Non-mutagenic Suppression of Enterocyte Ferroportin 1 by Chemical Ribosomal Inactivation via p38 Mitogen-activated Protein Kinase (MAPK)-mediated Regulation

EVIDENCE FOR ENVIRONMENTAL HEMOCHROMATOSIS*

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Chang-Kyu Oh1‡, Seong-Hwan Park2‡, Juil Kim‡, and Yuseok Moon1‡

From the 1Laboratory of Mucosal Exposome and Biomodulation, Department of Biomedical Sciences, Pusan National University School of Medicine, Yangsan 50612, South Korea and the 2Medical Research Institute, Pusan National University, Busan 46241, South Korea

Iron transfer across the basolateral membrane of an enterocyte into the circulation is the rate-limiting step in iron absorption and is regulated by various pathophysiological factors. Ferroportin (FPN), the only known mammalian iron exporter, transports iron from the basolateral surface of enterocytes, macrophages, and hepatocytes into the blood. Patients with genetic mutations in FPN or repeated blood transfusion develop hemochromatosis. In this study, non-mutagenic ribosomal inactivation was assessed as an etiological factor of FPN-associated hemochromatosis in enterocytes. Non-mutagenic chemical ribosomal inactivation disrupted iron homeostasis by regulating expression of the iron exporter FPN-1, leading to intracellular accumulation in enterocytes. Mechanistically, a xenobiotic insult stimulated the intracellular sentinel p38 MAPK signaling pathway, which was positively involved in FPN-1 suppression by ribosomal dysfunction. Moreover, ribosomal inactivation-induced iron accumulation in Caenorhabditis elegans as a simplified in vivo model for gut nutrition uptake was dependent on SEK-1, a p38 kinase activator, leading to suppression of FPN-1 expression and iron accumulation. In terms of gene regulation, ribosomal stress-activated p38 signaling down-regulated NRF2 and NF-κB, both of which were positive transcriptional regulators of FPN-1 transcription. This study provides molecular evidence for the modulation of iron bioavailability by ribosomal dysfunction as a potent etiological factor of non-mutagenic environmental hemochromatosis in the gut-to-blood axis.

Iron is an essential element required for all living organisms and an abundant transition metal. In addition, iron is essential for hemoglobin synthesis of erythrocytes to carry oxygen from the lungs to the rest of the body (1). Iron plays important roles in cell proliferation, and iron deficiency results in cell growth arrest, DNA damage, and cell apoptosis. Accurate regulation of iron intake, storage, and export is necessary for cellular iron homeostasis, and intracellular distribution is tightly managed by various endogenous regulators. Increased iron deficiency or decreased iron overload results in enhanced dietary iron absorption via the intestinal epithelium. In mammals, iron homeostasis is controlled at the level of iron uptake rather than excretion, making iron absorption across the intestinal epithelial cell a key control point for iron homeostasis (2). Most dietary iron absorption occurs in the duodenum (3). However, disruptions in iron homeostasis result in a variety of diseases of iron overload (hemochromatosis). The most important causes of this state are hereditary hemochromatosis, a genetic disorder, and transfusional iron overload from repeated blood transusions (4).

The major form of dietary non-heme iron transported into the gastrointestinal tract is ferrous iron (Fe2+). Ferric iron (Fe3+) must be converted to Fe2+ prior to absorption via various enzymes, including ferric reductase (5). Divalent metal transporter 1 (DMT1)3 transports iron across the apical membrane of the enterocyte and transfers iron into the cells (6, 7). Absorptive enterocytes also take up iron-containing heme from the diet. Although the mechanisms of heme transportation into enterocytes are less well understood, some candidates, including heme carrier protein 1 (HCPI), a membrane protein, are known to mediate iron-containing heme uptake into enterocytes, whereas free iron is released from the heme by heme oxygenase (HO) (8). Iron transferred into enterocytes is stored as ferritin or exported across the basolateral membrane. The intracellular iron in the enterocytes, which is an important source of plasma iron, is also transported to other tissues or organs, including the liver, bone marrow, and spleen, for iron storage or use (9–11). For export of intracellular iron from enterocytes to blood vessels, a sole known transporter, ferroportin (FPN), which is expressed in the basolateral membrane of enterocytes, membrane of macrophages, and sinusoidal sur-

3 The abbreviations used are: DMT, divalent metal transporter; HO, heme oxygenase; FPN, ferroportin; DON, deoxynivalenol; ANS, anisomycin; 15AcDON, 15-acetyl deoxynivalenol; Niv, nivalenol; IEC, intestinal epithelial cell; ARE, antioxidant response element; MARE, Maf recognition element; sMAF, small Maf protein.

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1 Both authors contributed equally to this work.

