Inhibition of nuclear factor-erythroid 2–related factor (Nrf2) by caveolin-1 promotes stress-induced premature senescence

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ABSTRACT **Reactive oxygen species (ROS) can induce premature cellular senescence, which is believed to contribute to aging and age-related diseases. The nuclear erythroid 2 p45–related factor-2 (Nrf2) is a transcription factor that mediates cytoprotective responses against stress. We demonstrate that caveolin-1 is a direct binding partner of Nrf2, as shown by the binding of the scaffolding domain of caveolin-1 (amino acids 82–101) to the caveolin-binding domain of Nrf2 (amino acids 281–289). Biochemical studies show that Nrf2 is concentrated into caveolar membranes in human and mouse fibroblasts, where it colocalizes with caveolin-1, under resting conditions. After oxidative stress, caveolin-1 limits the movement of Nrf2 from caveolar membranes to the nucleus. In contrast, Nrf2 is constitutively localized to the nucleus before and after oxidative stress in caveolin-1–null mouse embryonic fibroblasts (MEFs), which do not express caveolin-1. Functional studies demonstrate that caveolin-1 acts as an endogenous inhibitor of Nrf2, as shown by the enhanced up-regulation of NQO1, an Nrf2 target gene, in caveolin-1–null MEFs and the activation or inhibition of a luciferase construct carrying an antioxidant responsive element (ARE) after down-regulation of caveolin-1 by small interfering RNA or overexpression of caveolin-1, respectively. Expression of a mutant form of Nrf2 that cannot bind to caveolin-1 (Φ→A-Nrf2) hyperactivates ARE and inhibits oxidative stress–induced activation of the p53/p21$^{Waf1/Cip1}$ pathway and induction of premature senescence in fibroblasts. Finally, we show that overexpression of caveolin-1 in colon cancer cells inhibits oxidant-induced activation of Nrf2-dependent signaling, promotes premature senescence, and inhibits their transformed phenotype. Thus, by inhibiting Nrf2-mediated signaling, caveolin-1 links free radicals to the activation of the p53/senescence pathway.**

Monitoring Editor
Carl-Henrik Heldin
Ludwig Institute for Cancer Research

Received: Sep 14, 2012
Revised: Apr 19, 2013
Accepted: Apr 23, 2013

INTRODUCTION

Caveolae are 50- to 100-nm, flask-shaped invaginations of the plasma membrane enriched in cholesterol and glycosphingolipids. Caveolae can exist as individual invaginations or be found in detached, grape-like clusters and long tubular structures derived from the fusion of single caveolae. Although caveolae were originally believed to function as macromolecular transport vesicles (Matveev *et al.*, 2001), their role has expanded to include signal transduction, cellular metabolism, cholesterol homeostasis, endocytosis, tumor promotion, and tumor suppression (Galbiati *et al.*, 2001a,b; Williams *et al.*, 2005; Watanabe *et al.*, 2009). Caveolin-1 is a structural protein component of caveolae in most cell types (Kurzchalia *et al.*, 1992). The direct interaction with caveolin-1 generally results in the sequestration of a given signaling molecule within caveolar membranes and modulation of its signaling activity. These signaling proteins include G-protein α subunits, H-Ras, nitric oxide synthase, epidermal growth factor receptor, Src-like nonreceptor tyrosine kinases, protein kinase C, and protein kinase A.

This article was published online ahead of print in MBoC in Press (http://www.molbiolcell.org/cgi/doi/10.1091/mbc.E12-09-0666) on May 1, 2013.

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Abbreviations used: Cav-1, caveolin-1; MEFs, mouse embryonic fibroblasts; ROS, reactive oxygen species.

Reactive oxygen species (ROS) are highly reactive due to the presence of unpaired electrons. ROS represent natural byproducts of the normal metabolism of oxygen. They are formed in mitochondria and as intermediates in enzyme reactions as part of normal aerobic life. Oxygen radicals can also be overproduced in cells exposed to environmental stresses such as ionizing radiation, ultraviolet light, cigarette smoke, and air pollution. ROS are implicated in a variety of cellular processes, including proliferation, differentiation, host defense, and wound repair mechanisms. Excessive production of ROS is usually counteracted by both enzymatic and nonenzymatic antioxidants. Nuclear factor-erythroid 2–related factor 2 (Nrf2) is a key transcription factor that regulates the antioxidant defense system. It acts as a cellular sensor of oxidative stress and protects cells against injury provoked by endogenous and exogenous stresses (Wakabayashi *et al.*, 2010). Following oxidative stress, Nrf2 transactivates the transcription of genes carrying the antioxidant response element (ARE), which are involved in the detoxification process. Under resting conditions, Nrf2 levels are kept low by Kelch-like ECH-associated protein 1 (Keap1), which directly binds to Nrf2 and promotes its proteasomal degradation. After oxidative stress, Nrf2 dissociates from Keap1 and moves to the nucleus, where it transactivates ARE-containing antioxidant genes (Itoh *et al.*, 2004).

A redox imbalance occurs when oxidant levels overcome the cellular antioxidant defense mechanisms. This oxidative stress can damage macromolecules, including proteins, nucleic acids, and lipids (Yla-Herttuala, 1999; Marnett, 2000; Stadtman and Levine, 2000), and lead to cellular dysfunctions such as premature senescence (Chen and Ames, 1994; Chen *et al.*, 1998; Frippiat *et al.*, 2001, 2002; Martindale and Holbrook, 2002; Finkel, 2003). Premature senescent cells are characterized by irreversible cell cycle arrest and the acquisition of characteristic biochemical and morphological features, including increased p53 activity, increased $p21^{Waf1/Cip1}$ and p16 protein expression, hypophosphorylation of pRb, formation of histone macroH2A1 foci, and development of enlarged and flattened morphology (Dimri *et al.*, 1995; Wynford-Thomas, 1999; Black *et al.*, 2000; Lundberg *et al.*, 2000; Sherr and DePinho, 2000). Senescent cells remain viable and metabolically active for a long period of time, however, despite their inability to proliferate (Matsumura *et al.*, 1979). This is mainly attributed to the fact that senescent cells no longer respond to external stimuli, including both growth factors and apoptotic agents (Cristofalo *et al.*, 1989; Wang, 1995).

Cellular senescence is a basic cellular mechanism developed by organisms to prevent the propagation of cells with damaged DNA and potentially carrying oncogenic mutations. Therefore cellular senescence is considered a powerful tumor suppressor mechanism. However, senescent cells accumulate over time (Dimri *et al.*, 1995; Melk *et al.*, 2003; Kishi, 2004; Herbig *et al.*, 2006; Jeyapalan *et al.*, 2007) and are believed to contribute to aging and age-related pathologies (Campisi, 1997). In fact, the failure of senescent cells to proliferate and their ability to secrete factors that alter the tissue microenvironment contribute to reduced tissue function in aging organs. Thus understanding the molecular mechanisms that regulate stress-induced premature senescence of eukaryotic cells is fundamental for gaining insight into the aging process and age-related diseases, including cancer.

