Modulation of Nuclear Factor E2-related Factor-2 (Nrf2) Activation by the Stress Response Gene Immediate Early Response-3 (IER3) in Colonic Epithelial Cells

A NOVEL MECHANISM OF CELLULAR ADAPTATION TO INFLAMMATORY STRESS

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Background: Nrf2 has a dual role in tumorigenesis.

Results: Nrf2 activation in colonic epithelial cells is controlled by the stress response gene IER3. Loss of IER3 expression causes enhanced Nrf2 activity, thereby conferring ROS protection and apoptosis resistance.

Conclusion: By regulating Nrf2-dependent cytoprotection, IER3 exerts tumor suppressive activity.

Significance: Loss of control by IER3 favors the protumorigenic action of Nrf2.

Although nuclear factor E2-related factor-2 (Nrf2) protects from carcinogen-induced tumorigenesis, underlying the rationale for using Nrf2 inducers in chemoprevention, this antioxidative transcription factor may also act as a proto-oncogene. Thus, an enhanced Nrf2 activity promotes formation and chemoresistance of colon cancer. One mechanism causing persistent Nrf2 activation is the multifunctional stress response gene immediate early response-3 (IER3) has a crucial role under these conditions. We now demonstrate that colonic tissue from Ier3−/− mice subject of dextran sodium sulfate colitis exhibit greater Nrf2 activity than Ier3+/+ mice, manifesting as increased nuclear Nrf2 protein level and Nrf2 target gene expression. Likewise, human NCM460 colonocytes subjected to shRNA-mediated IER3 knockdown exhibit greater Nrf2 activity compared with control cells, whereas IER3 overexpression attenuated Nrf2 activation. IER3-deficient NCM460 cells exhibited reduced reactive oxygen species levels, indicating increased antioxidative protection, as well as lower sensitivity to TRAIL or anticancer drug-induced apoptosis and greater clonogenicity. Knockdown of Nrf2 expression reversed these IER3-dependent effects. Further, the enhancing effect of IER3 deficiency on Nrf2 activity relates to the control of the inhibitory tyrosine kinase Fyn by the PI3K/Akt pathway. Thus, the PI3K inhibitor LY294002 or knockdown of Akt or Fyn expression abrogated the impact of IER3 deficiency on Nrf2 activity. In conclusion, the interference of IER3 with the PI3K/Akt-Fyn pathway represents a novel mechanism of Nrf2 regulation that may get lost in tumors and by which IER3 exerts its stress-adaptive and tumor-suppressive activity.

Chronic inflammation is a major risk factor for cancer including colorectal cancer. It is meanwhile widely accepted that the persistent exposure of epithelial cells, such as enterocytes, to an inflammatory environment leads to molecular alterations that favor tumor development, e.g. in colitis-associated cancer (1, 2). A wide array of inflammatory cells are involved in this process secreting cytokines and chemokines, e.g. TNF-α or IL-6, which affect the epithelial integrity and phenotype. In addition, inflammation-associated carcinogenesis is initiated quite early by genetic alterations resulting from oxidative damage during chronic inflammation (3, 4) as well as by adaptive signaling pathways engaged by the stressed epithelium to cope with the oxidative burden. These pathways include the activation of the antioxidative transcription factor nuclear factor-E2 related factor-2 (Nrf2). Acting mainly as a key regulator of the cellular response to oxidative and metabolic stress (5), Nrf2 induces the expression of a great number of antioxidative and phase II enzymes as well as a number of genes involved in cell growth and survival (6). Thus, Nrf2 confers protection from early damage during inflammation, e.g. in DSS-induced colitis and prevents colorectal carcinogenesis upon DSS/azoxymethane treatment (7). However, based on the wide spectrum of its

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The abbreviations used are: Nrf2, nuclear factor E2-related factor-2; ARE, antioxidant response element; DCF, 2',7'-dichlorodihydro-fluorescein diacetate; DSS, dextran sodium sulfate; GCLC, glutamate-cysteine ligase catalytic subunit; IER3, immediate early 3; IEX-1, immediate early gene x-ray 1; Keap1, Kelch-like-Ech-associated protein-1; Luc, luciferase; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium salt; NQO1, NAD(P)H quinone oxidoreductase 1; qPCR, quantitative PCR; ROS, reactive oxygen species; SFN, sulforaphane; tBHQ, tert-butylhydroquinone; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand.