2 To whom correspondence should be addressed: Dept. of Biomedical Sciences, Pusan National University School of Medicine, Yangsan 50612, South Korea. Tel.: 82-51-510-8094; Fax: 82-55-382-8090; E-mail: moon@pnu.edu.
faces of hepatocytes, plays crucial roles in the efflux of iron to other tissues or cells (12–14). The iron exported through FPN is oxidized to Fe$^{3+}$ by hephaestin and then binds to transferrin for circulation in the blood stream (9, 11, 15, 16). FPN is encoded by two tissue-specific spliced transcripts, FPN1A and FPN1B, that produce the same protein. However, FPN transcripts differ in the presence or absence of 5’-iron-responsive elements (IREs), which leads to translational repression of FPN synthesis when iron is lacking. In particular, FPN1B is mainly expressed in enterocytes and erythroid precursors, which are able to export intracellular iron when iron deficiency occurs (17). In addition to posttranscriptional repression, transcriptional and posttranslational regulation are involved in FPN expression in other cells and tissues (18, 19). Patients with genetic mutations in the FPN gene develop hemochromatosis. There are two types of hereditary FPN diseases: macrophage-type and hepatic-type diseases. Loss-of-function mutations in FPN genes causes iron trapping in macrophages, high transferrin saturation, and hepatocellular iron overload (4).

Stress responses by ribosomal inactivation that cause mucosal insults are etiological factors of epithelial inflammatory diseases that have been investigated in various experimental models (20–22). Ribosome-inactivating xenobiotics such as deoxynivalenol (DON) belong to a large family of ribonucleolytic agents. A number of these xenobiotics can irreversibly cleave 28S ribosomal RNA at a single phosphodiester bond within a universally conserved sequence known as the sarcinecin loop (23). This cleavage leads to peptidyltransferase dysfunction and subsequent global translational arrest (24). This interference leads to a ribotoxic stress response that stimulates intracellular sentinel signaling pathways linked to the activation of cellular stress kinases, including MAPKs (25, 26). This process results in the expression of genes important for cellular homeostasis as well as genes essential to a variety of pathogenic processes involved in cell survival modulation, proliferation, and stress response (26, 27). Moreover, alimentary exposure to several ribosome-inactivating xenobiotics alters the intestinal mucosal integrity by interfering with transepithelial resistance, epithelial differentiation, and nutrient transporting, which are associated with gastrointestinal injuries, malnutrition, and weight loss (28–31). Depending on the degree of mucosal insult, ribosomal inactivators such as DON can interfere with the transport of sugars and minerals in different animal models (30, 32, 33). Moreover, some ribosome-inactivating xenobiotics are known to retard iron incorporation into circulatory erythrocytes, bone marrow, and the spleen, which is associated with erythropoietic injuries in animal models (34–36).

Iron overload has been shown to induce oxidative stress and DNA damage, which can lead to various disorders, including gastrointestinal distress (37–39). Patients with high dietary iron had a higher risk for injuries and inflammation in both the upper and lower gastrointestinal tracts (37, 38). In this study, ribosomal inactivation–insulted human enterocytes showed altered levels of intracellular iron accumulation. Iron transfer across the basolateral membrane of the enterocyte into circulation is the rate-limiting step in iron absorption, but any discordance in iron transfer from enterocytes to the circulation would lead to excessive iron accumulation and subsequent mucosal injuries (5, 39). Based on the assumption that iron transfer in the intestine is the main site of iron incorporation from dietary sources and that this can impact mucosal injuries, we investigated the effects of intestinal ribosomal inactivation on rate-limiting iron transport at enterocytes. This investigation provides new insights into iron regulation in the intestinal barrier and iron metabolism–associated disorders, including hemochromatosis in the gastrointestinal tract.

Results

Chemical Ribosomal Inactivation Induces Iron Accumulation in Various Cell Types by Suppression of the Iron Efflux Transporter FPN-1—Enterocytes, specifically duodenal villus cells, play crucial roles in managing iron homeostasis by regulating iron absorption from the diet. Under the assumption that enterocytes insulted by ribosomal inactivation altered iron absorption, we investigated the effects of ribosomal inactivation on intracellular iron levels in human enterocyte cells using HT-29 cells, which have more duodenal characteristics than other intestinal cell lines (3). To investigate the effects of ribosomal stress on iron transport in cells, we measured the intracellular iron levels by Prussian blue staining following ribotoxic stress induced by DON as a representative chemical ribosomal inactivator. 48 h and 72 h after DON exposure, cellular iron accumulation was increased in HT-29 human enterocytes (Fig. 1A). Among the iron transporters, FPN-1 protein, an iron-exporting transporter, was decreased by ribosome-inactivating DON in time- and dose-dependent manners (Fig. 1, B and C). Moreover, FPN-1 mRNA expression was also significantly suppressed in a time- and dose-dependent manner (Fig. 1, D and E), demonstrating that transcription was reduced in response to ribosomal inactivation, which occurs earlier than translational arrest, the well known intrinsic action of ribosomal inactivation. From the following experiments, chemical-induced ribosomal inactivation was achieved by treating cells with DON (500–1000 ng/ml). Similarly, other chemical ribosomal inactivators, including anisomycin (ANS), 15-acetyl DON (15AcDON), nivalenol (Niv), and T-2 toxin also enhanced intracellular iron accumulation in human enterocytes (Fig. 1F). Mechanistically, these ribotoxic chemical insults suppressed FPN-1 protein and mRNA expression in the same manner as DON (Fig. 1, G and H). In contrast with FPN-1, DMT-1 expression, a major iron-importing transporter, was not altered by ribosomal stress in human enterocytes (data not shown).