We previously found that caveolar membranes are key regulators of oxidative stress–induced premature senescence. We demonstrated that disruption of caveolae inhibits oxidative stress-induced premature senescence (SIPS) both in cell culture models and in vivo (Volonte *et al.*, 2002, 2009; Volonte and Galbiati, 2009, 2011; Dasari *et al.*, 2006; Bartholomew *et al.*, 2009). Because we also showed that caveolin-1 is an endogenous inhibitor of thioredoxin reductase 1 (TrxR1), an important antioxidant enzyme, and that inhibition of TrxR1 by caveolin-1 mediates SIPS (Volonte *et al.*, 2002, 2009; Volonte and Galbiati, 2009, 2011; Dasari *et al.*, 2006; Bartholomew *et al.*, 2009), we investigate here the role of caveolin-1 in the regulation of Nrf2-mediated antioxidant signaling and its functional significance in the context of premature senescence. We find that caveolin-1 is an endogenous inhibitor of Nrf2 and that the caveolin-1–mediated inhibition of Nrf2 promotes SIPS. Our data provide new insight into the molecular mechanisms that link oxidative stress to cellular senescence and age-related diseases.

RESULTS

Caveolin-1 directly binds to Nrf2

The scaffolding domain of caveolin-1 (CSD), which is represented by residues 82–101, mediates direct protein–protein interactions between caveolin-1 and a variety of signaling molecules carrying the caveolin-binding domain (CBD; ΦXΦXXXXΦ, ΦXXXXΦXXΦ, or ΦXΦXXXXΦXXΦ, where Φ represents an aromatic amino acid and X represents any amino acid; Couet *et al.*, 1997; Song *et al.*, 1997; Jagannadham *et al.*, 2002). Analysis of both human and mouse Nrf2 protein sequence indicates that Nrf2 has a putative CBD between amino acids 281 and 289 (Figure 1A). Thus, to investigate whether caveolin-1 is a binding partner of Nrf2, we performed pull-down assays using a series of caveolin-1 deletion mutants fused to glutathione *S*-transferase (GST). Figure 1B shows that Nrf2 is a caveolin-1–binding protein and that the scaffolding domain of caveolin-1 is sufficient for binding to Nrf2. To determine whether Nrf2 directly binds to caveolin-1, we performed GST pull-down assays using purified wild-type (WT)-Nrf2-histidine (His). We found that WT-Nrf2-His bound to caveolin 1 (residues 82–101)–GST (Figure 1C). Finally, we generated a mutant form of Nrf2 in which the aromatic residues within the CBD of Nrf2 were mutated to alanines (Φ→A-Nrf2). We show in Figure 1D that the ability of Φ→A-Nrf2 to bind to caveolin-1 (82–101) is dramatically compromised compared with WT-Nrf2. From these data, we conclude that the scaffolding domain of caveolin-1 (amino acids 82–101) directly binds to the CBD (amino acids 281–289) of Nrf2.

Nrf2 partially localizes to caveolar membranes under resting conditions. Caveolin-1 inhibits oxidant-induced translocation of Nrf2 to the nucleus

Because we observed direct interaction between caveolin-1 and Nrf2 in in vitro studies, we asked whether Nrf2 can localize to caveolae and the two proteins interact in cells. We show in Figure 2A that ~50% of endogenous Nrf2 is localized in caveolar membranes of WI-38 human diploid fibroblasts under resting conditions. These data were confirmed by coimmunoprecipitation studies in which Nrf2 was detected by immunoblotting analysis after immunoprecipitation of WI-38 cell lysates with an antibody probe specific for caveolin-1 under resting conditions (Figure 2B). Because Nrf2 mediates signaling after exogenous stress, we also subjected WI-38 cells to sublethal oxidative stress for 2 h and examined the localization of Nrf2 with respect to caveolar membranes over time. We found that Nrf2 was still partially localized into caveolar membranes 24 h after oxidative stress and completely exited caveolae after 48 h (Figure 2A). Moreover, we found that caveolin-1 interacted with Nrf2 at 24 h, but not 48 h, after oxidative stress (Figure 2B). Consistent with these data, immunofluorescence studies show that, under resting conditions, Nrf2 was localized in the nucleus and at the plasma membrane of WI-38 cells, where it colocalized with caveolin-1 (Figure 2C). At 48 h after oxidative stress, Nrf2 was only found in the nucleus (Figure 2C). Thus these data demonstrate that Nrf2 partially