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IER3 Controls Nrf2 Activation

actions and the cellular context, Nrf2 has a dual role in cancer (8).

On the one hand, Nrf2 has gained attention in chemoprevention because activation of Nrf2 by certain antioxidants such as sulforaphane and oltipraz leads to protection from toxic DNA damage and thereby from carcinogen-induced tumorigenesis (9). On the other hand, evidence has accumulated that Nrf2 exhibits also profound protumorigenic activity (10, 11), and a number of malignant tumors, including colonic (12–14) and pancreatic (15, 16) cancer, are known to exhibit an amplified activity of Nrf2.

Among the mechanisms leading to activation of Nrf2 in tumor cells, certain genetic and epigenetic alterations have been described, affecting mainly the regulation of Nrf2 by its inhibitor Kelch-like-Ech-associated protein-1 (Keap1) (17–20). Metabolic effects, e.g. through down-regulation of the citric acid cycle enzyme fumarate hydratase (21, 22) and deregulated signaling pathways quite common in tumorigenesis, also relate to Nrf2 activation, e.g. the PI3K/Akt pathway that controls the late induction phase of Nrf2 through interference with its Fyn kinase-dependent nuclear export (23, 24). In addition, persistent oxidative stress leads to an up-regulation of Nrf2 expression/activity, as well (25), a condition that exists in epithelial cells exposed to an inflammatory environment (26), e.g. in colonocytes from inflammatory bowel disease patients.

Using the well established DSS-colitis model in mice we have recently observed a dramatic gain in the inflammatory phenotype of the diseased colon (27) when mice are lacking the stress-inducible, multifunctional early response gene immediate early response-3 (IER3) (28, 29), also known as IEX-1 gene (30). Besides markedly increased leukocyte infiltrations in the colonic mucosa of DSS-treated mice, an aggravated impact on the crypt architecture and colonocyte morphology was noted if the Ier3 gene had been deleted (27) along with a greater incidence of tumor formation. In accordance with previous findings, the lack of IER3 expression is associated with a deregulation of the NF-κB and PI3K/Akt pathways (31–33) thereby impacting on tumorigenesis. A number of tumors (34–36) negatively correlate with IER3 expression thus pointing a tumor-suppressive action of this gene. Nambari et al. (36) reported down-regulation of colonic IER3 expression in a mouse colorectal cancer model as well as in patients with advanced colorectal cancer. IER3 has therefore gained attention during the last couple of years in terms of its use as novel biomarker in certain types of cancer (37, 38), particularly relating to its profound and variable effects on chronic inflammation and inflammatory carcinogenesis (27, 39, 40).

Addressing the involvement of Nrf2 in these processes, e.g. in colitis-associated cancer, we were interested whether IER3 affects Nrf2 activation and thereby adds to the adaptation of epithelial cells to oxidative stress, along with phenotype alterations paving the way for carcinogenesis. Cell culture-based studies and experiments with Ier3 knock-out mice demonstrate that IER3 controls Nrf2 activation in colonic epithelial cells and thereby cellular protection and survival. Accordingly, the loss of IER3 expression relates to a marked increase of Nrf2 activity along with a stress-adapted phenotype of these cells. Our findings provide a novel mechanism of Nrf2 regulation that may be affected in disease and account for the protumorigenic potential of Nrf2 on the one hand, and for the tumor-suppressive effects of IER3 on the other hand.

EXPERIMENTAL PROCEDURES

Chemicals and Reagents—LY294002 was from Calbiochem, tBHQ and SFN from Sigma, Killer-TRAIL from Enzo Life-Science/Alexis (Loerrach, Germany), and etoposide (Vepesid) from Bristol-Myers/Squibb.

