Nuclear factor erythroid 2-related factor 2 enhances carcinogenesis by suppressing apoptosis and promoting autophagy in nickel-transformed cells

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Nickel-containing compounds are widely used in industry. Nickel is a known human carcinogen that primarily affects the lungs. Proposed mechanisms of nickel-induced carcinogenesis include disruption of cellular iron homeostasis, generation of reactive oxygen species (ROS), and induction of autophagy via Stat3 signaling. However, the precise molecular mechanisms of nickel-induced carcinogenesis remain unclear. This study shows that the transcription factor Nrf2 is high in nickel-transformed cells (NiT cells). A critical role for Nrf2 in nickel-induced carcinogenesis is evidenced by extremely low expression levels of both antioxidant enzymes and antiapoptotic proteins (e.g. Bcl-2 and Bcl-xL) in NiT cells. Using similar approaches, we show that constitutively high Nrf2 expression inhibits apoptosis by up-regulating antioxidant enzymes and antiapoptotic proteins to increase autophagy via Stat3 signaling. These findings indicate that the Nrf2-mediated suppression of apoptosis and promotion of autophagy contribute to nickel-induced transformation and tumorigenesis.

This article has been withdrawn by the authors. The image from NiT cells treated with Ni2⁺ was previously published in Fig. 5H of Son et al. (2015) J. Biol. Chem. 290, 27090-27100, representing different experimental conditions. The first two lanes of the GAPDH immunoblot from Fig. 3H were reused in Figs. 4A and 7A. The ki67 and H&E panels from normal tissue in Fig. 8I were reused from Fig. 7C of Son et al. (2015) J. Biol. Chem. 290, 27090-27100) without attribution.

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beneficial or negative effects on human diseases such as cancer, liver disease, and neurodegeneration (17). By recycling nutrients, autophagy promotes tumor growth, metabolism, and survival (18). Autophagy is evidenced by the early appearance of large autophagic vacuoles in the cytoplasm (19–23). Beclin 1 and microtubule-associated protein 1 light chain 3 (LC3) are two critical components in autophagy. Beclin 1 is the mammalian orthologue of yeast Atg6/Vps 30 and is involved in the regulation of autophagy (24–28). The other key protein, LC3, is the mammalian homologue of yeast Atg8 and localizes to autophagosomal membranes after post-translational modifications. LC3 exists in two molecular forms as follows: LC3-I (18 kDa) is the cytosolic form, and LC3-II (16 kDa) is incorporated into autophagosome membranes (29, 30). The level of LC3-II directly correlates with the number of autophagosomes (30).

Nrf2 regulates antioxidant proteins to neutralize ROS, thereby regulating the cellular redox balance (31, 32). Under normal conditions, Nrf2 binds to Keap1 (Kelch-like ECH-associated protein 1) in the cytosol, which promotes the proteosomal degradation of Nrf2. Alternatively, under oxidative stress conditions, Nrf2 is freed from Keap1-mediated repression and is translocated to the nucleus, where it binds to antioxidant-response elements (AREs) in the promoter regions of genes encoding antioxidant proteins and detoxification enzymes to initiate their transcriptions (33). Constitutively high overexpression of Nrf2 protects cancer cells against oxidative and chemotherapeutic agents (34). Constitutively high expression of Nrf2 is evident in several human cancer cell lines and tumors (35–38). Furthermore, high Nrf2 levels correlate with chemoresistance (39). We explored the role of Nrf2 in cell death and autophagy in BEAS-2B and NiT cells. These results demonstrate that Nrf2 is a key regulator of ROS levels, autophagy, and cell survival in nickel-transformed cells.

Results

NiT cells are resistant to cell death, including apoptosis

To generate the nickel-transformed cell line, NiT, we continuously exposed BEAS-2B cells to Ni2+ (50 μM) for 4 months. A soft agar assay revealed that this exposure malignantantly transformed the cells (Fig. 1A). The NiT cells had a higher proliferative potential than the parental non-transformed BEAS-2B cells, during both short culture periods at high cell density (Fig. 1B) and long culture periods at low cell density (Fig. 1C). Ni2+ induced less cell death in NiT cells than in BEAS-2B cells (Fig. 1, D and F). An apoptosis assay showed that ~60% of the BEAS-2B cells were apoptotic following Ni2+ exposure. In contrast, ~40% of the NiT cells were apoptotic following the same Ni2+ exposure (Fig. 1, F and G). Furthermore, the cleavage of PARP and caspases 3 and 7, as well as the decrease in the levels of pro-caspase 8, were much more pronounced in the parental BEAS-2B cells compared with NiT cells. These results suggested that NiT cells resist cell death, including apoptosis.

NiT cells are sensitive to autophagy induction

Ni2+ treatment dramatically increased the conversion of LC3-I to LC3-II in NiT cells in a dose- and time-dependent manner, whereas this conversion was not as extensive in the parental BEAS-2B cells (Fig. 2, A and B). Treatment with bafilomycin A1, an inhibitor of autophagosome and lysosome fusion, led to increased LC3-II levels in both BEAS-2B and NiT cells. LC3-II levels were increased when the cells were treated with Ni2+ alone and bafilomycin A1, suggesting autophagic flux, rather than the obstruction of autophagosomes with lysosomes. Furthermore, treatment with Ni2+ in the presence of wortmannin, an inhibitor of autophagosome formation, led to increased LC3-II conversion of LC3-II was attenuated in parental BEAS-2B cells. Treatment, GFP-LC3 puncta dramatically in NiT cells but not in parental BEAS-2B cells (Fig. 2, D and E). To extend the study of autophagy, in these cells, we used a tandem fluorescent tagging LC3 system. When the transfected cells were exposed to Ni2+, both yellow (mCherry+/GFP+) (autophagosome) and red (mCherry+/GFP-) (autolysosome) puncta were increased in NiT cells, whereas only yellow low-intensity puncta were increased in normal cells (Fig. 2, F and G). These results indicate that, following Ni2+ exposure, autophagosomes fuse with lysosomes to generate autolysosomes in NiT cells but not in parental BEAS-2B cells.