As other major iron-absorbing cells, monocytes and hepatocytes were also assessed for the effects of ribosomal inactivation on iron accumulation. Differentiated U937 human monocytes (Fig. 2A) and Huh7 human hepatocytes (Fig. 2C) also showed intracellular iron accumulation in response to ribosomal inactivation in the same manner as human enterocytes. Ribosomal inactivation suppressed FPN-1 protein expression in both monocytes and hepatocytes (Fig. 2, B and D). Moreover, exposure to chemical ribosomal inactivation during the undifferentiated state also suppressed FPN-1 expression in human monocytes (Fig. 2E). Taken together, ribosomal stress induced intercellular iron accumulation in human enterocytes, monocytes (undifferentiated and differentiated), and hepatocytes.
by suppressing expression of the iron-exporting transporter FPN-1.

p38 MAPK Signaling Is Critical for FPN Suppression and Subsequent Iron Accumulation by Chemical Ribosomal Inactivation—Ribosomal inactivation stimulates intracellular sentinel signaling pathways that are linked to the activation of cellular stress kinases, including MAPKs, which modulate the expression of genes crucial for homeostasis as well as genes integral to a variety of pathogenic processes (26, 27). We evaluated the involvement of MAPK signals in FPN-1 expression in human IECs. When cells were pretreated with each inhibitor of three major MAPKs (ERK1/2, JNK1/2, and p38 MAPK), p38 inhibition dramatically restored FPN-1 protein and mRNA expression that had been suppressed by ribosomal inactivation (Fig. 3, A and B). p38 phosphorylation was transiently induced by ribosomal inactivation in a time- and dose-dependent manner (Fig. 3, C and D). In addition to protein and mRNA analysis, p38 inhibition also restored DON-suppressed FPN-1 expression in human IECs.
gene expression, visualized in human enterocytes using confocal microscopy (Fig. 3E). Taken together, these results indicate that p38 MAPK is positively involved in FPN-1 suppression in ribosomal inactivation-insulted intestinal epithelial cells.

Ribosomal Stress-activated p38 Signaling Regulates FPN-1-promoting NRF2 and NF-κB—Among the known transcription factors in FPN-1 transcription, nuclear factor erythroid 2-like (NRF2) binds to antioxidant response elements (AREs)/Maf recognition elements (MAREs) with small Maf protein (sMAF) within the FPN-1 promoter (-7007/-7016) (18, 40). NF-κB is also an important pro-inflammatory transcription factor within the FPN-1 promoter to induce FPN-1 expression (Fig. 4A) (41). In this study, we observed the effects of ribosomal inactivation on NRF2-mediated ARE transcription activity in human enterocytes. Ribosomal inactivation inhibited ARE transcription activity in a dose-dependent manner (Fig. 4B). Functionally, NRF2, as a crucial mediator of ARE-involved antioxidant responses, counteracted oxidative stress- or ribosomal inactivation-induced cell growth suppression in human IECs (Fig. 4C). Moreover, total and nuclear levels of NRF2 decreased in response to the ribosomal inactivator DON in enterocytes (Fig. 4, D and E). Other ribosomal inactivators also decreased the nuclear amount of NRF2 (Fig. 4F) and thus suppressed the ARE-linked transcription activity (Fig. 4G).

Because FPN-1 expression is regulated by ribosomal stress-activated p38 MAPK, we attempted to address the relationship between p38 activation and FPN-1-modulating NRF2 expression in ribosomal inactivation-insulted cells. Ribosomal inactiv-
vation suppressed both cytosolic and nucleic NRF2 expression, which were retarded in p38 MAPK-inhibited cells, indicating p38 MAPK-dependent NRF2 suppression (Fig. 5A). Confocal microscopy revealed that ribosomal inactivation-reduced nucleic NRF2 expression was significantly retarded by inhibiting p38 MAPK signals (Fig. 5B). To confirm that NRF2 is a key transcription factor for FPN-1 expression in ribosomal stress-exposed enterocytes, we checked the effects of NRF2 overexpression on FPN-1 expression under the stress of ribosomal inactivation. First, NRF2 overexpression enhanced ARE-linked transcriptional activity, whereas ribosomal inactivation suppressed the reporter activity (Fig. 5C). Moreover, ribosomal inactivation-repressed FPN-1 transcription activity using the human FPN-1 promoter-linked reporter plasmid was also enhanced in NRF2-overexpressed human IECs (Fig. 5D).