Cell Lines and Animals—Human NCM460 colonocytes (41) were purchased from INCELL Corp. (San Antonio, TX) and cultured as described (12). For the isolation of distal colonic organ cultures, gly96/Ier3−/− mice on C57BL/6 background or their wild-type littermates were used (27, 42).

Colon Organ Culture—A segment of the distal colon from gly96/Ier3−/− mice on C57BL/6 background or from their gly96/Ier3+/+ counterparts was removed, cut open longitudinally, and washed in PBS containing 100 μg/ml penicillin (Sigma) and 100 μg/ml streptomycin (Sigma). The colon was then further cut into segments of 1 cm², placed and incubated in 24-well flat-bottom culture plates containing 1 ml of fresh RPMI 1640 medium supplemented with penicillin and streptomycin at 37 °C for 16–24 h. Then, tBHQ was administered for various periods, or not, at a dose of 50 μM. Cultured cells were then subjected to preparation of nuclear extracts, total cell lysates, or RNA, as described (27). For validation of equal viability, colon organ cultures were analyzed in parallel by the MTS assay (CellTiter 96®; Promega) according to the manufacturer’s instructions. OD values were normalized to cellular protein content of each culture determined by the DC assay (Bio-Rad).

Western Blotting—Nuclear extracts or total cell lysates were prepared as described before (27, 43). After electrophoresis and semidry electroblotting onto PVDF membranes, the following primary antibodies were used for immunodetection: Nrf2 (Abcam); NQO1, GCLC, IER3, Hsp90, and lamin A/C (Santa Cruz Biotechnology); tubulin (Sigma) or Fyn; Akt, PARP1, and caspase-3 (Cell Signaling) at 1:500- to 1:1000-fold dilutions in blocking buffer (5% (w/v) nonfat milk powder in TBST (Tris-buffered saline (50 mM Tris-HCl, pH 7.6, and 150 mM NaCl) plus 0.05% Tween 20), or P-Akt (Cell Signaling Technology) at a 1:1000 dilution in 5% (w/v) bovine serum albumin in TBST. After incubation overnight at 4 °C, blots were exposed to the appropriate horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology) diluted (1:1000) in blocking buffer and developed using the Dura detection kit (Perbio Sciences, Bonn, Germany). Data acquisition was done with the Chemidoc-XRS™ gel documentation system (Bio-Rad) using Quantity One® software (Bio-Rad). Lamin A/C, tubulin, or Hsp90 served as loading controls.

RNA Preparation and Real-time PCR—Isolation of total RNA and reverse transcription into single-stranded cDNA were carried out as described (12). cDNA was subjected to real-time PCR (iCycler; Bio-Rad) using the SYBR Green assay (12) with gene-specific primers (all from Realtime Primers Biomol) at a final concentration of 0.2 μM. The cycling conditions were: 95 °C for 20 s/58 °C for 20 s/72 °C for 20s for 40 cycles.
shRNA Transfection and siRNA Treatment—NCM460 cells were stably transfected with an IER3 shRNA vector or the corresponding control shRNA vector (both from Qiagen) following the manufacturer’s protocol and using puromycin (Sigma) selection at 0.5 μg/ml. The functionality of IER3 shRNA was verified by qPCR analysis of IER3 mRNA (see Fig. 3c). For siRNA (Qiagen) transfection, cells grown in 12-well plates were submitted to lipofection using 6 μl of the HiPerfect reagent (Qiagen) and 150 ng/well siRNA. Under these conditions, NCM460 shRNA cells were treated with negative control siRNA (SI1027280), Nrf2 siRNA (SI03246614), Akt siRNA (SI02757244), or Fyn siRNA (SI02654729).

