmacon (Lafayette, CO, USA).

2.3. Cell treatments

Cells were cultured in hypoxia (2% O_2) or treated with $CoCl_2$ (100 μM), 5-aza-dC (1 μM), 5-Fluorouracil (20 μM, 50 μM), U0126 (10 μM), U0124 (10 μM) or PD98059 (20 μM) for 48 h unless stated otherwise.

2.4. Ras activation assay

Ras pull-down assay was performed using EZ-Detect™ Ras Activation Kit (Pierce) according to the manufacturer's instructions. Briefly, 106 cells were lysed in 0.5 ml of the lysis buffer and equivalents of 0.5 mg of total protein were incubated with the GST-protein binding domain (PBD) fusion protein (GST-Raf1-PBD) and loaded into preconditioned columns. The active Ras was affinity-purified by binding to glutathione and eluted from the glutathione resin using SDS–PAGE sample loading buffer. The eluate was resolved using SDS–PAGE and probed with the Ras-specific antibody.

2.5. Western blotting

Cells were lysed (20 mM Tris–HCl, 100 mM NaCl, 0.5% NP-40) supplemented with SIGMAFAST Protease Inhibitor Cocktail (Sigma). The samples were resolved by SDS–PAGE (6% or 12% gels) and transferred to PVDF membranes (Millipore Corp., Billerica, MA, USA). The membranes were blocked and incubated overnight at 4 °C with the indicated primary antibody followed by detection with the appropriate secondary HRP-conjugated antibody. Protein bands were visualized using ECL (Enzymatic Chemiluminescence) blotting detection system (Pierce).

2.6. Silencing assays

Cells (2.0×10^5 cells/well of 6-well plate) were transiently transfected with 100 pmol siRNA in Opti-MEM for 4 h using Lipofectamine2000 Reagent (Invitrogen). To maximize transfection efficiency, cells were transfected twice – 24 h (*HIF1A* -siRNA) or 48 h (*E2F1*-siRNA) apart. After transfection the treatment was restored. The RNA was extracted 48 h after the second transfection. Corresponding schedules were applied to non-targeting siRNA controls.

2.7. Plasmid transfection

Cells were cultured as described above and transfected using Lipofectamine2000 Reagent, with 4 μg of plasmid DNA (pcBNIP3, pCMV-RasN17 and pcDNA3) for 4 h. After transfection the treatment was restored and RNA was isolated 24 h later.

2.8. Quantitative polymerase chain reaction (real-time RT-PCR)

Total RNA was extracted from the cells using EZ-10 Spin Column Total RNA Mini-Preps Super Kit (BioBasic) followed by DNase I (Fermentas, Waltham, MA, USA) treatment. The RNA was reverse-transcribed by iScript cDNA Synthesis Kit (Bio-Rad Labs, Hercules, CA, USA). Quantitative PCR using appropriate TaqMan Gene Expression Assay and Viia7 or 7300 systems (Applied Biosystems).

2.9. Luciferase reporter assay

Cells (6×10^3 cells/well) were plated on white, 96-well plates (Greiner Bio-One GmbH, Frickenhausen, Germany), and transfected (4 h) with HRE-luciferase (0.4 μg DNA/well) using Lipofectamine2000 Reagent following 48 h of indicated treatment. Cells were lysed in GloLysis Buffer (Promega) and after 15 min of incubation Bright GloReagent (Promega) was added. Relative Light Units (RLU) were measured using Synergy H4 Hybrid Multi-Mode Microplate Reader (BioTek, Winooski, VT, USA).

2.10. MTS assay

Cells viability was determined using the CellTiter 96 AQueous Non-Radioactive Cell Proliferation Assay (Promega, Madison, WI,

USA). Briefly, 6×10^3 cells/well (96-well plate) were pretreated for 24 h with 5-aza-dC and next treated with indicated concentrations of 5-Fluorouracil for 48 h. The loss of the viability was calculated as percentages difference of the control sample (100%).

2.11. Data analysis

Gene expression data are relative quantities (RQ) of target gene mRNA normalized to endogenous control (GAPDH) mRNA. They are shown as an average RQ from at least two independent experiments ± standard deviation or RQ ± standard error (as indicated in figures descriptions). Statistical analysis involved Student's *t*-test.