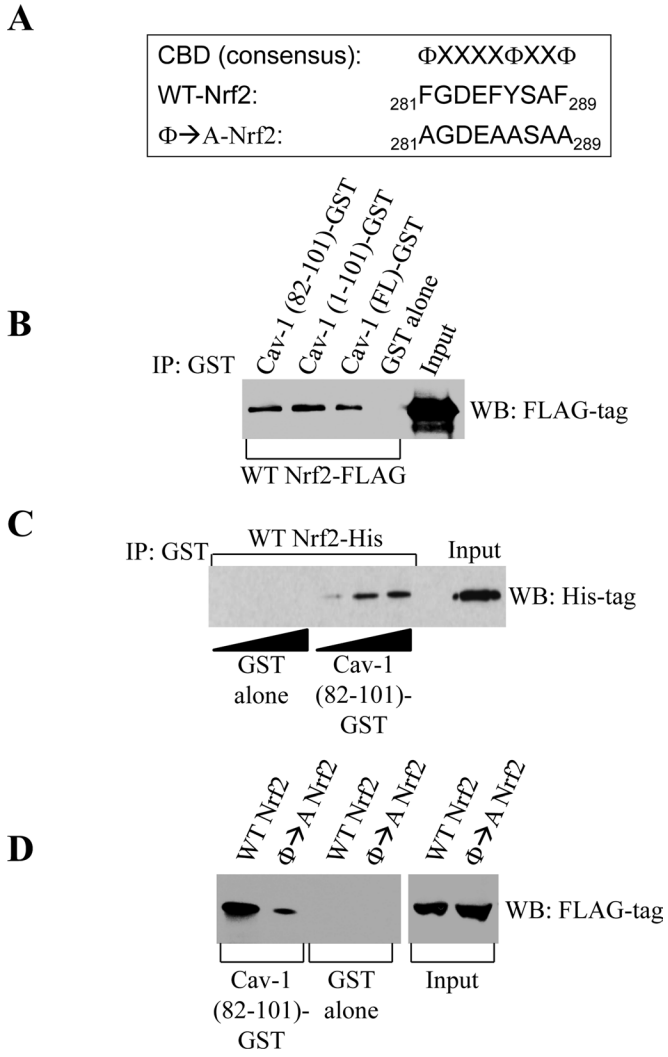

FIGURE 1: Nrf2 directly interacts with caveolin-1 in vitro. (A) Schematic diagram showing the consensus CBD and the CBD of Nrf2 (amino acids 281–289). A mutant form of Nrf2 in which the aromatic residues were substituted with alanines (Φ→A) is shown in the bottom line. Φ represents an aromatic amino acid and X represents any amino acid. (B) Caveolin-1–GST fusion protein pull-down assays were performed using cell lysates from NIH 3T3 cells transiently transfected with wild-type Nrf2-flag. (C) Pull-down assays between purified WT-Nrf2-His and increasing concentrations of affinity-purified GST alone or caveolin-1(residues 82–101)–GST. (D) Caveolin-1(residues 82–101)–GST fusion protein and GST-alone pull-down assays were performed using cell lysates from NIH 3T3 cells transiently transfected with either wild-type Nrf2-flag or Φ→A-Nrf2-flag. Right, total expression of WT-Nrf2-flag and Φ→A-Nrf2-FLAG.

resides in caveolar membranes under resting conditions and within the first 24 h after oxidative stress.

To independently corroborate these findings, we examined the expression, stability, and localization of Nrf2 in mouse embryonic fibroblasts (MEFs) derived from either wild-type or caveolin-1–null mice, which do not express caveolin-1. We found that oxidative stress marginally up-regulated Nrf2 mRNA (Supplemental Figure S1A) and strongly increased Nrf2 protein expression (Supplemental Figure S1B) in both wild-type and caveolin-1–null MEFs. In addition, Nrf2 had a half-life of ~20–30 min in both wild-type and caveolin-1–null MEFs under resting conditions (Supplemental Figure S1, C and D). Oxidative stress dramatically enhanced Nrf2 half-life to ~8 h in both wild-type and caveolin-1–null MEFs (Supplemental Figure S1, C and D). Similar to WI-38 cells, Nrf2 was equally distributed between caveolar membranes and the nucleus under resting conditions in wild-type MEFs, and 48 h were necessary for Nrf2 to completely exit caveolar membranes and move to the nucleus after oxidative stress (Figure 3, A and B). Of interest, Nrf2 was constitutively localized to the nucleus and not into caveolar membranes in caveolin-1–null MEFs before and after oxidative stress (Figure 3, A and B). These data were confirmed by fractionation experiments showing that Nrf2 was equally distributed in the membrane and nuclear fractions under resting conditions, and oxidative stress stimulated the relocalization of Nrf2 from the membrane to the nuclear fraction in wild-type MEFs (Supplemental Figure S2A). In contrast, Nrf2 was not found in the membrane fraction but was enriched in the nuclear fraction both before and after oxidative stress in caveolin-1–null MEFs (Supplemental Figure S2A). The fact that we did not see any significant expression of Nrf2 into the cytoplasmic fractions of both wild-type and caveolin-1–null MEFs is consistent with our immunofluorescence data in Figures 2 and 3 and the notion that Nrf2 undergoes rapid Keap1-mediated degradation in the cytoplasm under resting conditions and rapid translocation to the nucleus after oxidative stress. In support of this conclusion, we show that oxidative stress promoted the dissociation of Nrf2 from its negative regulator Keap1 as early as 24 h after oxidative stress in wild-type MEFs (Supplemental Figure S2B), which is consistent with the increased total Nrf2 protein expression 24 h after oxidant stimulation (Supplemental Figure S1B). Of importance, we did not detect interaction between caveolin-1 and Keap1 either before or after oxidative stress in MEFs (Supplemental Figure S2B), consistent with the known membrane localization of caveolin-1 and cytoplasmic localization of Keap1 in MEFs (Watai *et al.*, 2007). Taken together, these data show that caveolin-1 inhibits the translocation of Nrf2 to the nucleus, under resting conditions and during the early cellular response to sublethal levels of oxidants, by sequestering Nrf2 into caveolar membranes.

Caveolin-1 inhibits Nrf2-dependent signaling

Nrf2 activates cytoprotective signaling by transactivating antioxidative and other target genes containing an ARE. To directly address the functional consequence of the caveolar localization of Nrf2, we down-regulated endogenous caveolin-1 expression by small interfering RNA (siRNA) in NIH 3T3 fibroblasts (data not shown) and measured Nrf2 transcriptional activity using an ARE-based luciferase reporter assay. We found that down-regulation of caveolin-1 expression increased Nrf2 transcriptional activity by ~1.8-fold (Figure 4A). Consistent with these data, overexpression of caveolin-1 inhibited the ARE-containing reporter by approximately twofold (Figure 4A). NAD(P)H:quinone oxidoreductase 1 (NQO1) is a well-known Nrf2 target gene whose expression is activated by Nrf2 after oxidative stress (Thimmulappa *et al.*, 2002). To gain insight into the constitutive localization of Nrf2 into the nucleus in the absence of caveolin-1, we subjected wild-type and caveolin-1–null MEFs to sublethal oxidative stress for 2 h and evaluated NQO1 expression by reverse transcription (RT)-PCR before and 24 h after oxidative stress, when Nrf2 remains partially localized in caveolar membranes in caveolin-1–expressing cells (Figure 2A). We found that the oxidant-induced activation of NQO1 gene expression was dramatically enhanced in caveolin-1 null MEFs as compared with wild-type MEFs (Figure 4B). In support of increased signaling in the absence of caveolin-1 expression, we show that cell viability was enhanced in caveolin-1–null MEFs as compared with wild-type MEFs after treatment with