Because enhanced NRF2 as a crucial transcription factor can promote FPN-1 transcription, NRF2 overexpression restored FPN-1 protein expression that had been suppressed by ribosomal inactivation in human enterocytes (Fig. 5E).

We also investigated another p38-regulating transcription factor, NF-κB, because the κB binding site is also located in the FPN-1 promoter. A previous study reported that ribosomal inactivation suppressed activation of NF-κB signals (42). In human duodenal enterocytes, ribosomal inactivation suppressed p65 phosphorylation, one of the NF-κB subunits (Fig. 6A). We tested the involvement of NF-κB-linked signals in FPN-1 expression in human duodenal enterocytes. A chemical IκB kinase inhibitor (Bay 11-7082) or induction of a modified
A molecule that is a super-repressor protein (SR-I) constitutively sequestering NF-κB in the cytoplasm down-regulates FPN-1 expression (Fig. 6, B and C). Moreover, Bay 11-7082 or SR-I expression was shown to repress FPN-1 transcription activity in cells transfected with the human FPN-1 promoter-linked reporter plasmid (Fig. 6, D and E). This suppression was more prominent in the presence of the chemical ribosomal inactivator in human IECs. In terms of the signaling pathway, ribosomal inactivation-triggered p38 MAPK was assessed for its relationship with the NF-κB signal. Inhibition of p38 kinase attenuated p65 dephosphorylation by ribosomal inactivation, indicating that p38 MAPK is the upstream negative regulator of the NF-κB signal in human enterocytes under the chemical stress of ribosomal inactivation (Fig. 6F). Among the various negative regulators of the NF-κB signal, A20 was induced by chemical ribosomal inactivation, which was also dependent on the p38 MAPK-linked pathway in our model (Fig. 6G). Furthermore, genetic knockdown of A20 using its shRNA repressed ribosomal inactivation-induced p65 dephosphorylation (Fig. 6H), suggesting that p38 MAPK-induced A20 down-regulates NF-κB activation in human enterocytes. Taken together, ribosomal stress sup-

**FIGURE 4.** Chemical ribosomal inactivation suppresses the NRF2-linked signal in IECs. A, schematic of transcriptional regulation in the human FPN-1 promoter. The 5′ UTR of the human FPN-1 gene has two crucial transcriptional binding sites, ARE/MARE and a κB site, which are potent binding regions for NRF2 and NF-κB, respectively. B, HT-29 cells transfected with the ARE-containing reporter plasmid were treated with the indicated dose of DON for 2 h. The luciferase activity of the cellular lysate was measured according to the methods described under “Experimental Procedures.” ** and *** represents a significant difference from the 0 h group (**, p < 0.001; ***, p < 0.001). RLU, relative luciferase units. C, control or NRF2-expressing (NRF2 O/E) HT-29 cells were treated with DMSO, 400 μM H2O2, 1000 ng/ml DON, or 1000 ng/ml DON plus 400 μM H2O2 for 24 h. Cell viability was analyzed using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Different letters (a–g) over each column represent a significant difference between two groups (p < 0.05). D, HT-29 cells were treated with vehicle, 1000 ng/ml DON, 100 ng/ml ANS, 1000 ng/ml 15AcDON, 1000 ng/ml Niv, or 20 ng/ml T-2 toxin for 48 h. Cellular lysates were subjected to Western blotting analysis. Bottom panel, relative intensity of NRF2 protein per actin. *** represents a significant difference from the control group (p < 0.001). E, HT-29 cells were treated with vehicle or 500 ng/ml DON for the indicated time. Cells were fixed, immunostained, and visualized by confocal microscopy (original magnification, ×1800). Right panel, the relative density of nuclear NRF2 based on confocal microscopic observation. ** represents a significant difference from 0 h (p < 0.001). F, HT-29 cells were treated with vehicle, 50 ng/ml ANS, 500 ng/ml 15AcDON, 500 ng/ml Niv, or 10 ng/ml T-2 toxin for 30 min. Cells were fixed, immunostained, and visualized by confocal microscopy (original magnification, ×1800). Right panel, the relative density of nuclear NRF2 according to confocal microscopic observation. *, **, and *** represent a significant difference from the vehicle group (*, p < 0.05; **, p < 0.01; *** p < 0.001). G, HT-29 cells transfected with the ARE-containing reporter plasmid were treated with vehicle, 50 ng/ml ANS, 500 ng/ml 15AcDON, 500 ng/ml Niv, or 10 ng/ml T-2 toxin for 2 h. The luciferase activity of the cellular lysate was measured as described under “Experimental Procedures.” * and *** represent a significant difference from vehicle of each group (*, p < 0.05; *** p < 0.001). All results are representative of three independent experiments.
pressed FPN-1 expression via p38 MAPK-mediated A20 and subsequent down-regulation of the NF-κB signal. In conclusion, ribosomal inactivation suppressed expression of the iron-exporting transporter FPN-1 via p38-mediated suppression of NRF2 expression, one of the crucial transcription factors, as well as NF-κB signals.