Immunohistochemistry—Six-μm cryostat sections were mounted on uncovered glass slides, air-dried overnight at room temperature, fixed in chilled acetone (Merck) for 10 min, and air-dried again for 10 min. Then, slides were washed in PBS. To avoid nonspecific binding, sections were treated with 4% bovine serum albumin (BSA) (Serva, Heidelberg, Germany) for 20 min followed by incubation with the monoclonal rabbit Ser40-phospho-Nrf2 antibody (Abcam) used at 1:200 dilution in 1% BSA/PBS (50). After primary antibody incubation (overnight, 4 °C), sections were washed three times in PBS and then treated with EnVision peroxidase conjugates (DakoCytomation, Hamburg, Germany) for 30 min. Afterward, sections were washed three times in PBS. Then, peroxidase substrate reaction was performed with the AEC peroxidase substrate kit (DakoCytomation) according to the manufacturer’s instructions. Afterward, sections were washed in water, counterstained in 50% hemalaun (Merck), and mounted with glycerol gelatin. The same protocol was performed for negative controls using isotype-matched rabbit IgG. For TUNEL staining of apoptotic cells, the tissue sections were incubated with the in situ cell death detection enzyme/nucleotide mixture (Roche Diagnostics) for 1 h at 37 °C followed by the POD-converter for 30 min at 37 °C and then further processed as described above. For immunofluorescence microscopy, slides were incubated (1 h,
IER3 Controls Nrf2 Activation

room temperature) with a rat phycoerythrin-conjugated monoclonal F4/80 antibody (AbD Serotec, Düsseldorf, Germany, 1:100 dilution in 1% BSA/PBS) or a rabbit monoclonal P-Akt antibody (Cell Signalling, 1:100 dilution) followed by an anti-rabbit second antibody conjugated to Cy3 (1:100 dilution). Before examination, nuclei were counterstained with DAPI. The corresponding isotype-matched IgG antibodies were used as negative controls.

**Plasmid Transfection**—Cells were transfected with mock or IER3 expression plasmids (32) and with the control or ARE-Luc

![Figure 2. ler3 deficiency enhances Nrf2 activation in murine colonic tissue.](image-url)
plasmids (SABioscience/Qiagen) using the Effectene transfection reagent (Qiagen) following the manufacturer’s instructions.

**Dual Luciferase Assay**—ARE-driven reporter gene expression in cells was determined using the Nrf2 pathway detection kit (SABioscience/Qiagen) and as described recently (12).

**Caspase-3/-7 Activity Assay**—Apoptosis induced by Killer-TRAIL or etoposide was determined by the measurement of caspase-3/-7 activity (Promega) according to the manufacturer’s instructions and as described (12). All assays were done in duplicates. Caspase-3/-7 activity was normalized to the protein content of the analyzed cell lysates.

**Determination of Intracellular ROS**—Medium of cells was replaced by 1 ml of prewarmed PBS, and the cells were treated with 10 μM cellular ROS indicator 5-carboxy-2’,7’-dichlorodihydro-fluoresceine diacetate (c-H2DCF-DA; DCF) or the mitochondrial ROS (superoxide) detector MitoSOX Red (both from Invitrogen) dissolved in dimethyl sulfoxide, or with the vehicle alone for 20 min at 37 °C. Then, the labeled NCM460 cells were incubated in OptiMEM (PAA Laboratories, Cölbe, Germany) for several hours at 37 °C. Fluorescence was measured with the Techan Infinite 200 microplate reader.

**Colony Formation Assay**—Cells were seeded at a density of 200 or 500 cells/well on a 6-well plate. After 2 days, medium was exchanged, supplemented with tBHQ or without. After 2–3 weeks of culture with one weekly medium exchange, cells were washed twice with PBS, then fixed with methanol/acetic acid (3:1) for 5 min and stained with 0.1% (w/v) crystal violet. Plates were photographed using the Chemidoc-XRSTM transiluminator. Colonies >0.25-mm diameter were counted. The plating efficiency was calculated as the ratio of colony number/cells initially seeded.

**Statistical Analysis**—Data are presented as mean ± S.D. and were analyzed by student’s t test. A p value < 0.05 was considered statistically significant.