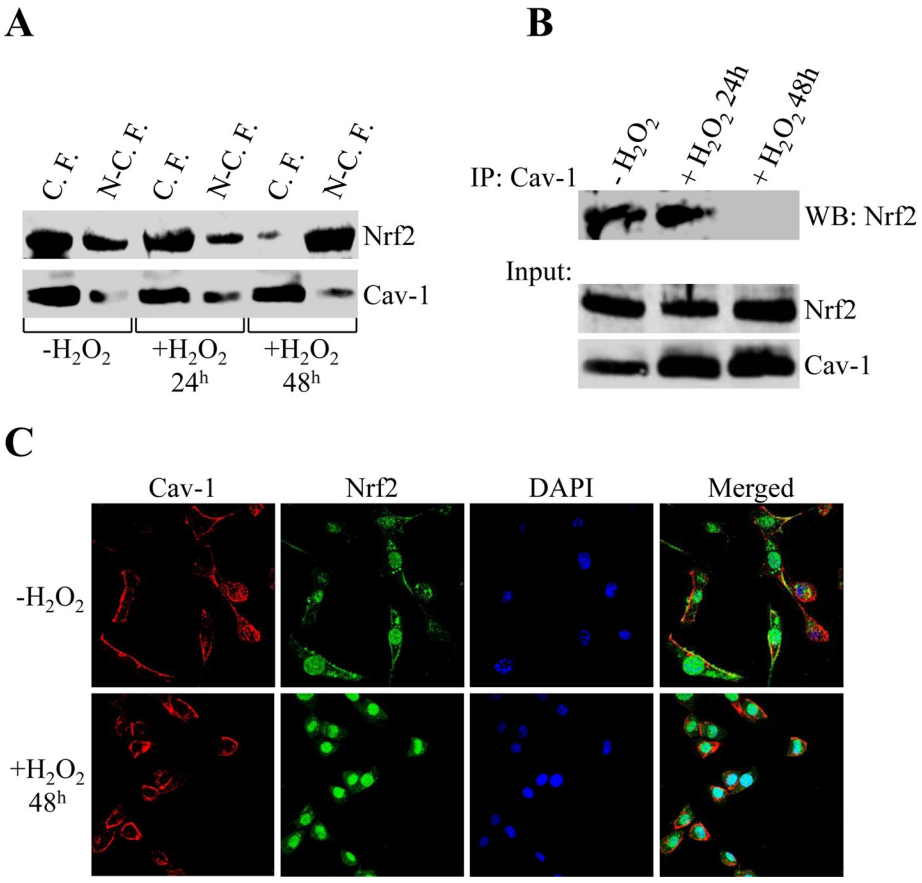

FIGURE 2: Nrf2 interacts with caveolin-1 in vivo. WI-38 human diploid fibroblasts were treated with sublethal doses of hydrogen peroxide (450 μM) for 2 h. Cells were then recovered in complete medium for different periods of time: (A) 24 and 48 h; (C) 48 h. Untreated cells ($-H_2O_2$) were used as control. (A) Cellular fractions containing caveolar membranes (C.F.) were isolated from cellular fractions containing the rest of cellular proteins (N-C.F.) by sucrose gradient centrifugation. Expression of caveolin-1 and Nrf2 into caveolar and noncaveolar fractions was determined by immunoblotting analysis using antibody probes specific for caveolin-1 (Cav-1) and Nrf2. (B) Cell lysates were immunoprecipitated using an antibody probe specific for caveolin-1 and immunoprecipitates subjected to immunoblotting analysis with anti-Nrf2 IgGs. Bottom, total expression of Nrf2 and caveolin-1. (C) Cellular localization of caveolin-1 and Nrf2, before and after oxidative stress as determined by immunofluorescence staining using antibody probes specific for caveolin-1 and Nrf2, which were fluorescently labeled using secondary antibodies. 4′,6-diamidino-2-phenylindole (DAPI) staining was performed to identify the nucleus. Representative confocal microscopy images are shown.

menadione, a free radical–generating compound that activates Nrf2 (Figure 4, C and D). Thus we conclude that the interaction of caveolin-1 with Nrf2 inhibits Nrf2-mediated signaling.

Inhibition of Nrf2 by caveolin-1 promotes oxidative stress–induced premature senescence

What is the functional consequence of the caveolin-1–mediated inhibition of Nrf2-dependent antioxidant signaling at the time cells are subjected to oxidative stress and within the first 24 h after oxidant stimulation? Sublethal oxidative stress induces premature senescence in cells (Chen and Ames, 1994; Chen *et al.*, 1998; Frippiat *et al.*, 2001, 2002; Martindale and Holbrook, 2002; Finkel, 2003). We previously showed that caveolin-1 mediates SIPS, as shown by the protection of caveolin-1–null MEFs against oxidative stress–induced premature senescence (Bartholomew *et al.*, 2009). The molecular mechanisms underlying such protection in the absence of caveolin-1 remain to be fully understood. Because Nrf2 is a master transcription factor and our data show that caveolin-1 inhibits Nrf2 by sequestering it into caveolar membranes, we asked whether the ability of caveolin-1 to inhibit Nrf2-dependent signaling at the time of oxidative stress and within the first 24 h after oxidative stress was one of the molecular mechanisms through which caveolin-1 allows oxidants to promote SIPS. To directly test this hypothesis, we took advantage of Φ→A-Nrf2, a mutant form of Nrf2 that cannot interact with caveolin-1 (Figure 1D). First, we show that the ability of Φ→A-Nrf2 to activate ARE is significantly higher, in the presence of caveolin-1, than that of WT-Nrf2 (Figure 5A), suggesting that a lack of interaction of Φ→A-Nrf2 with caveolin-1 activates Nrf2-dependent signaling. We then subjected vector alone–, wild-type Nrf2–, and Φ→A-Nrf2–expressing cells to sublethal oxidative stress for 2 h and examined the development of premature senescence 7 d after oxidative stress. We found that only the expression of the mutant form of Nrf2 that cannot bind to caveolin-1 and is constitutively active (Φ→A-Nrf2) protected cells from oxidative stress–induced premature senescence, as shown by reduced staining for senescence-associated β-galactosidase activity (SA-β-gal) and $p21^{Waf1/Cip1}$ protein expression (Figure 5, B–D). Thus caveolin-1 promotes SIPS in fibroblasts by inhibiting Nrf2.