Chemical Ribosomal Inactivation Induces Iron Accumulation in Caenorhabditis elegans via Regulation of FPN-1.1 Expression through SEK-1 Signals—C. elegans is a nematode with a simple epithelial lining barrier for both defense and nutrient absorption; therefore, it is a good model for mucosal stress responses, including ribosomal inactivation (43, 44). In this study, C. elegans was used as an in vivo model to confirm the iron accumulation in response to ribosomal inactivation in cell culture models. Ribosomal inactivation significantly increased gut epithelial iron accumulation in the wild-type N2 strain (Fig. 7A). FPN1.1, which is a critical iron exporter homologous to human FPN-1, was quantified in C. elegans following ribosomal inactivation. Although FPN-1.1 expression was reduced in the N2 strain following ribosomal inactivation, it was restored in the sek-1 mutant strain (AU1, a homolog of human MAP2K-activating p38 kinase) of C. elegans, indicating SEK-1-depen-
dent suppression of FPN-1.1 (Fig. 7B). Moreover, the length of *C. elegans* as a readout of nutrient-dependent growth was severely reduced in the ribosomal inactivation-insulted N2 strain; however, there was no significant shortening of the AU1 strain (Fig. 7C). Taken together, ribosomal inactivation-induced iron accumulation in *C. elegans* is dependent on SEK-1, a p38 kinase activator of suppression of FPN-1.1 expression, leading to growth suppression by ribosomal inactivation. These *in vivo* results support the assumption that ribosomal inactivation increases gut iron accumulation by reducing the expression of FPN-1 via p38 activation.

**Discussion**

Dietary iron absorption across intestinal epithelial cells is a key control point for iron homeostasis because the intracellular iron in enterocytes is an important source of plasma iron that is transported to other tissues or organs for iron storage or use. In this study, enterocytes insulted by ribosomal inactivation...
showed increased intracellular iron accumulation via decreased FPN-1 protein. DMT1 is a passage used to import luminal Fe\(^{2+}\) from food intake in the small intestine, and excessive Fe\(^{2+}\) is exported via the iron-exporting transporter FPN-1 for iron distribution via blood vessels to other organs or tissues. Ribosomal inactivation disrupted this iron homeostasis in enterocytes via regulation of the exporter, leading to intracellular accumulation in enterocytes. Ribosomal inactivation impairs iron efflux via suppression of FPN-1 expression, a crucial iron-exporting transporter in enterocytes. Ribosomal inactivation in human enterocytes leads to rapid down-regulation of NRF2 and NF-κB, which are positive transcription factors binding to the ARE and conserved κB binding sites within the human FPN-1 promoter (Fig. 8). Mechanistically, ribosomal inactivation activated p38 MAPK as an integral regulator of NRF2 expression and NF-κB activation, leading to reduced FPN-1 expression.

This is the first report of negative regulation of FPN-1 by p38 MAPK, although activation of p38 mediates FPN-1 induction by interleukin 1β (41). Several studies, including this one, have demonstrated inhibitory effects of p38 on NRF2 and NF-κB (45, 46). However, most studies indicate that p38 MAPK is positively associated with activation of NRF2 and NF-κB, which are crucial transcription factors involved in FPN-1 induction (47, 48). In response to mucosal insults leading to ribosomal inactivation, pharmacological inhibition of p38 MAPK enhanced expression and nuclear translocation of NRF2 as well as phosphorylation of p65, which can be explained by several different indirect activation mechanisms. First, genetic or pharmacological inhibition of p38 may trigger compensatory activation of other MAPKs, such as ERK1/2, which can be positively involved in activation of NRF2 and NF-κB without p38 activation (49, 50). Second, blocking of p38 can enhance production of reactive oxygen species (ROS), triggering antioxidative mediators via the NRF2-linked signal. In particular, ROS-sensing p38 can induce apoptosis of ROS-mediated transformed cells and prevent ROS accumulation, leading to carcinogenic effects (51). Therefore, p38 suppression may cause ROS accumulation and subsequent activation of ARE-promoting NRF2. Ribosomal inactivation can also increase ROS production (52, 53), and subsequent p38 activation may counteract ROS-mediated action, which would limit further activation of NRF2-linked responses. However, cytotoxic levels of ribosomal inactivation...
may produce excessive ROS and subsequent antioxidative responses via NRF2 to overcome its cytotoxicity (54). In our previous report (55), ribosomal inactivation suppressed proliferation of human enterocyte via cell cycle arrest. In this study, ROS- or ribosomal inactivation-induced cell growth retardation was attenuated by overexpression of ARE-promoting NRF2 (Fig. 4C), suggesting that NRF2 is not only a critical positive regulator of FPN-1 expression but also a potent survival-related factor against growth suppressive actions of the ribosomal inactivation or ROS. Therefore, suppression of NRF2 by ribosomal inactivation may contribute to reduced viability of enterocytes as well as iron accumulation under chemical stress. Because intracellular iron overload can cause cell growth arrest or cytotoxicity, a reduction in NRF2 accounts well for the iron accumulation and subsequent suppression of cell growth in ribosome-insulted enterocytes. Although we demonstrated the mechanism of iron accumulation via NRF2 regulation by ribosomal inactivation, some questions still remain regarding signaling modulation. First, the effects of ribosomal inactivation and p38 activation on other transcription factors need to be addressed because NRF2-interacting proteins such as sMAF can promote transcription of ARE-promoted genes (56). In addition, biochemical modification of NRF2-linked signaling molecules such as phosphorylation or ubiquitin-mediated degradation can be important factors influencing gene regulation by ribosomal inactivation. NRF2 is not only important for iron metabolism but also for the general antioxidant response. NRF2 is usually restrained in the cytoplasm in association with Keap1, which can degrade quickly in response to oxidative and oncogenic stress (57). Under oncogenic and oxidative stress, free NRF2 translocates into the nucleus for activation of antioxidative stress-related enzymes such as HO-1, NQO-1, or GSTA-2, which is crucial for cellular defense under the effects of toxicants or carcinogens (58). In this study, cellular stress caused by ribosomal inactivation retarded NRF2 activation, making cells more susceptible to oxidative and oncogenic insults.