Autophagy plays opposite roles in normal and NiT cells

The combination treatment of nickel with the autophagy inhibitors sortomannin or 3-methyladenine (3-MA) produced a greater reduction in cell viability and enhanced apoptosis when compared with the nickel-only treatment in NiT cells, whereas cell viability was enhanced and apoptosis was reduced in parental BEAS-2B cells (Fig. 3, A and B). Note that single agent treatments with either wortmannin or 3-methyladenine did not alter cell viability or apoptosis (Fig. 3, A and B, and supplemental Fig. 1). These findings indicate that Ni2+-induced autophagy in NiT cells is involved in cell survival, whereas autophagy promotes cell death in the parental BEAS-2B cells. Ni2+-induced cell death was significantly enhanced in autophagy-defective beclin 1-deficient NiT cells when compared with NiT cells transfected with the control shRNA (Fig. 3C). Knockdown of beclin 1 by beclin 1 shRNA transfection did not alter cell viability or apoptosis in either the BEAS-2B or NiT cells (supplemental Fig. 2). Interestingly, inhibition of autophagy in NiT cells by both genetic (beclin 1 inhibition) and pharmacological (bafilo-
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mycin A1) approaches increased Ni\(^{2+}\)-induced apoptosis over that observed in NiT cells transfected with the control shRNA or in untreated NiT cells, respectively (Fig. 3, D and E). However, these phenomena were not observed in the normal BEAS-2B cells (Fig. 3, D and E). Inhibiting the apoptosis signaling cascade, both pharmacologically (using the caspase inhibitor, Z-VAD; Fig. 3G) or genetically (by overexpressing Bcl-2; Fig. 3H), led to an enhancement of autophagy in Ni\(^{2+}\)-exposed NiT cells. The inhibition of apoptosis by Z-VAD was confirmed by annexin V/PI staining (Fig. 3F), and the effects of Bcl-2 overexpression on cell viability were verified by MTT assay (supplemental Fig. 3). These results suggest that in Ni\(^{2+}\)-exposed NiT cells autophagy inhibition enhances apoptosis and that inhibition of apoptosis enhances autophagy.

High expression of Nrf2 plays a critical role in the survival of NiT cells

Nrf2 regulates intracellular ROS levels in response to oxidative stimuli and toxic substances (43). We investigated whether Nrf2 is involved in apoptosis resistance in NiT cells. NiT cells

Figure 1. NiT cells have a high proliferative potential and are apoptosis-resistant. Non-transformed BEAS-2B cells were exposed to Ni\(^{2+}\) for 4 months. Transformed cells (NiT) were selected, and their transformation was confirmed by soft agar assay (A). BEAS-2B or NiT cells (0.1 \times 10^4) were seeded into a 96-well plate, and their proliferation rates were analyzed at the indicated times using MTT (B). BEAS-2B and NiT cells (0.2 \times 10^6) were seeded into 10-cm culture dishes and incubated for 7 days. Each day, the total number of cells in each dish was obtained using a cell counter (C). BEAS-2B and NiT cells were exposed to Ni\(^{2+}\) (0 – 2 mM) for 24 h, and cell morphology was visualized microscopically (D). Following treatment of BEAS-2B and NiT cells as described in C, cell viability was assessed using MTT (E), and apoptosis was assessed using annexin V/PI staining following by flow cytometry (F). Graphic representation of the early apoptotic cells (annexin V\(^+/\)PI\(^-\)) is presented (G). BEAS-2B and NiT cells were exposed to increasing concentrations (0 – 2 mM) of Ni\(^{2+}\) for 24 h, and protein levels of PARP subunits, caspase 8, and c-caspase 3/7 were assessed by Western blot analysis using the indicated antibodies; GAPDH was used as a loading control (H). Photomicrographs presented are representative images for each experimental design. Data presented graphically are the mean ± S.E. of triplicate samples or of three independent experiments, with significant differences indicated as follows: *, p < 0.05; **, p < 0.01; and ***, p < 0.001, identified by ANOVA and Scheffe’s test.
that Nrf2 plays an important role in the apoptosis resistance of caspases 3 and 7 (Fig. 4, Ni2D). Inhibition of Nrf2 expression also increased lines (Fig. 4 after 1 h) and a second Nrf2 target, HO-1, was up-regulated protein NQO1 was dramatically up-regulated up to the 24-h C, A1 (100 nM) or wortmannin (100 nM) for 1 h prior to treatment with Ni2D). To analyze autophagic flux, the cells were pre-incubated with bafilomycin (0–24 h), and the levels of LC3-I and LC3-II were assessed by Western blot analysis (C). BEAS-2B and NiT cells were transfected with the mCherry-EGFP-LC3 plasmid and treated with Ni2D (2 mM). The yellow puncta (mCherry/GFP) were visualized using a fluorescence microscope (F), and the number of puncta/cell was quantified (E). Photomicrographs are representative images of each experiment. Data presented graphically are the mean ± S.E. of triplicate samples or of three independent experiments, with significant differences from the vehicle control indicated as *, p < 0.05; **, p < 0.01, as identified by ANOVA and Scheffe’s test.

High Bcl-2 and Bcl-xL expression levels contribute to the resistance of NiT cells to cell death