Caveolin-1/Nrf2–mediated cellular senescence inhibits colon cancer cell growth

Cellular senescence is a powerful tumor suppressor mechanism. In fact, cancer cells need to bypass senescence in order to form a relevant tumor mass. Thus, to put the caveolin-1/Nrf2/senescence signaling into a pathological context, we asked whether inhibition of Nrf2 by caveolin-1 occurs in colon cancer cells and whether oxidative stress–induced senescence is antitumorigenic in these cells. For these studies, we used HCT116 colon cancer cells, which are known to express caveolin-1 and in which Nrf2 signaling has been linked to tumor growth (Kim *et al.*, 2011). First, we evaluated the consequences of down-regulating caveolin-1 expression for Nrf2 signaling. We show that sublethal oxidative stress activated an ARE-containing luciferase reporter in HCT116 colon cancer cells transfected with scrambled siRNA (Figure 6A). Down-regulation of caveolin-1 expression by siRNA in HCT116 cells activated ARE even in the absence of oxidative stress (Figure 6A). Consistent with these data, we found that the up-regulation of heme oxygenase 1 (HO1) expression, an Nrf2 target, by sublethal oxidative stress was inhibited in HCT116 cells stably overexpressing caveolin-1 (Figure 6B). Taken together, these data indicate that caveolin-1 acts as an inhibitor of Nrf2 signaling also in colon cancer cells. We then asked whether overexpression of caveolin-1 in colon cancer cells, in addition to inhibiting Nrf2, can promote premature senescence after oxidative

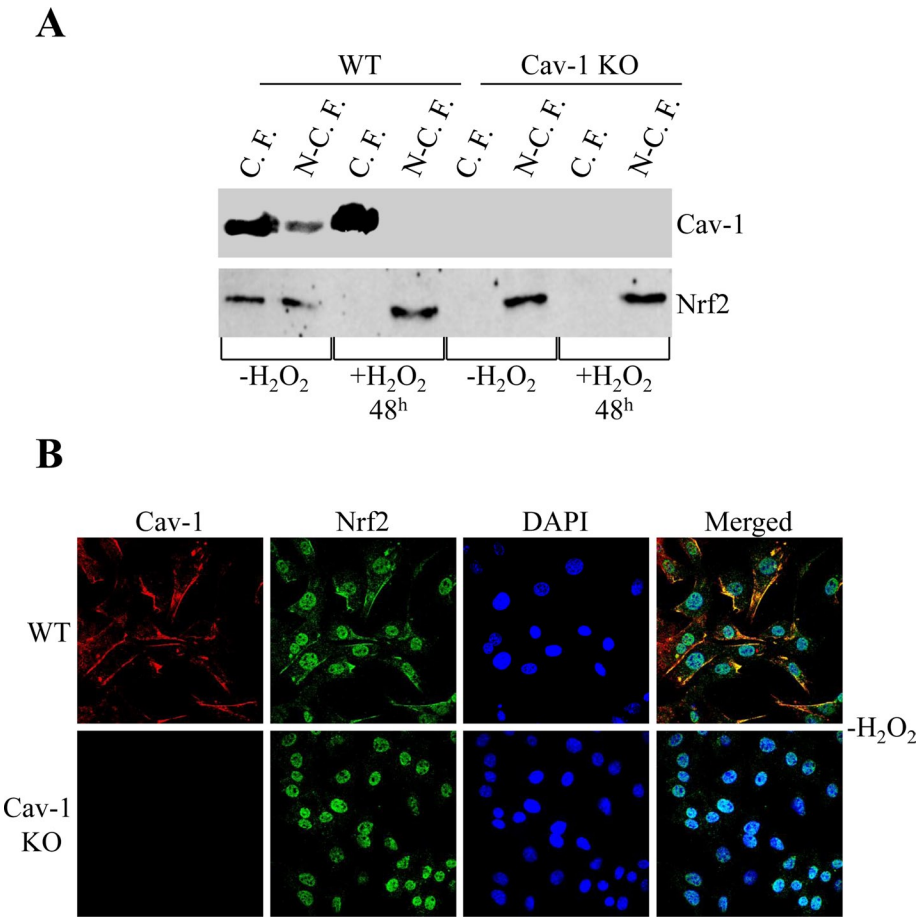

FIGURE 3: Caveolin-1 expression is necessary for the caveolar localization of Nrf2. Mouse embryonic fibroblasts were derived from WT and caveolin-1–null (Cav-1 KO) mice. (A) Cells were treated with sublethal doses of hydrogen peroxide (150 μM) for 2 h. Cells were then recovered in complete medium for 48 h. Untreated cells ($-H_2O_2$) were used as control. Cellular fractions containing caveolar membranes (C.F.) were isolated from cellular fractions containing the rest of cellular proteins (N-C.F.) by sucrose gradient centrifugations. Expression of caveolin-1 and Nrf2 into caveolar and noncaveolar fractions was determined by immunoblotting analysis using antibody probes specific for caveolin-1 (Cav-1) and Nrf2. (B) The cellular localization of caveolin-1 and Nrf2 before oxidative stress was determined by immunofluorescence staining using antibody probes specific for caveolin-1 and Nrf2, which were fluorescently labeled using secondary antibodies. DAPI staining was performed to identify the nucleus. Representative confocal microscopy images are shown.

stress. Parental and caveolin-1–overexpressing HCT116 cells were treated with sublethal concentrations of hydrogen peroxide for 2 h. Five days after oxidative stress, cellular senescence was determined by SA-β-gal staining and immunoblotting using antibody probes specific for $p21^{Waf1/Cip1}$ and p16. We show in Figure 7, A–C, that the stable overexpression of caveolin-1 in HCT116 cells potentiated the oxidative stress–induced up-regulation of $p21^{Waf1/Cip1}$ and p16 expression and promoted the development of SA-β-gal–positive cells. These data indicate that caveolin-1 inhibits Nrf2 and mediates SIPS in colon cancer cells. Of interest, enhanced SIPS by the overexpression of caveolin-1 is not limited to colon cancer cells but also occurs in nontransformed cells, as shown by increased oxidant-induced up-regulation of $p21^{Waf1/Cip1}$ and p16 expression (Supplemental Figure S3A) and accumulation of SA-β-gal–positive cells (Supplemental Figure S3B) 3 d after oxidative stress in wild-type MEFs overexpressing caveolin-1. Finally, because Nrf2 can facilitate cell growth in cancer cells (Kim *et al.*, 2011), premature senescence is a tumor suppressor mechanism, overexpression of caveolin-1 inhibits Nrf2, and caveolin-1-mediated inhibition of Nrf2 leads to premature senescence, we asked whether overexpression of caveolin-1 in colon cancer cells is antitumorigenic. We show in Figure 7, D and E, that the transformed phenotype of HCT116 cells, as assessed by growth in soft agar, was inhibited by the overexpression of caveolin-1 after oxidative stress as compared with parental HCT116 cells. These data are consistent with growing evidence suggesting that Nrf2 signaling may be beneficial to cancer cells and suggest that caveolin-1–mediated inhibition of Nrf2 inhibits cell transformation by promoting premature senescence.