3. Results and discussion

3.1. Ras pathway activity is essential for 5-aza-dC mediated expression of BNIP3

The expression of *BNIP3* in human cancers is highly variable and the significance of this remains to be established. Ras activation is prevalent in CRC, and for that reason DLD-1, HT-29 and DKs-8 [23] cell lines were chosen for our detailed studies. DLD-1 cells exemplify cancer cells in which activating mutation G13D of the *KRAS* oncogene coincides with virtually undetectable levels of *BNIP3* mRNA. Indeed, treatment of DLD-1 cells with DNA methyltransferase inhibitor, 5-aza-dC, resulted in ample expression of *BNIP3* within 48 h of exposure at 1 μM (Fig. 1A - left panel). This is consistent with the activity of this agent in the context of colorectal [5] and pancreatic cells [6]. Importantly, we show that this effect is strictly dependent on the $KRas^{G13D}$, as DKs-8 isogenic cells (expressing only wt *KRAS* allele) are unable to express *BNIP3* under these conditions (Fig. 1A - right panel). While mutant Ras controls the activities of methyltrasferases in various cellular contexts [24], the methylation of *BNIP3* locus in DLD-1 cells was not a result of the KRas activity, as disruption of the mutant allele sequence in these cells (DKs-8 variant) obliterated *BNIP3* expression, regardless of 5-aza-dC treatment.

Since MEK/MAPK pathway has been implicated as therapeutic target in colorectal cancers [25] as well as in regulation of *BNIP3* silencing [26] it is of interest whether this pathway also controls *BNIP3* expression in CRC cells. Here we show that in DLD-1 and HT-29 cells pre-treated with 5-aza-dC *BNIP3* expression can be reduced by PD98059 and U0126, two inhibitors of MEK1/2, in both CRC cell lines, by about 90% and 60%, respectively, while an inactive analogue (U0124) was ineffective (Fig. 1C). Similarly direct inhibition of Ras activity in DLD-1 cells by transient expression