Members of the Bcl-2 family of proteins are well-known regulators of apoptosis. To investigate whether two antiapoptotic Bcl-2 proteins, Bcl-2 and Bcl-xL, are involved in the resistance of NiT cells to cell death, we analyzed the Bcl-2 and Bcl-xL expression levels in BEAS-2B and NiT cells. We found that NiT cells have higher basal levels of Bcl-2 and Bcl-xL than the non-transformed parental cells (Fig. 5A). In dose and time course experiments, treatment with Ni2+ reduced Bcl-2 and Bcl-xL expression to much lower levels in BEAS-2B cells than in NiT cells (Fig. 5, B and C). To determine the effect of disrupting Bcl-2 and Bcl-xL with pro-apoptotic proteins in the two cell lines, we treated them with the Bcl-2 family inhibitor ABT-263. As expected, this treatment effectively enhanced PARP cleavage and Bim expression in both cell lines, following Ni2+ exposure (Fig. 5D, upper panel). Treatment with ABT-263 also led to a greater reduction in cell viability following Ni2+ exposure in both cell lines (Fig. 5D, lower panel). These cell viability results are consistent with those obtained using the genetic knock-down approaches of Bcl-2 and Bcl-xL, (Fig. 5E). These results showed important functional roles for Bcl-2 and Bcl-xL in cell resistance to death. It has been reported that Nrf2 up-regulates the expressions of Bcl-2 and Bcl-xL (44, 45). To investigate whether Nrf2 up-regulates Bcl-2 and Bcl-xL expression in NiT cells, we transfected the cells with Nrf2-
specific siRNA. As anticipated, the siRNA-mediated knock-down of Nrf2 reduced expression levels of Bcl-2 and Bcl-xL (Fig. 5F). Recently, our group found one consensus ARE and six putative AREs in the 3.6-kb Bcl-xL promoter and two putative AREs in the 8-kb Bcl-2 promoter (12). We used ChIP analysis to investigate whether Nrf2 up-regulates the transcription of Bcl-2 and/or Bcl-xL in BEAS-2B and NiT cells by binding to these sequences. Our analysis revealed that Nrf2 binding to the ARE-containing regions of the Bcl-2 (R1, −278 to −2769) and Bcl-xL (F1, −2992 to −2984)
promoters was higher in NiT cells than in BEAS-2B cells (Fig. 5G). The ChIP analysis coupled with quantitative real-time PCR also revealed that the extent of Nrf2 binding to the Bcl-2 ARE R1 and Bcl-xL ARE F1 was greater in NiT cells compared with BEAS-2B cells, where Nrf2 binding was minimal (Fig. 5H). These results suggested that the high expression levels of antiapoptotic proteins in NiT cells at least partially result from Nrf2-mediated up-regulation of transcription in NiT cells.

Nrf2 regulates intracellular ROS levels in NiT cells

Intracellular ROS levels serve as an important determinant of cell fate upon exposure to internal or external stimuli (46). We used several methods to measure the intracellular ROS levels of non-transformed BEAS-2B and NiT cells to determine whether the levels of these species differ between the two cell lines. First, we recorded the ESR spectra of BEAS-2B and NiT cells using DMPO for free radical trapping. We detected a 1:2:2:1 quartet ESR signal for the BEAS-2B cells. In contrast, the ESR signal for the NiT cells was extremely weak (supplemental Fig. 4A). The ESR signal was increased in Ni²⁺/H₁₁₀₀₁-exposed BEAS-2B cells but not in NiT cells (Fig. 6A). Next, we stained the cells with CM-H₂DCFDA and analyzed fluorescence intensity using flow cytometry, fluorescence microscopy, or fluorescence spectroscopy (supplemental Fig. 4, B–D). In all of these experiments, the fluorescence intensity was significantly lower in NiT cells than in non-transformed BEAS-2B cells, indicating that NiT cells contain lower levels of ROS. Importantly, these fluorescence intensities were significantly increased only in Ni²⁺-exposed BEAS-2B cells, whereas the fluorescence signal was only slightly increased in NiT cells (Fig. 6, A–D). These results suggested that NiT cells have a higher ROS scavenging potential than normal BEAS-2B cells. We also used Western blot analysis to evaluate whether higher levels of antioxidant enzymes could account for the lower ROS levels detected in NiT cells compared with BEAS-2B cells. The major antioxidant enzymes catalase and superoxide dismutases (SODs) 1 and 2 were in fact expressed to a greater extent in NiT cells than in BEAS-2B cells (Fig. 6E). Furthermore, when we exposed the cells to Ni²⁺, the expression levels of catalase, SOD1, and SOD2 decreased in a dose-dependent manner in both BEAS-2B and NiT cells; however, NiT cells retained higher levels of these enzymes than BEAS-2B cells did (Fig. 6F). Moreover, pharmacological inhibition of ROS scavengers in NiT cells, by the addition of catalase inhibitor 3AT, the SOD1 inhibitor LCS-1, or the SOD2 inhibitor 2ME, led to a greater reduction in viability following Ni²⁺ exposure (Fig. 6G). Collectively, these results suggest that the high expressions of antioxidant enzymes in NiT cells allows them to maintain low ROS levels and contributes to their resistance to apoptosis following Ni²⁺ exposure.
To investigate the role of Nrf2 in maintaining low intracellular ROS levels in NiT cells, we transfected these cells with Nrf2-specific siRNA. Suppression of Nrf2 expression in NiT cells resulted in elevated intracellular ROS levels, as determined by fluorescence microscopy (Fig. 6H), fluorescence spectroscopy (Fig. 6L), and ESR (Fig. 6J and K). Nrf2 knockdown also reduced the expression levels of catalase, SOD1, and SOD2 (Fig. 6L) as well as the activities of catalase and SOD (Fig. 6M and N). These results suggest that Nrf2 up-regulates the levels of antioxidant enzymes in NiT cells.
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A

BEAS-2B
Ni²⁺ 2 mM

NIT cells

ROS level (fold)

Ni²⁺ (2 mM) − +

***

B

BEAS-2B

Events

Events

Mean fluorescence intensity

EM

E

F

10^6 10^7 10^8 10^9

Ni²⁺ (mM) 0 0.5 1 2 0 0.5 1 2 0

Catalase
SOD1
SOD2
GAPDH

BEAS-2B
NIT cells

Ni²⁺ (2 mM) − + + + +

3AT (10 mM) − − + −

LSC-1 (10 μM) − − − +

2ME (1 μM) − − − −

D

E

H

NIT cells

Cont siRNA

Nrf2 siRNA

DCF fluorescence (Arbitrary units)

Ni²⁺ (2 mM) − +

***

F

G

H

I

DCF fluorescence (Arbitrary units)

Cont siRNA Nrf2 siRNA

Ni²⁺ (2 mM) − +

**

K

RO S level (fold)

Cont Nrf2 siRNA siRNA

L

M

N

CAT activity (mole/min/mg)

SOD activity (U/mg protein)