DISCUSSION

Our understanding of oxidative stress–induced premature senescence is limited by unknowns at the cellular and molecular levels, resulting in ineffective pharmacological intervention designed to minimize the potential detrimental effects of SIPS. Thus a clearer understanding of the complex role SIPS plays is critical for elucidating the mechanism behind organismal aging. In addition, as our population ages, the incidence of age-related diseases will increase. Therefore revealing the basic science behind the mechanisms leading to senescence becomes increasingly necessary for improving public health. Nrf2 is a key transcription factor that regulates an adaptive stress response system. The interaction of Nrf2 with Keap1 in the cytoplasm leads to degradation of Nrf2. As a result, basal expression of Nrf2-regulated genes is maintained at low levels. According to the current paradigm, this is the main mechanism that prevents Nrf2-dependent signaling under resting conditions. Following oxidative stress, Nrf2 escapes degradation and translocates to the nucleus, where it activates the expression of several cytoprotective genes that enhance cell survival. Our data challenge the current paradigm that Nrf2 signaling is inhibited only by the Keap-1–mediated degradation of Nrf2. We show that Nrf2 is a novel direct binding partner of caveolin-1; Nrf2 is expressed, in addition to the nucleus, at the plasma membrane in caveolar membranes under resting conditions; and the interaction of caveolin-1 with Nrf2 inhibits Nrf2 signaling. Because we did not find any interaction between caveolin-1 and Keap1 before or after oxidative stress in fibroblasts (Supplemental Figure S2B), we propose a novel model in which the sequestration of Nrf2 in caveolar membranes contributes to the inhibition of Nrf2-dependent signaling under resting conditions independently of Keap1-mediated degradation of Nrf2 in the cytoplasm.

The timing of the caveolin-1–mediated localization of Nrf2 in caveolar membranes is intriguing. Our data show that, under resting conditions, approximately half of Nrf2 is localized in caveolar membranes and interacts with caveolin-1, whereas the other half is in the nucleus. Twenty-four hours after treatment with sublethal doses of hydrogen peroxide, Nrf2 remains localized in caveolar membranes,

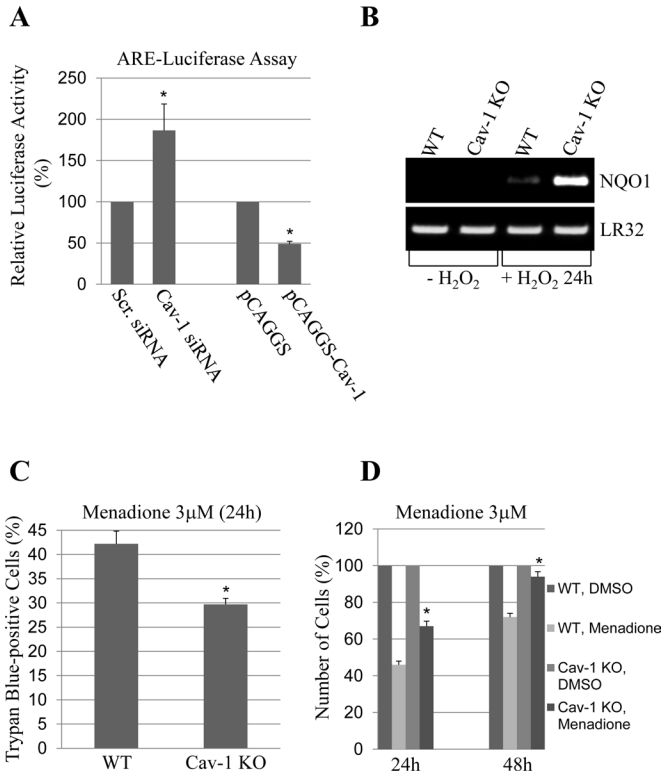

FIGURE 4: Effect of caveolin-1 expression on Nrf2-dependent signaling. (A) NIH 3T3 cells were transiently transfected with caveolin-1 siRNA to down-regulate caveolin-1 expression or with the expression vector pCAGGS-caveolin-1 to overexpress caveolin-1. Transfection with scrambled siRNA and the pCAGGS vector alone was done as a control. Twenty-four hours later, cells were transfected with a luciferase-based construct containing an ARE. Luciferase activity was quantified 48 h later. Values represent means ± SEM; $n = 4$; *$p < 0.001$. (B) MEFs were derived from WT and caveolin–1 null (Cav-1 KO) mice. Cells were treated with sublethal doses of hydrogen peroxide (150 μM) for 2 h. Cells were then recovered in complete medium for 24 h. Untreated cells (–H_2O_2) were used as control. The expression level of NQO1 was determined by RT-PCR using NQO1-specific primers. LR32 amplification was used as a control. (C, D) WT and caveolin-1 KO MEFs were treated with 3 μM menadione for 24 h (C) or 24 and 48 h (D). In C, the percentage of trypan blue–positive cells was calculated. In D, the percentage of total number of cells was determined. Values in C and D represent means ± SEM; $n = 6$; *$p < 0.001$.

but its nuclear localization is increased due to dissociation of cytoplasmic Nrf2 from Keap1 and movement of this fraction of cytoplasmic Nrf2 that escapes Keap1-mediated degradation to the nucleus. Forty-eight hours after oxidant stimulation, Nrf2 localization in the nucleus is further enhanced due to the exit of Nrf2 from caveolae and translocation to the nucleus. In the absence of caveolin-1 expression, Nrf2 localization in the nucleus is increased, as compared with caveolin-1–expressing cells, both under resting conditions and 24 h after oxidative stress due to a lack of sequestration of Nrf2 in caveolar membranes. At 48 h after oxidative stress, Nrf2 accumulation in the nucleus is comparable in wild-type and caveolin-1–null MEFs due to the exit of Nrf2 from caveolar membranes in wild-type cells. Our data also demonstrate that oxidative stress up-regulated Nrf2 mRNA and total protein levels and increased the half-life of the protein Nrf2 both in wild-type and caveolin-1–null MEFs, indicating that the difference between caveolin-1–expressing and caveolin-1–lacking cells is in the cellular localization of Nrf2 rather than total Nrf2 protein expression. Of interest, we found that caveolin-1 inhibits Nrf2-dependent signaling under resting conditions and during the first 24 h after treatment with sublethal concentrations of hydrogen peroxide, and expression of Φ→A-Nrf2, a mutant form of Nrf2 that cannot bind to caveolin-1 and is constitutively active, inhibits SIPS. Taken together, these data show that the caveolin-1–mediated sequestration of Nrf2 in caveolar membranes promotes premature senescence by preventing excessive antioxidative signaling during the early phase of the cellular stress response, when cell fate decisions are made.