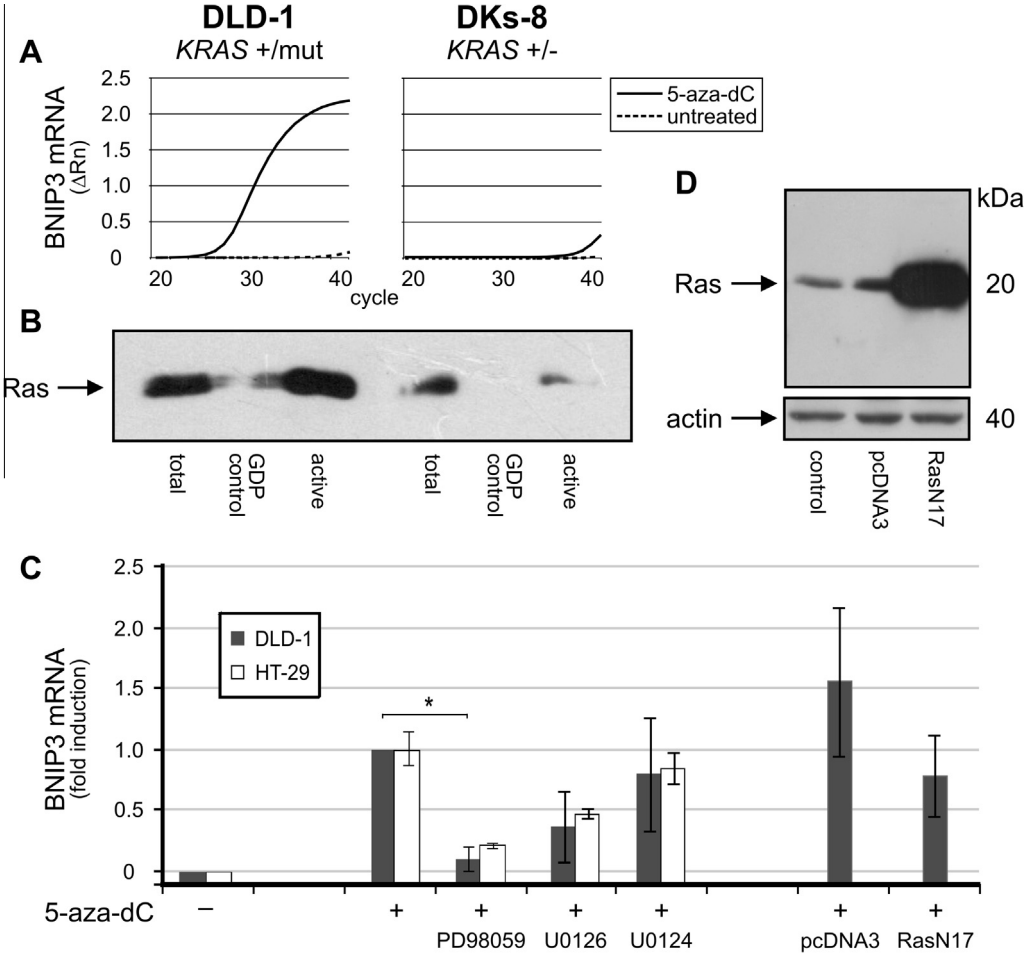

Fig. 1. The MEK pathway is essential for restoration of BNIP3 expression by 5-aza-dC. *BNIP3* expression can be restored by 5-aza-dC (1 μM) in DLD-1 cells (A-left panel, C-dark bars) carrying G13D activating mutation (*mut*) of KRas protein (B) and in HT-29 (C-empty bars). Disruption of the mutant allele sequence in DKs-8 cells, reflected by lower Ras activity (B), obliterate *BNIP3* expression (A-right panel) regardless of treatment with methylation inhibitor 5-aza-dC. Inhibition of MEK/MAPK pathway (C) by PD98059 (20 μM) and U0126 (10 μM), but not U0124 (10 μM) control reagent, down-regulates *BNIP3* mRNA expression in both cell lines. Similarly, transfection of DLD-1 cells with plasmid pCMV-RasN17 expressing dominant negative form of Ras, reflected by the overexpression of Ras protein detected by panRas antibody (D) decreases the *BNIP3* mRNA expression in comparison to an empty vector (pcDNA3). (A) deltaRn vs cycle graphs of representative *BNIP3* real-time RT-PCR assay; $n = 9$. (B) Representative blot of active Ras pull-down; $n = 2$. (C) Fold induction (average RQ ± standard deviation) of at least two independent experiments; $p < 0.005$. (D) Representative Western blots of Ras and actin (serving as a loading control) of two independent experiments.

of RasN17 (Fig. 1D), the dominant negative mutant of Ras, resulted in 50% decrease in *BNIP3* mRNA level in comparison to cells transfected with empty vector pcDNA3(Fig. 1C). Thus, here we present that in CRC cell line Ras-pathway is essential for 5-aza-dC-mediated expression of *BNIP3*.

Moreover, the dominant role of Ras in this setting raises the questions of the potential implication of agents targeting Ras pathway on the status of *BNIP3* in CRC cells and about the related biological and therapeutic consequences.

3.2. The secondary role of HIF-1 in hypoxic BNIP3 regulation in CRC cells

BNIP3 overexpression has been attributed mainly to the activation of HIF-1 transcription factor in the regions of hypoxia commonly occurring in solid tumors and related to resistance to hypoxia-induced cell death. However, our research have revealed that BNIP3 expression can be increased in normoxia, as illustrated by examples of cells harboring activated Ras pathway [17,27]. It should be noted that Ras pathway may activate HIF-1 in normoxia [28] resulting in a potential interdependence and synergy. To understand these events in more detail and in the context of $KRas^{G13D}$ expressing CRC cells we first tested the effects of HIF-1 and hypoxia on 5-aza-dC-treated cells positive (DLD-1) and negative (DKs-8 cells) for mutant *KRAS*. As expected, under normoxia the expression of the HIF-1-α subunit was undetectable by Western blotting in either cell line. Exposure of these cells to cobalt chloride (100 μM), a known stabilizer of HIF-1-α protein led to predictable and almost uniform increase in HIF-1 expression and activity in both cell lines, regardless of their differential levels of Ras activity (Fig. 2A, B). $CoCl_2$ and mild microenvironmental hypoxia (2% O_2) also consistently triggered HIF-1 transcriptional activity against HRE-Luciferase reporter in the case of both cell lines. Notably, neither levels of *HIF1A* nor activities of HIF-1 were significantly affected by the exposure to 5-aza-dC (Fig. 2B).