Cont Nrf2

siRNA siRNA

N

Cont Nrf2 siRNA

siRNA

*
Nrf2 induces autophagy by activating transcription of Stat3 in NiT cells

To investigate signaling pathways of Nrf2 on autophagy induction, we analyzed Stat3 levels. Compared with non-transformed BEAS-2B cells, total and phosphorylated Stat3 levels were dramatically higher in NiT cells than in parental BEAS-2B cells (Fig. 7A). Treating NiT cells with stat3, a pharmacological Stat3 inhibitor, or genetic knockdown Stat3 with siRNA blocked the Nrf2-mediated increase in LC3-II levels as well as the number of cells containing GFP-LC3 puncta (Fig. 7B and C). To further investigate the role of Stat3 in autophagic activity, we used plasmid DNA to overexpress Stat3 in BEAS-2B cells (Fig. 7D). Overexpressing Stat3 led to significantly higher LC3 puncta, following Ni²⁺ exposure (Fig. 7, E and F). These results indicate that Stat3 plays an important role in inducing autophagy in response to Ni²⁺ exposure. We further investigated whether Stat3 levels are regulated by Nrf2 in BEAS-2B cells. When we used siRNA to suppress Nrf2 expression in NiT cells, the levels of Stat3 and phosphorylated Stat3 were sharply reduced (Fig. 7G). To determine whether Nrf2 transcriptionally activates Stat3 in our model, we performed ChIP analysis. Nucleotide sequence analysis of the 3-kb Stat3 promoter revealed the presence of five consensus AREs as follows: one in the forward strand and four in the reverse strand (Fig. 7H). The ChIP analysis demonstrated that, in Nrf2 cells, Nrf2 binds to ARE F1 (-41 to -32) and ARE R1 (-1731 to -1713) in the Stat3 promoter (Fig. 7I). Interestingly, the binding to these AREs was much greater in BEAS-2B cells (Fig. 7J). Quantitative analysis further supported this conclusion that Nrf2 directly activates autophagy in NiT cells.

Nrf2 plays a critical role in the transformation and malignancy

We next used a genetic knockdown approach to investigate whether Nrf2 is involved in Ni²⁺-induced carcinogenesis. Soft agar and clonal assays revealed that, following prolonged Ni²⁺ exposure, suppressing Nrf2 expression in BEAS-2B cells whether by siRNA or shRNA transfection significantly impaired colony growth (Fig. 8, A–C). A shRNA-mediated knockdown of Bcl-2 also impeded colony growth in cells following prolonged Ni²⁺ exposure (Fig. 8D). When we injected the NiT cells that had been exposed to Ni²⁺ for 4 months into mice, Ni²⁺-induced tumor volume was dramatically increased than unexposed parental control cells (Fig. 8, E and F), whereas body weight remained similar to unexposed parental control cells (Fig. 8G). Interestingly, BEAS-2B cells that had been transfected with Nrf2 shRNA prior to the prolonged Ni²⁺ exposure produced smaller tumors than those that had been transfected with control shRNA prior to this exposure (Fig. 8H). To investigate whether Nrf2 may play a role in lung tumorigenesis in humans, we examined the expression levels of Nrf2 in tumor tissues and adjacent normal tissues obtained from lung cancer patients (Fig. 8I). The expression level of Nrf2 was dramatically higher in lung tumor tissues when compared with adjacent normal tissues from the same patients (Fig. 8I). The mitotic index of tumor tissues was confirmed by Ki67 and hematoxylin and eosin (H&E) staining (Fig. 8I). Overall, in vitro, in vivo, and clinical data suggest that the Nrf2 plays a pivotal role in the development of lung adenocarcinoma.

Discussion

Industrial or environmental exposure to nickel compounds is associated with a higher incidence of human lung cancer (1–3). Nickel has a potent mutagenic activity on DNA (7, 47) and could transform normal cells by acting on a number of target molecules, including iron-transport proteins and iron-sulfur clusters involved in hypoxia (5, 48). Nrf2 regulates its carcinogenic activities by transcriptionally activating antioxidant enzymes as well as several redox-related proteins (31, 44, 45). Constitutive activation of Nrf2 contributes to malignant transformation, and increased expression of Nrf2 has been observed in a variety of tumor cells (38, 50). Previously, our group reported that Nrf2 is highly expressed in metal-transformed cells and that this may play a critical role in metal-induced carcinogenesis (11, 12). Our immunohistochemical results presented in this study show that Nrf2 expression was markedly higher in lung tumor tissues from patients with a 40-year smoking history when compared with that of the adjacent normal tissues (Fig. 8I). In addition, although BEAS-2B human lung bronchial epithelial cells that had been chronically exposed to Ni²⁺ produced tumors in mice, BEAS-2B cells in which Nrf2 expression had been suppressed prior to the Ni²⁺ exposure produced smaller tumors (Fig. 8H). Silencing Nrf2 expression also impaired colony formation by BEAS-2B cells that had been chronically exposed to Ni²⁺ (Fig. 8, A–C). Collectively, these results support the path-