**RESULTS**

**Greater Nrf2 Activation in the Colon of Ier3<sup>-/-</sup> Mice**—To analyze the status of Nrf2 activation, tissue sections from the...
distal colon of DSS-treated ler3+/− and ler3−/− mice exhibiting a similar extent of macrophage infiltration, as detected by anti-F4/80 immunofluorescent staining (Fig. 1a), but different amounts of apoptotic cells, as shown by TUNEL staining (Fig. 1b). As shown in Fig. 1c, a much stronger staining in the crypt structures of DSS-treated ler3−/− mice was observed compared with DSS-treated wild-type mice. This intensive staining is particularly seen in the nuclei of enterocytes within severely inflamed areas of the colonic mucosa, exhibiting intense staining for macrophages with F4/80 (Fig. 1a). The number of apoptotic crypt cells visualized by TUNEL staining (Fig. 1b) was lower in the colon tissue from ler3−/− mice compared with ler3+/+ mice. Next, nuclear extracts from distal colon tissue from DSS-treated ler3−/− or ler3−/+ mice were analyzed by Western blotting for the presence of Nrf2 protein, indicative of Nrf2 activation. As shown in Fig. 1d (top panel), nuclear extracts from DSS-treated ler3-deficient mice contained much greater amounts of Nrf2 (appearing as a 100-kDa band) than the extracts from DSS-treated wild-type mice. Further, Western blot analysis detected greater expression level of the Nrf2 target genes Nqo1 and Gclc (Fig. 1d, middle panel) in ler3−/− colonic tissue, thus indicating enhanced Nrf2 activation.

To confirm greater Nrf2 activation in ler3-deficient tissue, colon organ cultures from untreated ler3+/− or ler3−/− mice were treated with the Nrf2 inducer tBHQ (50 μM) for 16 h. As shown in Fig. 2a, an increase of Nrf2 protein level is seen
nuclear extracts after tBHQ treatment that was stronger in the absence of Ier3 expression. Moreover, Western blotting and qPCR analyses detected expression level of the Nrf2 target genes \( \text{Nqo1} \) and \( \text{Gclc} \) (Fig. 2, a and b), which were greater in Ier3\(^{-/-}\) than in Ier3\(^{+/+}\) colonic cells.

MTS assay revealed that the viability of colonic tissues in the untreated organ culture was not significantly different between both mouse genotypes (Fig. 2c), and after treatment with tBHQ a slightly greater number of viable cells was present in the culture from Ier3\(^{-/-}\) mice. Likewise, PARP1 and caspase-3 Western blots revealed only slight differences in apoptosis during colon organ culture (Fig. 2d) and indicated somewhat lower apoptosis in the Ier3\(^{-/-}\) colonic culture.

\*Nrf2 Activation Is Controlled by IER3 in Human Colonic Epithelial Cells—To verify that IER3 controls Nrf2 activation in human colonic epithelial cells, representing a mechanism that may be affected in early carcinogenesis, and to study the impact of IER3 directly, we made use of the human colonic epithelial cell line NCM460. Overexpression of IER3 in NCM460 cells strongly suppressed the activation of Nrf2, as shown by decreased nuclear Nrf2 protein level, both in unstimulated and tBHQ-stimulated cells (Fig. 3a). In contrast, no such differences were seen when analyzing Nrf2 protein level in cytosolic extracts (Fig. 3a). ARE-luciferase assays detected a marked decrease in the induction of Nrf2-driven transcription by tBHQ (16 h) when NCM460 cells overexpressed IER3 (Fig. 3b). Under these conditions, the expression of the Nrf2 target genes NQO1 and GCLC was also decreased, as shown by qPCR and Western blot analyses (Fig. 3, c and d). When investigating SFN as another potent Nrf2 inducer, a similar inhibitory effect of IER3 overexpression on Nrf2 activation was noted (Fig. 3, a–d).

To study the effect of IER3 deficiency on Nrf2 activation, NCM460 cells were used stably expressing IER3-shRNA. As shown in Fig. 4, the induction of Nrf2 in NCM460 cells by tBHQ was increased in IER3-deficient cells. Western blotting detected greater amounts of Nrf2 protein in nuclear extracts from IER3-shRNA-expressing NCM460 cells compared with control shRNA NCM460 cells (Fig. 4a). ARE-luciferase reporter assays revealed an
IER3 Controls Nrf2 Activation