Interestingly, demethylation of *BNIP3* in DLD-1 cells renders this gene susceptible to further up-regulation in the presence of either $CoCl_2$ or hypoxia. Again, these effects were strictly dependent on the $KRas^{G13D}$, as expressing only wt *KRAS* allele DKs-8 cells were not induced to express *BNIP3* under these conditions, in spite of containing ample levels of active *HIF1A* (Fig. 2C, B). Moreover, the combined stimulation of *BNIP3* by KRas and pharmacological hypoxia remained susceptible to MEK inhibitors: PD98059 and U0126 in both DLD-1 and HT-29 cells and the RasN17 mutant in DLD-1 cells (Fig. 2D).

Since HIF-1 may mediate some of the regulatory effects of the activated Ras pathway we chose to assess more directly the requirement for HIF-1 activity in KRas dependent regulation of *BNIP3*. To accomplish this, DLD-1 cells were transfected with siRNA directed against *HIF1A* and marked and specific down-regulation of this transcript was achieved, regardless of cell exposure to 5-aza-dC, $CoCl_2$ or hypoxia (Fig. 3A, B). Simultaneously, we assessed the levels of *BNIP3* mRNA under similar conditions and observed that in 5-aza-dC-treated DLD-1 cells the silencing of *HIF1A* removed the hypoxia/HIF-1-dependent increase in *BNIP3* mRNA (Fig. 3A, B), but not the baseline expression of this gene, which is regulated by mutant $KRas^{G13D}$ (Fig. 3C, B). Therefore, we did not detect a particularly close correlation between *HIF1A* levels in DLD-1 cells and the status of *BNIP3* mRNA (e.g. in 5-aza-dC-treated cells under normoxia or in untreated cells under hypoxia). Moreover, silencing of *HIF1A* mRNA had no effect on the baseline *BNIP3* expression upon demethylation, and only counteracted induction of *BNIP3* by hypoxia or $CoCl_2$.