As shown in this study, NRF2 suppression by ribosomal inactivation via p38 MAPK would retard the antioxidative response, which is essential for cellular survival. In diverse mucosal injury models, epithelial NF-κB is also involved in survival and protective actions, including wound healing responses, by inreac-
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...ing cellular proliferation (59, 60). Moreover, epithelial NF-κB promotes the reconstitution of injured mucosal monolayers via up-regulation of target genes such as inducible nitric oxide synthase and cyclooxygenase-2, which are strong mediators of epithelial migration to the site of injury (61, 62). Thus, it can be speculated that the wound healing and epithelial survival processes during mucosal inflammation are interfered with in response to NF-κB suppression by ribosomal inactivation (63). However, in terms of inflammation, ribosomal inactivation-suppressed NF-κB signaling in gut epithelial cells can contribute to enhanced tolerance of mucosal inflammation by gut microbes. Our previous report demonstrated that pre-exposure to ribosomal inactivation attenuated endotoxin-induced pro-inflammatory signals in gut epithelial cells (64, 65). As mentioned above, despite the increased tolerance of pro-inflammatory stress via decreased NF-κB, retardation of pro-survival signals in association with low NF-κB and NRF2 signals indicates inefficient epithelial protection against luminal insult in the intestine.

In terms of the interorgan network, FPN-1 is also regulated by hepcidin, which is secreted from the liver to the blood in response to pro-inflammatory cytokines, hepatocellular iron loading, and endoplasmic reticulum stress (66–68). Hepcidin binds to FPN-1 in the duodenum, liver, or macrophages and induces internalization and proteasome-dependent degradation of FPN-1, leading to phosphorylation of FPN-1 in the membrane and internalization to the cytoplasm for degradation through the JAK-STAT pathway or other pathways (14, 69, 70). Moreover, DMT-1, which imports divalent metals (iron, zinc, manganese, copper, cobalt, and nickel (71–75)) is also regulated by hepcidin like FPN-1, depending on the status of inflammation and iron levels (76, 77). Therefore, it is necessary to assess the effects of ribosomal inactivation on hepcidin-mediated iron metabolism using in vivo models. C. elegans has well-developed iron metabolism-related proteins, most of which are homologous with human iron metabolism-related proteins; namely, SMF-3 for DMT-1, FTN-1 and 2 for ferritin, and FPN.1–1 for FPN-1 (78). C. elegans can thus be considered a suitable model for in vivo experiments for the investigation of iron metabolism.

Recycling of iron is as important as daily absorption of dietary iron for maintaining homeostasis. The iron from destroyed RBCs is reabsorbed by circulating macrophages via phagocytosis, after which macrophages export iron for recycling in erythropoiesis (79). According to our results, ribosomal inactivation was able to disrupt this recycling by down-regulating FPN-1 expression in human monocytes (Fig. 2, A, B, and E). As a result, chronic insults by ribosomal inactivation would contribute to iron deficiency-associated anemia. Accordingly, further investigation of macrophage regulation of systemic iron metabolism is warranted. Iron is a necessary metal nutrient required by all living organisms for the transportation of oxygen. Accordingly, its absorption and recycling is physiologically important for the maintenance of homeostasis. However ribosomal inactivation would interfere with the systemic bioavailability of iron from the diet and disturb the recycling of iron by suppressing the iron exporter FPN-1 in macrophages and hepatocytes. In addition to defects in bioavailability and recycling of iron, our mechanistic investigation demonstrated that ribosomal inactivation down-regulated NRF2-linked signals, which are essential for the induction of antioxidative defense genes in tissues and cells. Therefore, extended ribosomal inactivation is not beneficial for the maintenance of cellular integrity because cells would be exposed to accumulated oxidative and oncogenic stress. This study provides novel insights into the effects of organellar dysfunction on iron metabolism as well as NRF2-linked defense against oxidative stress in the biological system.