Figure 6. NiT cells have low ROS levels due to the Ni2+-mediated up-regulation of antioxidant enzymes. To measure ROS levels, cell suspensions were prepared from BEAS-2B and NiT cells and incubated in the absence or presence with Ni²⁺ (2 mM) for 6 h. The generation of a 1:2:2:2 quartet ESR signal and the signal intensity of DMPO-OH was demonstrated (A). ROS levels in BEAS-2B and NiT cells were also examined by flow cytometry (B), fluorescence microscopy (C), and fluorescence spectroscopy (D) after staining with CM-H2DCFDA (5 μM) for 30 min. Basal levels of catalase and SODs in BEAS-2B and NiT cells were assessed by Western blot analysis (B). The effects of Ni²⁺ (0–2 mM) on the levels of antioxidant enzymes in BEAS-2B and NiT cells were also assessed by Western blot analysis (F). To demonstrate inhibition, NiT cells were incubated with Ni²⁺ (2 mM) for 24 h in the presence or absence of 3AT (10 mM), L-10 (10 μM), or 2ME (1 μM), and cell viability was assessed using MIT (G). To examine the involvement of Nrf2 on ROS levels in NiT cells, we transfected these cells with Nrf2-specific siRNA and then evaluated intracellular ROS levels by fluorescence microscopy (H), fluorescence spectroscopy (I), or ESR (J and K). NiT cells were transfected with Nrf2-specific siRNA, and the expression levels of (L) and activities (M and N) of the indicated antioxidant enzymes were examined by Western blot analysis. The ESR spectrometer settings were as follows: frequency, 9.8 GHz; power, 39.91 milliwatts; modulation frequency, 100 kHz; receiver gain, 5.02 × 10⁴; time constant, 40.96 ms; modulation amplitude, 1.00 G; scan time, 60 s; and magnetic field, 3451 ± 100 G. All spectra shown are an accumulation of 16 scans. Photomicrographs are representative images of each experimental design. Results are shown as the mean ± S.E. of three separate experiments. *, p < 0.05; **, p < 0.01, and ***, p < 0.001 indicate significant differences determined by ANOVA and Scheffe’s test. GAPDH was used as a loading control.
Figure 7. Elevated Stat3 expression is involved in the sensitivity of NiT cells to autophagy. The basal expression levels of phosphorylated and total Stat3 in BEAS-2B and NiT cells were evaluated by Western blot analysis (A). NiT cells were incubated with Ni2+ (2 mM) for 24 h in the presence or absence of stattic (10 μM), and the LC3 levels and the number of cells containing GFP-LC3 puncta were assessed by Western blot analysis and fluorescence microscopy, respectively (B). NiT cells were transfected with siRNA to knock down Stat3, and the levels of LC3 were assessed by Western blot analysis, and the number of cells containing GFP-LC3 puncta were determined by fluorescence microscopy (C). BEAS-2B cells were transfected with a Stat3 plasmid, and the overexpression of Stat3 was confirmed by Western blot analysis (D). The Stat3-overexpressing BEAS-2B cells were exposed to various concentrations of Ni2+ (0–2 mM) for 24 h, and the levels of LC3 (E) or the number of cells containing GFP-LC3 puncta (F) were evaluated by Western blot analysis and fluorescence microscopy, respectively. To investigate the relationship between Stat3 and Nrf2, NiT cells were transfected with Nrf2-specific siRNA. After a 12-h transfection, the expression levels of phosphorylated Stat3 and total Stat3 were assessed by Western blot analysis (G). In addition, chromatin was immunoprecipitated from BEAS-2B and NiT cells with an anti-Nrf2 antibody. Nucleotide sequence analysis of the 3-kb Stat3 promoter revealed the presence of five consensus AREs (H). The binding of Nrf2 to the Stat3 promoter was assessed by normal real-time PCR (I) or quantitative real-time PCR (J) with primers specific for the ARE-containing regions of the promoter. Graphic data are the mean ± S.E. of three independent experiments, with significant differences indicated as *, p < 0.05; **, p < 0.01, and ***, p < 0.001, determined by ANOVA and Scheffe’s test. GAPDH and β-actin were used as loading controls.
Figure 8. Nrf2 plays a critical role in Ni\textsuperscript{2+}-induced malignant transformation and carcinogenesis. BEAS-2B cells were exposed to Ni\textsuperscript{2+} (50 µM) for 4 months. The cells were harvested each month, and a soft agar assay was performed (A). NIT cells were transfected with siRNA or shRNA specific for Nrf2, and a clonal assay (B) or soft agar assay (C) was performed, respectively. A soft agar assay was also performed for NIT cells following transfection with shRNA specific for Bcl-2 (D). BEAS-2B cells that had been exposed to Ni\textsuperscript{2+} (50 µM) for 4 months were injected subcutaneously (1 × 10\textsuperscript{6} cells/site) into 6-week-old male athymic nude mice. After 2 months, each tumor was dissected, and the tumor volume (E and F) and body weight (G) were measured. After transfection with Nrf2-specific shRNA, BEAS-2B cells were exposed to Ni\textsuperscript{2+} (50 µM) for 4 months. The cells were then injected subcutaneously (1 × 10\textsuperscript{6} cells/site) into 6-week-old male athymic nude mice. After 2 months, each tumor was dissected, and the tumor volume was measured (H). Normal and tumor tissues were obtained from lung adenocarcinoma patients (stage IA or IIA) with a smoking history of more than 40 years. After fixation with 4% paraformaldehyde, Nrf2 expression (upper) and proliferation (middle) were examined by immunohistochemical staining. The photomicrographs are representative images of nine samples (I). Graphic data are the mean ± S.E. of three independent experiments, with significant differences indicated as *, p < 0.05; **, p < 0.01; and ***, p < 0.001 identified by ANOVA and Scheffe’s test.
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The biological significance of Nrf2 signaling in nickel-induced carcinogenesis. To understand underlying molecular mechanisms by which Nrf2 contributes to nickel-induced carcinogenesis, we generated NiT cells by continuously exposing BEAS-2B cells to 50 μM Ni^{2+} for 4 months (51). As anticipated, Nrf2 expression was increased in NiT cells when compared with the parental BEAS-2B cells (Fig. 4A). Furthermore, in NiT cells, the expression of Nrf2 was accompanied by resistance to apoptosis (Fig. 4E). NiT cells also had a higher proliferative potential than their parental cell line (Fig. 1). In exploring a possible link between high Nrf2 expression and apoptosis resistance, we found that, compared with BEAS-2B cells, NiT cells have lower ROS levels (Fig. 6, A–D, and supplemental Fig. 4) and higher levels of the antioxidant enzymes catalase, SOD1, and SOD2 (Fig. 6E). The high expression of antioxidant enzymes is also involved in the resistance of NiT cells to apoptosis (Fig. 6G). Because siRNA-mediated knockdown of Nrf2 in NiT cells enhanced the generation of ROS and attenuated the expression and activities of catalase, SOD1, and SOD2, we conclude that Nrf2 tightly regulates antioxidant enzymes and contributes to maintaining low levels of ROS in these cells (Fig. 6, H–N). Furthermore, compared with BEAS-2B cells, NiT cells express high levels of the antiapoptotic proteins Bcl-2 and Bcl-xL, contributing to the resistance of NiT cells to apoptosis (Fig. 5, A–E). ChIP analysis revealed that Nrf2 binding to the ARE-containing regions of Bcl-2 or Bcl-xL promoter was greater in NiT cells than in BEAS-2B cells (Fig. 5, G and H). Moreover, siRNA-mediated knockdown of Nrf2 expression in NiT cells abolished the expression of Bcl-2 and Bcl-xL (Fig. 6F). The antiapoptotic proteins by Nrf2 might play an important role in the apoptosis signaling in NiT cells. The suppression of apoptosis and activated autophagy contribute to cell survival and carcinogenesis of NiT cells (Fig. 7, A–F). As shown in Fig. 3, NiT cells have lower ROS levels and resist apoptosis. In addition, the Nrf2-induced up-regulation of Stat3 in NiT cells directly results from Nrf2-mediated transcription (Fig. 7B). Moreover, Stat3-dependent autophagy contributes to cell survival in NiT cells. The cell survival function of Stat3-dependent autophagy is consistent with other researcher’s findings. For example, Stat3-dependent induction of autophagy was found to play an important role in taxol resistance in human colorectal cancer cells (61), and inhibition of Stat3-dependent autophagy enhances capsaicin-induced apoptosis in human hepatocellular carcinoma cells (62). These results suggest that autophagy and apoptosis have a negative feedback loop in cancer cells and that the regulations of these mechanisms are important for chemotherapeutic strategies.