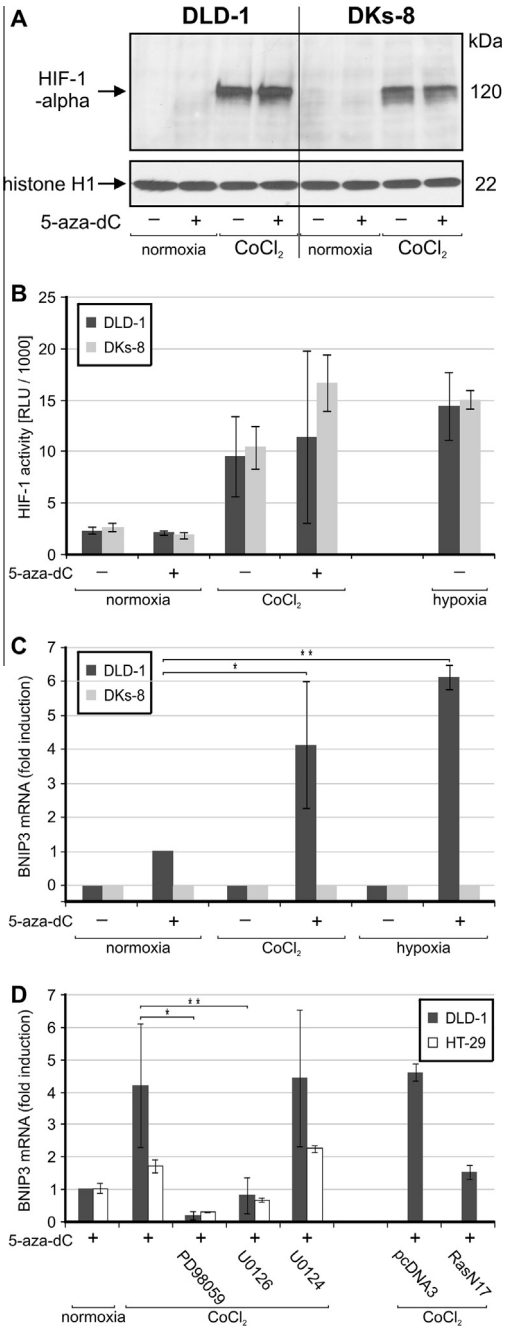

Fig. 2. Ras activity is essential for restoration of *BNIP3* expression by 5-aza-dC in hypoxia. Hypoxia (2% O_2) or pharmacological hypoxia ($CoCl_2$, 100 μM) up-regulates 5-aza-dC-induced (1 μM) *BNIP3* expression in DLD-1 cells (C). Disruption of the mutant *KRAS* allele sequence in DKs-8 cells, prevents expression of *BNIP3* by 5-aza-dC (C – grey bars), despite of HIF-1-α expression (A) and HIF-1 transcription factor activity (B). Similarly, inhibition of MEK/MAPK pathway (D) by PD98059 (20 μM) and U0126 (10 μM), but not U0124 (10 μM) control reagent, in DLD-1 and HT-29 CRC, as well as transfection of DLD-1 cells with plasmid pCMV-RasN17 (RasN17) expressing dominant negative form of Ras, but not control pcDNA3 vector, down-regulate $CoCl_2$-stimulated expression of *BNIP3* mRNA (D – right side). (A) Representative of 3 independent Western Blots of HIF-1-α and histone H1 (a loading control). (B) Relative luciferase units (RLU) measured in Luciferase based assay using HRE-luciferase vector with promoter consisting of 3 consecutive hypoxia responsive elements (HRE). Representative result of 3 independent experiments. (C) Fold induction (Average RQ ± standard deviation) of at least 5 independent real time RT-PCR experiments, $^{*}p < 0.02$, $^{**}p < 0.03$. (D) Fold induction (average RQ ± standard deviation) of 3 (DLD-1) or 2 (HT-29) independent real time RT-PCR experiments, $^{*}p < 0.0005$, $^{**}p < 0.005$.