Experimental Procedures

Cell Culture Conditions and Reagents—HT-29, HepG2, and U937 were purchased from the American Type Culture Collection (Manassas, VA) and cultured in RPMI 1640 medium (Welgene, Daegu, South Korea) supplemented with 10% (v/v) heat-inactivated FBS (Welgene), 50 units/ml penicillin, and 50 μg/ml streptomycin (Welgene) in a 5% CO2 humidified incubator at 37°C. The number of cells was counted by exclusion of trypan blue dye (Sigma-Aldrich, St. Louis, MO) using a hemacytometer. DON, ANS, 15AcDON, NIV, T-2 toxin, and SP600125 were purchased from Sigma-Aldrich. SB203580 was purchased from Calbiochem (Merck Millipore, Billerica, MA). U0126 was purchased from assay design (Enzo Life Science, Plymouth Meeting, PA). All other chemicals were purchased from Sigma-Aldrich.

Construction and Transient Transfection of Plasmids—NC16 Pcdnna3.1-FLAG-NRF2 was purchased from Addgene. The FLAG-SR-1xκBα expression vector has been described previously (64, 80). The human FPN-1 promoter (+2774/+236) was amplified using Prime Star Taq (TAKARA, Shiga, Japan) with human genomic DNA, cloned into the CloneJET PCR cloning kit (Thermo Fisher Scientific, Waltham, MA) followed by excision at the BglII (Enzymonics, Daejeon, South Korea) site, and then transferred into the pGL4.14 [luc2/hygro] vector (Promega, Fitchburg, WI). The primers were as follows: CGT TCT TGA AAT TTG CCT GTA ACA C and AGC CTT GGG CAA AAA GAC TAC AAC G. The ARE-luciferase vector was kindly provided by Donna D. Zhang (University of Arizona, Tucson, AZ). HT-29 cells were transfected with a mixture of plasmids and OmicsFect transfection reagent (Oomics Bio, Taipei, Taiwan) according to the protocols of the manufacturer. The efficiency of transfection was maintained at 40–50% and confirmed by expression of a pMX-GFP vector.

Prussian Blue Staining in Vitro—Cells were fixed with 4% paraformaldehyde for 10 min and then washed with PBS twice. Next, cells were stained with a mixture of 10% potassium ferrocyanide (Sigma-Aldrich) and 20% hydrochloric acid for 30 min at room temperature and then washed with PBS twice. Cells were subsequently counterstained with nuclear fast red (Sigma-Aldrich) for 10 min and washed five times with PBS. Sections were examined using a Moticam Pro 205A (Motic, Hong Kong, China).

Quantitation of Intracellular Iron by Colorimetric Ferrozine Assay—Methods for the quantitation of intracellular iron by colorimetric ferrozine assay have been described previously (81). Cells were lysed with 200 μl of 50 mM NaOH for 2 h on a shaker in a humidified atmosphere. Aliquots of cell lysates were mixed with 100 μl of 10 mM HCl (the solvent of the iron stan-
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Cells were washed with cold PBS and lysed with passive lysis buffer (Promega), after which cell lysates were centrifuged at 13,475 × g for 10 min. The supernatant was collected, isolated, and stored at −80 °C until being assessed for luciferase activity. The measurement of luciferase activity was described previously (42).

Luciferase Assay—Cells were washed with cold PBS and lysed with passive lysis buffer (Promega), after which cell lysates were centrifuged at 13,475 × g for 10 min. The supernatant was collected, isolated, and stored at −80 °C until being assessed for luciferase activity. The measurement of luciferase activity was described previously (42).

Cell Viability Assay—Colorimetric analysis of cell growth was performed with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (20 μl from 5 mg/ml stock solution) was added to the cells for 2 h. The supernatant was removed, and the pellet was dissolved with 100 μl of DMSO. The optical density was read at 560 nm with the background at 670 nm subtracted. The optical density was directly correlated with the quantity of cells.

C. elegans Culture Conditions and Chemical Treatment—Wild-type C. elegans, N2, and C. elegans with mutated sek-1 (homology with MAPKK), AU1 (derived from N2 through ethyl methanesulfonate mutagen exposure), were kindly provided by the Caenorhabditis Genetics Center (University of Minnesota, St. Paul, MN). C. elegans was maintained on a streptomycin-resistant Escherichia coli OP50 spread nematode growth medium plate (50 Mm NaCl, 1.7% agar, 0.25% peptone, 1 mM CaCl2, 5 μg/ml cholesterol, 1 mM MgSO4, and 25 mM KPO4 in D2O) at 24 °C. Streptomycin-resistant E. coli OP50 was kindly provided by Seung-Jae Lee (Postech, Pohang, South Korea). The number of C. elegans was counted by suspension in M9 buffer (22 mM KH2PO4, 42 mM Na2HPO4.8 6mM NaCl, and 1 mM MgSO4 in D2O). C. elegans were synchronized with a mixture of 500 μl of 5 N NaOH, 1 ml of household bleach (Yohanclorox, Seoul, South Korea), and 5 ml D2O. Synchronized eggs were seeded on an nematode growth medium plate without OP50 overnight, after which L1 were collected with M9 buffer. C. elegans were washed with M9 buffer, counted for seeding on a nematode growth medium plate (A600 = 0.6), and spread.