**FIGURE 7. Increased clonal growth of IER3-deficient NCM460 cells.** NCM460 cells stably transfected with control or IER3 shRNA were seeded at a density of 200 or 500 cells/well on a 6-well plate and cultured for 1–2 weeks in the absence or presence of 50 μM tBHQ. Then, cells were fixed and stained with crystal violet. Visualized colonies with a diameter of ≥0.25 mm were counted, and the plating efficiency was calculated. Representative results (left panel) of four independent experiments performed in duplicates are shown, and the evaluation was carried out using the mean values ± S.D. (error bars, right panel) from these duplicate experiments. * indicates statistical significance between the IER3 shRNA- and control shRNA-expressing cells.

enhanced Nrf2 activation in NCM460 cells expressing IER3 shRNA (Fig. 4b), and qPCR or Western blot analysis revealed a more pronounced induction of the Nrf2 target genes NQO1 and GCLC by tBHQ (Fig. 4, c and d). Likewise, the effect of SFN on Nrf2 activation and Nrf2 target gene expression was enhanced by the IER3 deficiency in NCM460 cells (Fig. 4, a–d), as well.

**IER3-deficient NCM460 Cells Exhibit Lower Intracellular ROS Levels Depending on Nrf2**—The increased activation of Nrf2 in IER3-deficient cells could be either the consequence of an enhanced ROS formation through the previously reported modulatory effect of IER3 on the respiratory chain (44), or in turn, the greater Nrf2 activity along with the expression of anti-oxidative target genes may neutralize intracellular ROS. We therefore analyzed the amount of intracellular ROS by staining with DCF. As shown in Fig. 5a, NCM460 cells subject of tBHQ treatment (24 h) exhibited decreased DCF staining compared with cells without tBHQ. When overexpressing IER3, this tBHQ-dependent decrease of DCF staining in NCM460 cells was abrogated, indicating interference of IER3 with cellular ROS neutralization. In support of this, IER3 shRNA-expressing NCM460 cells exhibited lower staining with DCF compared with control shRNA NCM460 cells (Fig. 5b). When preincubated with tBHQ for 24 h, DCF staining was further decreased in NCM460 IER3 shRNA cells more strongly than in control cells (Fig. 5b). No such effects were observed in NCM460 shRNA transfectants when stained with MitoSOX Red, a dye that detects exclusively mitochondrial superoxides generated through the respiratory chain (Fig. 5c). Thus, the effect of IER3 deficiency primarily affects the antioxidant and thereby ROS-neutralizing activity exerted by Nrf2. This is underscored by the finding that the knockdown of Nrf2 by siRNA elevated the DCF staining intensity in NCM460 IER3 shRNA cells and abrogated the enhanced neutralizing effect of tBHQ in these cells (Fig. 5d) compared with control shRNA cells.

NCM460 Cells Gain Protection from Apoptosis Induction through IER3 Deficiency in a Nrf2-dependent Fashion—By means of caspase-3/-7 assays it could be shown that NCM460 cells stably expressing IER3 shRNA are less sensitive to TRAIL or anticancer drug (etoposide)-induced apoptosis compared with control shRNA-expressing NCM460 cells. In the presence of tBHQ, the sensitivity to both apoptotic stimuli was more strongly reduced in IER3 shRNA-expressing NCM460 cells than in control shRNA cells (Fig. 6a). When knocking down Nrf2 expression in the NCM460 shRNA transfectants by siRNA, the sensitivity to both apoptotic stimuli was enhanced, in particular in IER3 shRNA NCM460 cells, and the more resistant phenotype of IER3 shRNA-expressing NCM460 cells could not be appreciated any more (Fig. 6b).

**Elevated Colony Formation of NCM460 Cells through IER3 Deficiency in a Nrf2-dependent Fashion**—Clonogenicity assays (Fig. 7) revealed that NCM460 cells stably transfected with IER3 or control shRNA exhibit clonal growth to a similar extent (plating efficiency 21.8 ± 8.4 versus 20.6 ± 9.0%) when cultured without additive. When treated with tBHQ, IER3 shRNA cells were capable of forming more colonies than control shRNA NCM460 cells (plating efficiency 42.1 ± 9.6 versus 25.6 ± 4.3%).