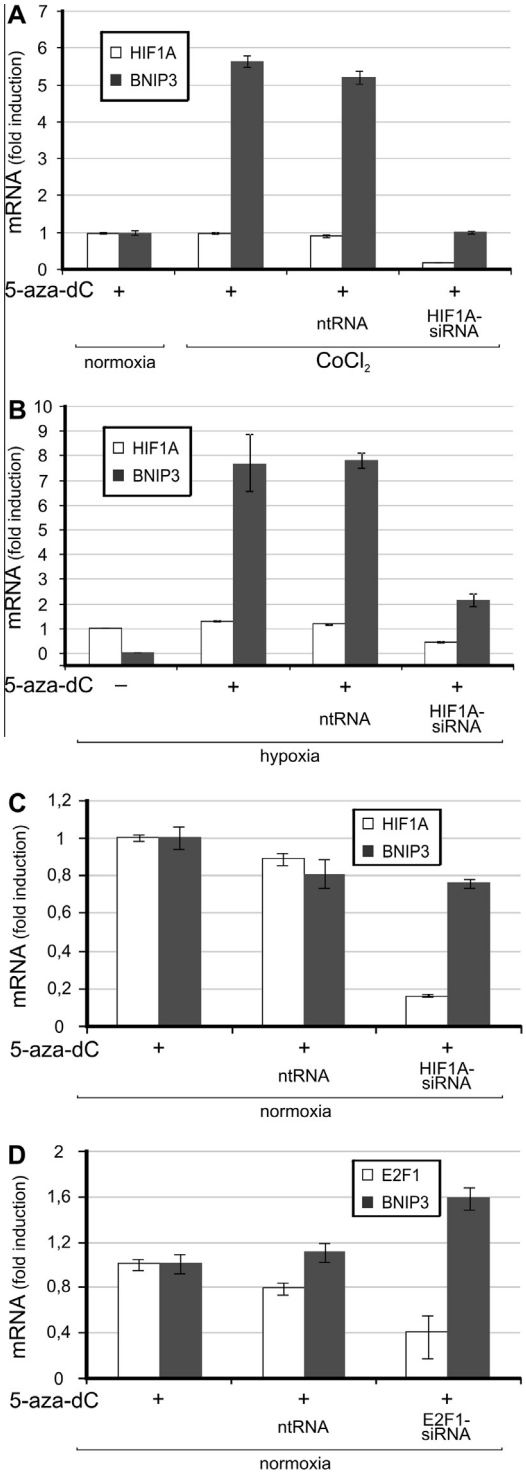

Fig. 3. *E2F1* and *HIF1A* are unnecessary for 5-aza-dC-induced *BNIP3* expression. Hypoxia (2% O_2) or pharmacological hypoxia ($CoCl_2$, 100 μM) up-regulates 5-aza-dC-induced (1 μM) *BNIP3* expression in DLD-1 (A, B – dark bars). Silencing of *HIF1A* (A, B – empty bars) by specific siRNA, as measured by real-time RT-PCR assay (A, B – empty bars), decreases the expression of *BNIP3* up-regulated by hypoxia (B) or pharmacological hypoxia ($CoCl_2$) (A), but not the baseline expression regulated by Ras. Silencing of *E2F1* or *HIF1A* in normoxia (C, D – empty bars) does not result in any significant down-regulation of *BNIP3* expression (C, D – dark bars) comparing to non-targeting siRNA (ntRNA). Graphs are showing the representative Fold induction (RQ ± standard error) of 2 independent real-time RT-PCR experiments.

Collectively, these results suggest that while *BNIP3* is a regulatory target of the hypoxia response pathway, the upregulation of KRas activity may be a necessary pre-condition for this effect to materialize in the molecular environment of human CRC cells.

3.3. BNIP3 regulation in $KRas^{G13D}$ expressing CRC cells is independent of E2F1

The composition of the transcriptional machinery that translates signals generated by the activated Ras/MEK pathway into up-regulation of *BNIP3* mRNA is presently unknown. Since the role of HIF-1 in this regard can be excluded, we turned our attention to E2F1. While E2F1 itself can be up-regulated by the Ras/MEK pathway [29], *BNIP3* can be induced by ectopic expression of E2F1, and its expression have been suggested to depend on endogenous E2F1 under hypoxic conditions [14]. In view of these observations, we asked whether *E2F1* is relevant for *BNIP3* expression by the rapidly dividing DLD-1 cancer cells driven by oncogenic $KRas^{G13D}$. To explore this, we decided to silence *E2F1* expression using appropriate siRNA. That resulted in over 60% inhibition of the respective mRNA. Surprisingly, this down-regulation did not decrease the levels of *BNIP3* mRNA in demethylated DLD-1 cells, but instead produced a modest increase (Fig. 3D). Thus, participation of E2F1 in the Ras-mediated *BNIP3* induction in DLD-1 cells is presently unlikely.

3.4. Impact of BNIP3 regulation on CRC cell survival

Although BNIP3 is often classified as a proapoptotic protein [30], DLD-1 cells expressing *BNIP3* (upon treatment with 5-aza-dC) do not display any signs of cell death under standard culture conditions. Up-regulation of *BNIP3* has been linked to increased responsiveness of cancer cells to signals inducing cell death, including cytotoxic ligands and chemotherapy [7,17,19,20,31,32]. However, presence of BNIP3 has been also implicated in resistance to hypoxia-induced cell death as well as to etoposide [9] or temozolomide [10,11] and the vector-induced overexpression of BNIP3 has been shown to increase growth rate of MCF-7 breast adenocarcinoma cells [33].