Prussian Blue Staining of C. elegans—C. elegans were collected using M9 buffer, centrifuged at 190 × g for 2 min, and then washed with M9 buffer twice. Washed C. elegans were fixed with 40% isopropanol overnight at 4 °C and then washed with M9 buffer. C. elegans were permeabilized with 0.1% Triton X-100 in M9 buffer overnight at 4 °C and washed with M9 buffer twice. Next, C. elegans were stained with a mixture of 10% potassium ferrocyanide (Sigma-Aldrich) and 20% hydrochloric acid overnight at 4 °C and then washed with M9 buffer twice. Stained C. elegans were dropped onto glass slides, covered with glass, and examined using a Moticam Pro 205A microscope (Motic).

Statistical Analysis—Data were analyzed using Sigma Stat for Windows (Jandel Scientific, San Rafael, CA). Student’s t test was used to compare two groups of data, whereas analysis of variance was used to compare multiple groups, and pairwise comparisons were made using the Student-Newman-Keuls method. Data not meeting normality assumptions were subjected to Kruskal-Wallis ANOVA by ranks, and pairwise comparisons were then made with the Student-Newman-Keuls method.

Author Contributions—Project design and hypotheses were made by C. O. and Y. M. C. O., J. K., and S. P. conducted the experiments and analyzed the data. Y. M. and S. P. prepared the manuscript. Y. M. supervised the overall project.

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dard FeCl3) and 100 μl of iron-releasing reagent (a freshly mixed solution of equal volumes of 1.4 M HCl and 4.5% (w/v) KMnO4 in H2O). These mixtures were incubated for 2 h at 60 °C within a fume hood because chlorine gas is produced during the reaction (82). After the mixtures had cooled to room temperature, 30 μl of the iron-detection reagent (6.5 mM ferrozine, 6.5 mM neocuproine, 2.5 mM ammonium acetate, and 1 mM ascorbic acid dissolved in water) was added to each tube. After 30 min, 280 μl of the solution in each tube was transferred into a well of a 96-well plate, and the absorbance was measured at 550 nm on a microplate reader (VERSAMax tunable microplate reader, Molecular Devices, Sunnyvale, CA). The iron content of the samples was calculated by comparing its absorbance with that of a range of standard concentrations of equal volume that had been prepared in a way similar to that of the sample (a mixture of 100 μl of FeCl3 standards (0–300 μM) in 10 mM HCl, 100 μl 50 mM NaOH, 100 μl of releasing reagent, and 30 μl of detection reagent). The intracellular iron concentration determined for each of a well cell culture was normalized against the protein content of that well.

Reverse Transcription and Real-time PCR—Methods for RNA extraction, conventional and real-time PCR, and analysis have been described previously (42). The 5′ forward and 3′ reverse complement PCR primers for amplifying each gene were as follows: human FPN-1 (5′-TAT TTC GGG ATG GAA CTT GG-3′ and 5′-ACC ACA TTT TCG ACG TAG CC-3′), human GAPDH (5′-TCA ACG GAT TTG GTC GTA TT-3′ and 5′-CTG TGG TCA TGA GTC AG-3′), C. elegans fpn-1 (5′-ATT CGA TAA CCT CGC CGC AT-3′ and 5′-GGA TGG CAG ACT CGG CTC AG-3′), and C. elegans act-1 (5′-CCA AGA GAG GTA TCC TTA CC-3′ and 5′-CTT GGA TGG CGA CAT ACA TG-3′). All experiments included three replicates, and each independent experiment was repeated three times.

Western Immunoblotting Analysis—Expression of proteins was assessed by Western immunoblotting analysis using goat polyclonal anti-FPN-1 and rabbit polyclonal anti-β-actin antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) and rabbit monoclonal anti-human NRF2, rabbit polyclonal anti-phospho-p38, rabbit polyclonal anti-phospho-p65, rabbit polyclonal anti-phospho-IkBα, and rabbit polyclonal anti-IκBα antibodies (Cell Signaling Technology, Beverly, MA). Monoclonal anti-FLAG was purchased from Sigma-Aldrich. The process of Western blotting analysis was described previously (42).

Confocal Microscopy—Cells were cultured in glass-bottom culture dishes (SPL Life Science, Pocheon, South Korea). The process of confocal microscopy was described in previous report (42).
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