**The Greater Nrf2 Activity in IER3-deficient Cells Depends on the PI3K/Akt Pathway**—In accordance with previous findings (33), IER3 affects Akt activation. Immunofluorescence microscopy detected stronger staining of P-Akt in the crypt structures of DSS-treated IER3−/− mice compared with DSS-treated IER3+/+ mice (Fig. 8a). Moreover, Western blot analysis detected increased P-Akt levels in total cell extracts of colonic epithelial cells from organ cultures of IER3−/− mice (Fig. 8b) either subject to no treatment or tBHQ treatment (4 h) compared with colon organ culture from IER3+/+ mice. Greater levels of P-Akt were similarly seen in tBHQ-treated as well as in untreated NCM460 cells when expressing IER3-shRNA (Fig. 8c), thus confirming the
modulating effect of IER3 on the PI3K/Akt pathway. An increased P-Akt level was also seen in IER3-deficient cells after SFN treatment (Fig. 8c) even though SFN per se does not induce Akt phosphorylation that much, as seen in control shRNA NCM460 cells. To elucidate whether the enhanced Nrf2 activity in IER3-deficient cells relates to the PI3K/Akt pathway, ARE-luciferase assays were conducted. When treated with the PI3K inhibitor LY294002, the enhancement of Nrf2 induction in IER3-shRNA NCM460 cells was diminished as shown by the decreased ARE-dependent luciferase levels (Fig. 8d) which did not differ between both cell lines any more. Likewise, after treatment with Akt-siRNA, the increasing effect of the IER3 deficiency in NCM460 cells on Nrf2-dependent luciferase expression was much less pronounced compared with control siRNA-treated cells (Fig. 8e).

The Tyrosine Kinase Fyn Relates to the Impact of the IER3 Deficiency on Nrf2 Activation—Akt plays an important role in the late phase of Nrf2 activation through inhibition of the nuclear export of Nrf2 that is promoted by the tyrosine kinase Fyn (45). As shown by Western blot analysis, nuclear extracts of tBHQ-treated (8 h) or untreated colon organ cultures from Ier3/H11002/H11002 mice contain less Fyn protein than nuclear extracts of colon organ cultures from Ier3/H11001/H11001 mice (Fig. 9a). Likewise, nuclear Fyn protein levels were decreased in IER3 shRNA NCM460 cells compared with control shRNA NCM460 (Fig. 9b). This was seen in untreated as well as in tBHQ- or SFN-treated (8 h) cells when Fyn reaccumulated in the nuclei at a greater level. Therefore, we next elucidated whether inhibition of Fyn abrogates the stronger Nrf2 activity in IER3-deficient NCM460 cells. As shown by ARE-luciferase assay, the
siRNA-mediated knockdown of Fyn increased the Nrf2 activity in tBHQ- or SFN-treated (16 h) control shRNA NCM460 cells (Fig. 9c), but not in IER3 shRNA NCM460 cells. Most notably, the greater Nrf2 activity in IER3-deficient NCM460 cells compared with IER3-proficient cells was much less pronounced after Fyn siRNA treatment (Fig. 9c).

DISCUSSION

In addition to certain genetic alterations (3, 4), colitis-associated carcinogenesis involves many other molecular events taking place in the colonic epithelium which can initiate malignant transformation (2). Among these, Nrf2 has an important role in switching a normal and homeostatic phenotype into a malignant one. Even though Nrf2 has beneficial effects in anticancer protection through its antioxidative and detoxifying activity which is the rationale for current attempts of using Nrf2 induction in chemoprevention (9), this transcription factor exerts also considerable tumor promoting effects when being persistently activated (11, 46). In fact, tumor cells gain profound antiapoptotic protection and growth advantages from persistent Nrf2 activation, relying on the wide spectrum of cytoprotective target genes of Nrf2 as well as genes involved in the ubiquitin/proteasome pathway (12, 47, 48). In common with many other tumor entities, colorectal cancer cells are characterized by higher Nrf2 activity and exhibit Nrf2-dependent resistance to death ligands and anticancer drugs (12, 26, 49).