In order to test the relevance of BNIP3 expression for chemosensitivity of CRC cells we assayed viability of DLD-1 or DKs-8 cells treated with 5-Fluorouracil (5-FU) in the presence of 5-aza-dC, restoring *BNIP3* expression in DLD-1, but not DKs-8 cells. Treatment with 5-FU resulted in 65% viability loss of DLD-1 cells, while 5-aza-dC-mediated up-regulation of *BNIP3* decreased that number by nearly 20% (Fig. 4A). Additionally, transfection of DLD-1 cells with BNIP3-expressing vector halved the loss of the viability induced by 5-FU (Fig. 4B, C). Notable, treatment of DKs-8 cells, unable to re-express *BNIP3*, or transfection of DLD-1 with an empty vector had no impact on cell viability, indicating KRas-dependent and *BNIP3*-mediated resistance to 5-FU. Therefore, these results suggests that, a potential chemosensitizer, 5-aza-dC may exert an opposite effect in CRC cells harboring $KRas^{G13D}$ mutation, increasing BNIP3-dependent resistance to 5-FU.

Collectively, our study reveals a novel interplay between two regulatory layers by which colorectal cancer cells control the expression of *BNIP3*, a gene that may negatively regulate their survival. In addition, this is the first study to verify the role of HIF-1 in the regulation of 5-aza-dC-mediated *BNIP3* expression in term of human cells harboring the endogenous mutation of the *KRAS* oncogene. Thus, *BNIP3* is initially silenced by methylation, but upon re-expression remains under predominant control of Ras and the related MEK/MAPK pathway (as presented in DLD-1 and HT-29 cells). This pathway along with hypoxia stimulates *BNIP3* expression, possibly influencing vulnerability of CRC cells to the oncogenic, microenvironmental and therapeutic stress, exemplified by substantial chemoresistance to 5-FU.

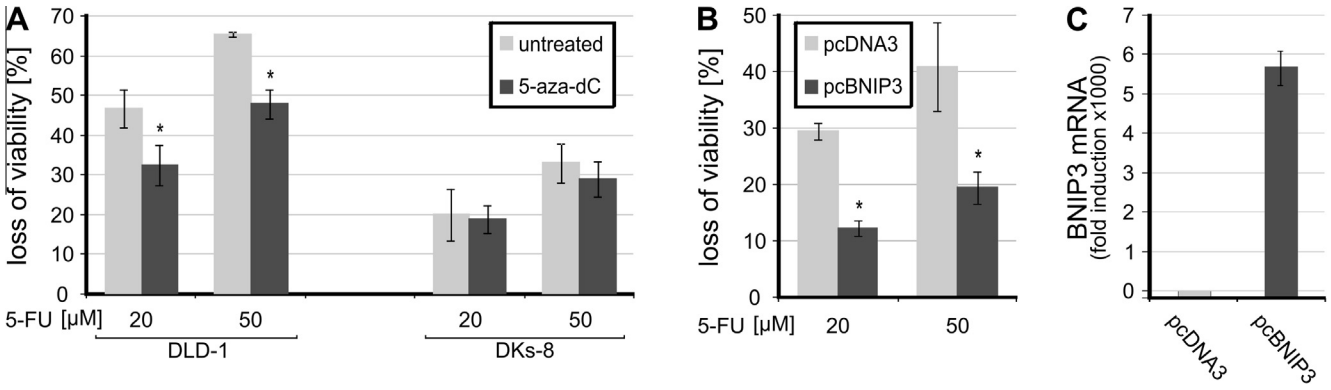

Fig. 4. 5-Aza-induced *BNIP3* expression renders resistance to 5-Fluorouracil. 5-aza-dC-induced (1 μM) expression of *BNIP3* in DLD-1 (A-left side), but not 5-aza-dC treatment of DKs-8 cells (A-right side) results in decreased sensitivity to 5-FU as measured by MTS Assay (A). Additionally, transient transfection with pcDNA3-BNIP3 vector (pcBNIP3), but not control vector (pcDNA3), results in increased number of viable cells observed upon 5-FU treatment (B), which is correlated with BNIP3 expression (C). Averages ± standard deviation of 3 (A) or 2 (B) independent experiments, $^{*}p < 0.05$. (C) Fold induction (RQ ± standard error) of 2 independent real-time RT-PCR experiments.

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