Overall, our study demonstrates a role for Nrf2 in regulating apoptosis and autophagy, which is mediated by its ability to induce expression of antioxidant enzymes (e.g. catalase, SOD1, and SOD2), antiapoptotic proteins (e.g. Bcl-2 and Bcl-xL), and Stat3, in nickel-induced carcinogenesis (Fig. 9). Constitutive overexpression of Nrf2 enhances the up-regulation of antioxidant enzymes and antiapoptotic proteins in the transformed cells. These phenomena allow Ni^{2+}-transformed cells to maintain low intracellular ROS levels and resist apoptosis. In addition, the Nrf2-induced up-regulation of Stat3 in NiT cells potentiates autophagy. Activation of autophagy inhibits apoptosis signaling in NiT cells. The suppression of apoptosis and promotion of autophagy contribute to cell survival and carcinogenesis of the Ni^{2+}-transformed cells. Collectively, our findings provide new insight into the molecular mechanisms underlying nickel-induced autophagy and apoptosis and point toward a possible effective therapeutic strategy for metal-induced carcinogenesis.
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Experimental procedures

Chemicals and reagents

Dulbecco’s modified Eagle’s medium (DMEM) and fetal bovine serum (FBS) (catalog nos. 11995 and 16000, respectively, Gibco BRL); the Bcl-2 family protein inhibitor ABT-263 (4-((4′-chloro-4,4-dimethyl-3,4,5,6-tetrahydro-[1,1′-biphenyl]-2-yl)methyl)piperazin-1-yl)-N-(4-((4-morpholino-1-(phenylthio)butan-2-yl)amino)-3-((trifluoromethyl)sulfonyl)phenyl)sulfonyl)benzamide) (Active Biochemicals Co., Ltd.); the SOD1 inhibitor LCS-1 (catalog no. 567417, Calbiochem); the catalase inhibitor 3AT; the Mn-SOD inhibitor and 2ME (mercaptoethanol); the Bcl-2 family protein inhibitor ABT-263 (451193, Sigma). After a 24-h incubation, 5 mg/ml in PBS) was added to each well of a 96-well plate and incubated for another 4 h at 37 °C. Following the addition of acidic isopropyl alcohol, the absorbance at 570 nm was recorded using an EL800 Microplate Reader (BioTek®, Winooski, VT).

FITC-Annexin V Assay

The apoptosis assay was performed as described previously (9). Antibodies used include the following: monoclonal antibody specific for Nrf2 (SC-365949, Santa Cruz Biotechnology); rabbit monoclonal anti-catalase antibody (NB100-79910, Novus Biologicals); anti-Cu/Zn-SOD (07-403, Millipore); anti-Mn-SOD (06-984, Millipore); mouse anti-GAPDH monoclonal antibody (A0839, GeneScript); anti-Bcl-xl (catalog no. 2762, Cell Signaling); anti-Bcl-2 (catalog no. 2876, Cell Signaling); anti-p-Stat3 (catalog no. 9145, Cell Signaling); anti-Stat3 (catalog no. 9139, Cell Signaling); anti-c-caspase 3 (catalog no. 9664, Cell Signaling); anti-p-Stat3 (catalog no. 9145, Cell Signaling); anti-c-caspase 7 (catalog no. 8438, Cell Signaling); anti-p-Stat3 (catalog no. 9145, Cell Signaling); and anti-c-caspase 3 (catalog no. 9664, Cell Signaling). The expression levels of Nrf2 in NiT cells leads to up-regulation of antiapoptotic proteins (e.g. Bcl-2 and Bcl-xl) and antioxidant enzymes (e.g. catalase and SODs), causing them to be less sensitive to apoptotic signaling. Nrf2 also activates Stat3, which leads to increased autophagy. Both the suppression of apoptosis and the promotion of autophagy increase cell survival, apoptosis resistance, and oncogenicity. Negative regulation between apoptosis and autophagy in response to Ni2+ stimuli enhances the carcinogenic potential and survival of transformed cells.

Measurement of cellular ROS levels

The electron spin resonance (ESR) assay was performed using a Bruker EMX spectrometer (Bruker Instruments, Billerica, MA) and a flat cell assembly, as described previously (65). NiT cells and normal BEAS-2B cells (1 × 10^6) were cultured overnight, harvested, and mixed with DMPO (50 mM; catalog no. D5766, Sigma). The Acquisit program was used for data acquisition and analysis (Bruker Instruments). For fluorescence
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microscopy image analysis, the cells (2 × 10^4) were seeded onto glass coverslips placed in the wells of a 24-well plate and incubated overnight. Attached cells were then exposed to CM-H2DCFDA (5 μM; catalog no. C6827, Thermo Fisher Scientific) for 30 min. After washing with PBS, the coverslips were mounted onto microscope slides, and the cells were observed with a fluorescence microscope (Carl Zeiss, Germany). To determine the fluorescence intensity of the DCFDA signal, the cells (10,000 cells/well) were seeded into a 96-well plate and incubated overnight. Cells were treated with the cells (10,000 cells/well) were seeded into a 96-well culture plate and incubated overnight. Cells were treated with CM-H2DCFDA (5 μM) for 30 min. After two washes with PBS, the cells were fixed with 4% paraformaldehyde for 10 min at room temperature. Excess formaldehyde was quenched with glycine for 5 min. The cells were lysed, and nuclei were digested with micrococcal nuclease provided in the kit. The sheared chromatin was diluted (1:10) and immunoprecipitated with 2 μg of an anti-Nrf2 or a control IgG antibody. DNA-protein complexes were eluted from the protein A/G-agarose beads using a spin column, and cross-links were reversed by incubating with NaCl at 65 °C. The relative levels of Nrf2 bound to the ARE-containing regions of the Bcl-2, Bcl-xL, and Stat3 promoters were quantified using the Myq™ Single-Color Real-Time PCR Detection System (Bio-Rad) with SYBR Green PCR master mix (4472903, Thermo Fisher Scientific). PCR reactions were performed using a Mastercycler ep realPlex (Eppendorf, Hamburg, Germany).