While this work was being completed, an article appeared describing the inhibitory role of caveolin-1 in Nrf2-mediated signaling (Li *et al.*, 2012). However, the authors did not prove a direct interaction between caveolin-1 and Nrf2 in vitro, did not assess the functional role of caveolin-1 in Nrf2-dependent signaling in the context of oxidative stress–induced premature senescence, and did not investigate the tumor-suppressive properties of the caveolin-1–mediated inhibition of Nrf2 in cancer cells, as we have done in this article. Moreover, in contrast to our findings, these authors found an interaction between caveolin-1 and Nrf2 in both the cytoplasm and nucleus of lung epithelial Beas-2B cells and between caveolin-1 and Keap1. This discrepancy can be explained by the unconventional localization of caveolin-1 in Beas-2B cells (Li *et al.*, 2012). Whereas caveolin-1 is expressed at the plasma membrane and intracellular Triton-insoluble caveolar membranes in most cell types and cell lines, including WI-38 fibroblasts and mouse embryonic fibroblasts used in the present study, caveolin-1 appears to localize to the cytoplasm and nucleus of Beas-2B cells. Thus it is possible that caveolin-1 contributes to the inhibition of Nrf2-mediated signaling by either sequestering Nrf2 in caveolar membranes (this article) or promoting the formation of the Keap1-Nrf2 complex (Li *et al.*, 2012), depending on the cellular context.

One of the effects that oxidative stress has in eukaryotic cells is the induction of premature senescence. Our findings demonstrate that inhibition of Nrf2 by caveolin-1 contributes to limiting the cellular antioxidant response after oxidative stress, leading to activation of the p53/$p21^{Waf1/Cip1}$ pathway and development of premature senescence. These results are consistent with data showing that caveolin-1 expression is up-regulated, whereas Nrf2 has a decreased function in senescent cells and that silencing of Nrf2 leads to premature senescence (Volonte *et al.*, 2002; Dasari *et al.*, 2006; Kapeta *et al.*, 2010). Of interest, Nrf2-mediated signaling plays an important role in the resistance to oxidative stress in long-lived mice (Leiser and Miller, 2010), and Nrf2 activation is believed to contribute to the extension of lifespan in glutathione transferase mGSTA4–null mice (Singh *et al.*, 2010). We previously showed that caveolin-1 expression activates p53. The murine double minute (Mdm2) oncogene promotes degradation of p53. Data show that the inhibition of Nrf2 can suppress Mdm2 expression, which results in activation of p53 signaling (You *et al.*, 2011). Because we found that the oxidant-induced activation of the p53 pathway is inhibited in cells expressing a mutant form of Nrf2 that cannot bind to caveolin-1, our data suggest that inhibition of Nrf2 by caveolin-1 may be one of the molecular mechanisms by which caveolin-1 activates p53 and promotes premature senescence.

We previously showed that caveolin-1 expression is up-regulated by cigarette smoking, a source of oxidative stress, and that caveolin-1–null mice were protected against cigarette smoke–induced premature senescence of lung fibroblasts and development of pulmonary emphysema (Volonte *et al.*, 2009). Because we show here that caveolin-1–mediated inhibition of Nrf2 promotes

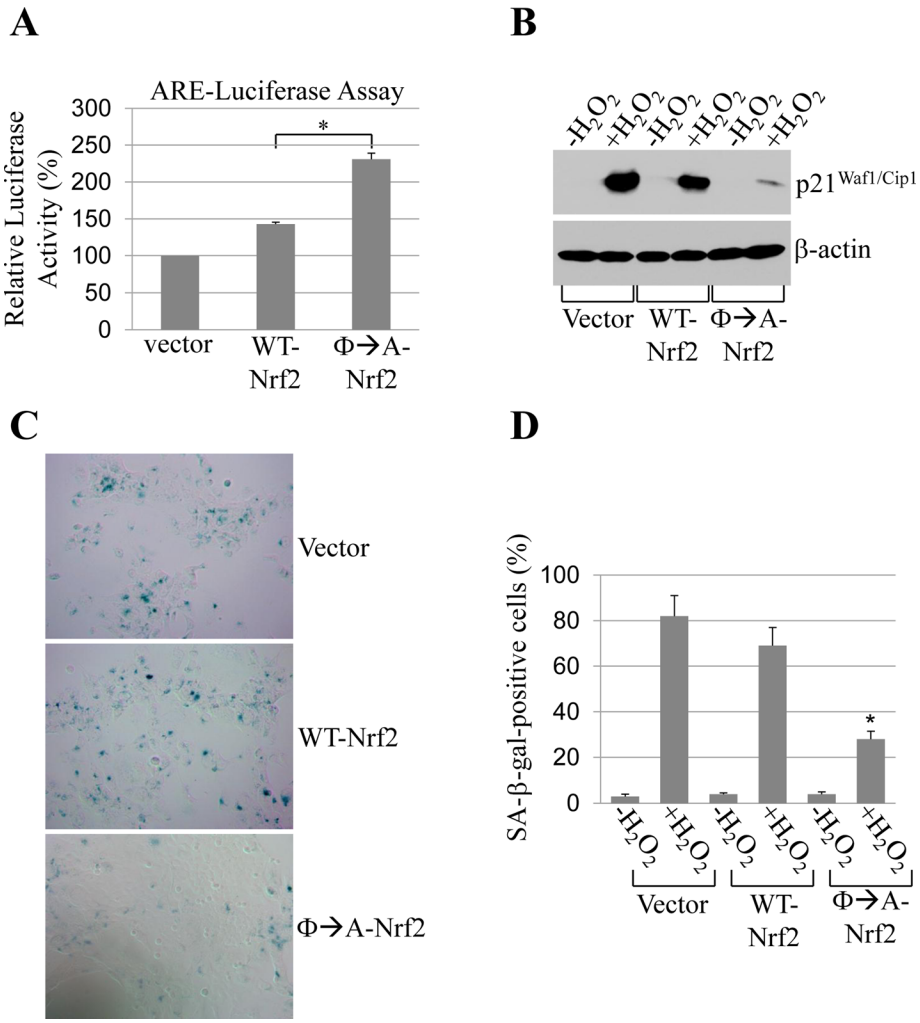

FIGURE 5: Effect of expression of Φ→A-Nrf2 on SIPS. Vector alone, wild-type Nrf2 (WT-Nrf2), and Φ→A-Nrf2 were stably transfected in NIH 3T3 cells. (A) Clones stably expressing either WT-Nrf2 or Φ→A-Nrf2 were then transfected with a luciferase-based construct carrying an ARE. Expression of the ARE-containing luciferase construct in NIH 3T3 cells stably carrying the vector alone was used as control. Luciferase activity was quantified 48 h later. Values represent means ± SEM; $n = 4$; *$p < 0.001$. (B–D) Clones stably expressing vector alone, WT-Nrf2, or Φ→A-Nrf2 were treated with sublethal doses of hydrogen peroxide (150 μM) for 2 h. Cells were then recovered in complete medium for 7 d. Untreated cells ($-H_2O_2$) were used as control. In B, the expression level of $p21^{Waf1/Cip1}$ was determined by immunoblotting analysis using an antibody probe specific for $p21^{Waf1/Cip1}$. Immunoblotting with anti–β-actin IgGs was done as a control. In C and D, cells were subjected to senescence-associated β-galactosidase activity staining. Representative images are shown in C, and a quantitative analysis is shown in D. Values in D represent means ± SEM; $n = 3$; *$p < 0.001$.