Still, the mechanisms leading to deregulated Nrf2 activity in tumor cells are not completely understood. Besides established gene mutations or epigenetic alterations affecting the Nrf2 inhibitory protein Keap1 or Nrf2 directly (17, 18), metabolic conditions and persistent oxidative stress play a role, as well (25, 50). During the latter, certain signaling pathways are involved,
IER3 Controls Nrf2 Activation

e.g. the PI3K/Akt pathway. An amplification of this pathway occurs, e.g. through mutations of the PTEN tumor suppressor or forced Ras/Raf and ERK1/2 activities. Another mechanism in this context relies on the control of the PI3K/Akt pathway by the stress response gene IER3 (33). As we demonstrated in this study, IER3-deficient cells (here NCM460 colonocytes or murine colonic tissue) are characterized by an enhanced level of P-Akt, and a consequence of this Akt amplification is an increased activation of Nrf2 that manifests particularly during a persistent oxidative challenge, e.g. during chronic inflammation. Accordingly, this IER3-dependent control of Nrf2 activation is seen in colonic tissue from mice suffering from DSS colitis. Whereas in Ier3-proficient mice the disease phenotype is mild, the animals with abrogated Ier3 expression had a more severe inflammatory disease activity (27).

Besides an amplified NF-κB activity found in the diseased tissues (27) we now detected also an exaggerated activation of Nrf2 if Ier3 expression had been ablated. This increasing effect through the Ier3 deficiency on Nrf2 is not merely an indirect one resulting from the stronger inflammatory/oxidative stress, but is obviously caused by the direct interference of Ier3 with the activation of Nrf2. Our results indicate that the control of Nrf2 activation by IER3 relates to its already documented negative impact on Akt (33), and through the inhibition of Akt, IER3 favors the inhibitory effect of Fyn on Nrf2 activation (24, 45). As we could demonstrate in human NCM460 colonocytes, the activation status of Nrf2 is clearly affected by IER3 expression. The impact of IER3 was noted with the activation of Nrf2 by TβH which itself increases the phosphorylation of Akt, but the modulatory effect of IER3 was also noted when analyzing SFN as Nrf2 activator which is rather a weak inducer or sometimes even an inhibitor of Akt (51, 52). Thus, IER3 provides a general control of Nrf2 activation through its impact on Akt, and IER3 deficiency amplifies Nrf2 activators which themselves do not substantially affect Akt.

Even though Nrf2 is primarily protective from DSS-induced colitis (7), avoiding early tissue damage and thereby colitis-associated cancer, its persistent activation later on may be detrimental and protumorigenic through Nrf2-dependent stress adaptation of the colonic epithelium. In this way, IER3 modulates a signal pathway having a crucial role in oncogenesis, and a loss of this modulation through IER3 deficiency may contribute to cancer initiation. This may apply also to other inflammation-induced cancers, e.g. pancreatic cancer that could develop from chronic pancreatitis, as the Nrf2-modulating effect of IER3, its expression then is part of a compensatory mechanism. A compromised function of IER3 or even the loss of its expression as it is evident in several types of cancer (28, 29), including colon cancer (36), would therefore unleash these pathways and favor a tumorigenic phenotype through the prosurvival effects of NF-κB and also of Nrf2 (11, 43, 56). This would be of particular importance as it has been recently reported that Nrf2 is a target gene of p65/p50 in leukemic cells (57), but we did not see an impact of IER3 on Nrf2 relating to this p65/RelA effect. Instead, representing itself a p65/p50 target gene (28, 31), IER3 delivers an inhibitory activity from NF-κB to Nrf2, an effect that might add to the recently reported (58) direct attenuation of Nrf2 by p65/RelA. On the other hand, NF-κB certainly triggers inflammatory stress, including ROS production, leading to Nrf2 activation (25, 59). Thus, IER3 as modulator of both NF-κB and Nrf2 would balance the cross-talk between both transcription factors, and a failure in this modulation contributes to oncogenesis initiated by chronic inflammation, e.g. in colitis-associated cancer. Given this unique function of IER3, its expression may indeed serve as valuable biomarker (37, 38) for the prognosis and responsiveness of certain types of cancer.

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