**GFP-LC3 and mCherry-E GFP-LC3 puncta formation assays**

The number of cells containing GFP-LC3 puncta was quantified as described elsewhere (66). The number of red-positive (mCherry+/GFP−) and yellow-positive (mCherry+/GFP+) puncta from 25 cells was scored using the ImageJ software (National Institutes of Health, Bethesda, MD). Briefly, NiT cells and non-transformed BEAS-2B cells were transfected with GFP-LC3 or mCherry-E GFP-LC3 plasmids (catalog no. 672080, Addgene). The transfected cells were seeded in 6-well culture plates (0.5 × 10^6 cells/well) and incubated overnight. Attached cells were then exposed to various inhibitors for 24 h. Fluorescence-positive nuclei were detected using a fluorescence microscope (Carl Zeiss, Jena, Germany).

**shRNA and siRNA-mediated knockdown**

Four unique 29-mer shRNAs (G311194, OriGene Technologies, Inc.) targeting Nrf2 (hsa-shRNA ID: s9491, Ambion), Bcl-2 (siRNA ID: 214532, Ambion), Bcl-xL (siRNA ID: 120716, Ambion), Stat3 (siRNA ID: 116558, Ambion), and control siRNA (AM4611, Ambion) were utilized. NiT cells were seeded in 6-well culture plates and transfected with siRNA duplexes (50 nm) using the Lipofectamine™ RNAiMAX transfection reagent (13778150, Thermo Fisher Scientific) according to the manufacturer’s instructions. Twenty-four hours after transfection, the cells were harvested and plated in 96-well culture plates for selection. Expression of constructs into the selected colonies was checked by immunoblot analysis.

**Anchorage-independent colony growth assays**

The soft agar assay was performed as described previously (67). Briefly, 0.5% agar (30391023, Thermo Fisher Scientific) (3 ml) in DMEM supplemented with 10% FBS was spread onto each well of a 6-well culture plate. A suspension (1 ml) containing NiT cells or BEAS-2B cells (1 × 10^6) was mixed with 0.5% agar (2 ml) and plated on top of the 0.5% agar layer. The plates were incubated at 37 °C in 5% CO₂ for 2 months, and colonies larger than 50 μm in diameter were counted using a light microscope (Thermo Fisher Scientific).

**Chromatin immunoprecipitation (ChIP) assay**

The ChIP assay was performed using a kit (26156, Thermo Fisher Scientific). Briefly, 90% confluent NiT cells or non-transformed BEAS-2B cells were used for the assay. DNA and proteins were cross-linked by incubating the cells with 1% formaldehyde for 10 min at room temperature. Excess formaldehyde was quenched with glycine for 5 min. The cells were lysed, and nuclei were digested with micrococcal nuclease provided in the kit. The sheared chromatin was diluted (1:10) and immunoprecipitated with 2 μg of an anti-Nrf2 or a control IgG antibody. DNA-protein complexes were eluted from the protein A/G-agarose beads using a spin column, and cross-links were reversed by incubating with NaCl at 65 °C. The relative levels of Nrf2 bound to the ARE-containing regions of the Bcl-2, Bcl-xL, and Stat3 promoters were quantified using the Myq™ Single-Color Real-Time PCR Detection System (Bio-Rad) with SYBR Green PCR master mix (4472903, Thermo Fisher Scientific). PCR reactions were performed using a Mastercycler ep realPlex (Eppendorf, Hamburg, Germany).

**Immunohistochemical staining**

Tumor tissues (stage IA or IIA) with a 40-year smoking history were procured from the University of Kentucky Markey Cancer Center. Tumor tissues were fixed with 4% paraformaldehyde at 4 °C for 24 h, embedded in paraffin, and sectioned (5 μm thickness). The tissue sections were deparaffinized, rehydrated, and processed for immunohistochemical staining using a kit (PK6100, Vector Laboratories). Briefly, the sections were incubated in 3% H₂O₂ in distilled water to block endogenous peroxidase activity. After antigen retrieval, the sections were blocked with normal serum for 20 min and then incubated with primary antibodies for 1 h. Sections incubated with nonspecific mouse or rabbit serum IgGs served as negative controls. After washing with PBS, the sections were incubated with biotinylated secondary antibodies for 30 min. The sections were then washed twice with PBS, incubated with ABC reagent for 30 min, and developed in 3,3′-diaminobenzidine solution until the desired staining intensity was achieved.

**Statistical analysis**

All data are expressed as means ± S.E. For multiple comparisons, one-way analysis of variance (ANOVA) was performed using IBM SPSS Statistics 21 software (IBM Corp., Armonk, NY). Values of p < 0.05 were considered statistically significant.

**Author contributions**—Y.-O. S. designed the study and wrote the paper. P. P. performed the ChIP, soft agar, and xenograft assay shown in Figs. 5, 7, and 8. S. P. D. and Z. Z. generated transformed cells and performed autophagy assay shown in Figs. 1 and 2. X. S. conceived and coordinated the study and revised the paper.
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chemoresistance and radioresistance and promotes tumor growth. *Molecular cancer therapeutics* **9**, 336–346

41. Jiang, T., Chen, N., Zhao, F., Wang, X. J., Kong, B., Zheng, W., and Zhang, D. D. (2010) High levels of Nrf2 determine chemoresistance in type II endometrial cancer. *Cancer Res.* **70**, 5486–5496

42. Komatsu, M. (2011) Potential role of p62 in tumor development. *Autophagy* **7**, 1088–1090

43. He, X., Chen, M. G., and Ma, Q. (2008) Activation of Nrf2 in defense against cadmium-induced oxidative stress. *Chem. Res. Toxicol.* **21**, 1375–1383

44. Niture, S. K., and Jaiswal, A. K. (2013) Nrf2-induced antiapoptotic Bcl-xL protein enhances cell survival and drug resistance. *Free Radic. Biol. Med.* **57**, 119–131

45. Niture, S. K., and Jaiswal, A. K. (2012) Nrf2 protein up-regulates antiapoptotic protein Bcl-2 and prevents cellular apoptosis. *J. Biol. Chem.* **287**, 9873–9886

46. Diehn, M., Cho, R. W., Lobo, N. A., Kalisky, T., Dorie, M. J., Kulp, A. N., Qian, D., Lam, J. S., Ailles, L. E., Wong, M., Joshua, B., Kaplan, M. J., Wapnir, I., Dirbas, F. M., Somlo, G., Garberoglio, C., Paz, B., Shen, J., Lau, S. K., Quake, S. R., Brown, J. M., Weissman, I. L., and Clarke, M. F. (2009) Association of reactive oxygen species levels and radioresistance in cancer stem cells. *Nature* **458**, 780–783

47. Ke, Q., Davidson, T., Chen, H., Kluz, T., and Costa, M. (2006) Alterations of histone modifications and transgene silencing by nickel chloride. *Carcinogenesis* **27**, 1481–1488

48. Costa, M., Davidson, T. L., Chen, H., Ke, Q., Zhang, P., Yan, Y., Huang, C., and Kluz, T. (2005) Nickel carcinogenesis: epigenetics and hypoxia signaling. *Mutat. Res.* **592**, 79–88

49. Moi, P., Chan, K., Asunis, I., Cao, A., and Kan, Y. W. (1994) Isolation of NF-E2-related factor 2 (Nrf2), a NF-E2-like basic leucine zipper transcriptional activator that binds to the tandem NF-E2/AP1 repeat of the beta-globin locus control region. *Proc. Natl. Acad. Sci. U.S.A.* **91**, 9110–9114

50. Stacy, D. R., Ely, K., Massion, P. P., Yarbrough, W. G., and Hallahan, D. E. (2004) The JAK/STAT signaling pathway. *J. Biol. Chem.* **278**, 20,979–20,988

51. Pan, J. J., Chang, Q. S., Wang, X., Son, Y. O., Liu, J., Zhang, Z., Bi, Y. Y., and Shi, X. (2011) Activation of Nrf2 in nickel-transformed BEAS-2B cells. *Toxicol. Appl. Pharmacol.* **255**, 153–160

52. Chan, F. K., Shisler, J., Bixby, J. G., Felices, M., Zheng, L., Appel, M., and Kluz, T. (2005) Nickel carcinogenesis: epigenetics and hypoxia signaling. *Mutat. Res.* **571**, 75–82

53. He, X., Chen, M. G., and Ma, Q. (2008) Activation of Nrf2 in defense against cadmium-induced oxidative stress. *Chem. Res. Toxicol.* **21**, 1375–1383

54. Rawlings, J. S., Rosler, K. M., and Harrison, D. A. (2004) The JAK/STAT signaling pathway. *J. Cell Sci.* **117**, 1281–1288

55. Son, Y. O., Hitron, J. A., Pan, J., Zhang, Z., and Shi, X. (2010) Roles of intracellular Ca2+- and H2O2-dependent apoptosis signaling in p53- and p53-mediated pathways in skin epidermal cell line. *Cancer Res.* **70**, 626–636

56. Groner, B., Lucks, P., and Borghouts, C. (2008) The function of Stat3 in tumor cells and their microenvironment. *Semin. Cell Dev. Biol.* **19**, 341–350

57. Yang, X., Wang, S., Ouyang, Y., Tu, Y., Liu, L., Wang, A., Li, H., Zhi, H., and Lu, F. (2016) STAT3-dependent autophagy enhanced capsaicin-induced apoptosis in human colorectal cancer cells. *Int. J. Oncol.* **48**, 177–186

58. Wang, Y., Li, H., Huang, H., Liu, S., Mao, X., Wang, S., Wong, S. S., Xia, Z., and Irwin, M. G. (2016) Cardioprotection from emulsified isoflavone post-conditioning is lost in rats with streptozotocin-induced diabetes due to the impairment of Brk1/Nrf2/STAT3 signalling. *Clin. Sci.* **130**, 801–812

59. Xue, H., Yuan, G., Guo, X., Liu, Q., Zhang, J., Gao, X., Guo, X., Xu, S., Li, T., Shao, Q., Yan, S., and Li, G. (2016) A novel tumor-promoting mechanism of IL6 and the therapeutic efficacy of tocilizumab: hypoxia-induced IL6 is a potent autophagy initiator in glioblastoma via the p-STAT3-MIR155–3p-CREBRF pathway. *Autophagy* **12**, 1129–1152

60. Zhang, Z., Wang, A., Zhang, P., and Lu, F. (2016) STAT3-dependent TXNDC17 expression is a potential resistance through inducing autophagy in human CRC cells. *Sci. Rep.* **6**, 5784

61. Zhang, X. M., Chen, Y. Z., Yang, K., Hu, C., Liao, N., and Kluz, T. (2016) Inhibiting ROS-STAT3 signaling induces apoptosis in human skin cancer cells. *Cancer Res.* **76**, 744–755

62. Zhang, X. M., Chen, Y. Z., Chen, G., Luo, J., Bi, Y., and Kluz, T. (2016) Hypoxia-induced autophagy initiators in glioblastoma via the p-STAT3-MIR155–3p-CREBRF pathway. *Autophagy* **12**, 1129–1152

63. Pan, J. J., Chang, Q. S., Wang, X., Son, Y. O., Lee, J. C., Hitron, J. A., Cheng, S., Budhraja, A., Zhang, Z., Lan, G., Lee, J. C., and Shi, X. (2011) The dual roles of c-Jun NH2-terminal kinase signaling in Cr(VI)-induced apoptosis in JB6 cells. *Toxicol. Sci.* **119**, 335–345

64. Son, Y. O., Wang, X., Hitron, J. A., Zhang, Z., Cheng, S., Budhraja, A., Ding, S., Lee, J. C., and Shi, X. (2011) Cadmium induces autophagy through ROS-dependent activation of the LKB1-AMPK signaling in skin epidermal cells. *Toxicol. Appl. Pharmacol.* **255**, 287–296

65. Son, Y. O., Wang, L., Poyil, P., Budhraja, A., Hitron, J. A., Zhang, Z., Lee, J. C., and Shi, X. (2012) Cadmium induces carcinosogenesis in BEAS-2B cells through ROS-dependent activation of PI3K/AKT/GSK-3β/β-catenin signaling. *Toxicol. Appl. Pharmacol.* **264**, 153–160