SIPS, we can speculate that the caveolin-1–mediated inhibition of Nrf-2-dependent signaling after cigarette smoking may limit the antioxidant response of lung cells and lead to their senescent phenotype, which helps to explain the development of pulmonary emphysema. This scenario is consistent with data showing that Nrf2 depletion in mice enhances susceptibility to cigarette smoke–induced emphysema (Rangasamy *et al.*, 2004) and patients with emphysema have a decline in Nrf2-dependent signaling (Malhotra *et al.*, 2008).

Although Nrf2 has a beneficiary cytoprotective effect in normal cells, emerging evidence suggests that overactive Nrf2 signaling in several types of cancer cells may promote cell growth and contribute to their resistance to oxidative stress and therefore chemotherapy. Because cancer cells need to escape the barrier represented by cellular senescence in order to produce a clinically relevant tumor mass, cellular senescence is considered antitumorigenic. We show that overexpression of caveolin-1 in colon cancer cells inhibits Nrf2-dependent signaling, promotes SIPS, and inhibits their transformed phenotype. Thus caveolin-1–mediated inhibition of Nrf2 signaling in colon cancer cells appears to act in an antitumorigenic manner under conditions of oxidative stress that promote senescence. Our results support data showing that RNA interference–mediated knockdown of Nrf2 in colon cancer cells suppressed tumor growth (Kim *et al.*, 2011). We propose that the regulation of Nrf2-mediated signaling by caveolin-1 may control the fine balance that exists between the positive and negative effects of cellular senescence on cancer and aging, respectively. Additional work is necessary to fully understand the mechanisms through which this balance is regulated and whether therapeutic interventions aimed at altering the caveolin-1/Nrf2 signaling can enhance tumor suppression without accelerating aging and/or age-related phenotypes or slow the aging process without necessarily promoting tumor initiation and/or progression.

MATERIALS AND METHODS

Materials

Antibodies and their sources were as follows: anti–caveolin-1 immunoglobulin G (IgG; polyclonal antibody [pAb] N-20), anti-p21 IgG (pAb), and anti-p16 IgG (pAb C-20 or M-156) were from Santa Cruz Biotechnology (Santa Cruz, CA); anti–hexahistidine tag IgG (monoclonal antibody [mAb]) was from Clontech (Mountain View, CA); anti-Nrf2 IgG (mAb) was a gift from K. Itoh (Hirosaki University, Hirosaki, Japan); anti-HO1 IgG (mAb HO-1-1) was from Enzo Life Sciences (Farmingdale, NY); anti-FLAG IgG (mAb M2) was from Sigma-Aldrich (Saint Louis, MO); anti-Keap1 IgG (pAb 10503-2-AP) was from ProteinTech Group (Chicago, IL); horseradish peroxidase–conjugated goat anti-mouse and anti-rabbit secondary antibodies were from Pierce (Rockford, IL). Note that a Nrf2 doublet was sometimes detected by the anti-Nrf2 mAb only in mouse embryonic fibroblasts but not in human WI-38 cells. Additional studies are required to determine the nature and the functional significance of the fainter Nrf2 band that migrates more slowly in SDS–PAGE than the major Nrf2 band of ~70 kD in MEFs. All other biochemicals used were of the highest purity available and were obtained from regular commercial sources.

Cell culture and oxidative stress

WI-38 human diploid fibroblasts (American Type Culture Collection, Manassas, VA) were grown in MEM supplemented with glutamine,

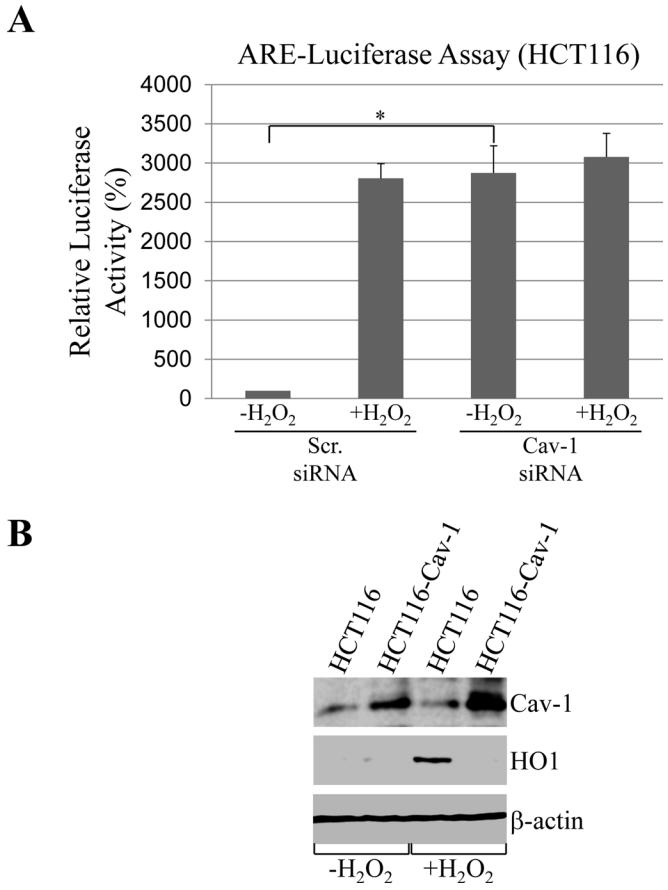

FIGURE 6: Caveolin-1 inhibits Nrf2-dependent signaling in HCT116 cells. (A) HCT116 colon cancer cells were transiently transfected with caveolin-1 siRNA to down-regulate caveolin-1 expression. Transfection with scrambled siRNA was done as a control. Twenty-four hours later, cells were transfected with a luciferase-based construct containing an ARE. After 24 h, cells were treated with sublethal doses of hydrogen peroxide (150 μM) for 2 h. Cells were then recovered in complete medium for 24 h. Untreated cells ($-H_2O_2$) were used as control. Luciferase activity was quantified. Values represent means ± SEM; $n = 4$; *$p < 0.001$. (B) HCT116 cells were stably transfected with pCAGGS–caveolin-1 to overexpress caveolin-1 (HCT116-Cav-1). HCT116 and HCT116-Cav-1 were treated with sublethal doses of hydrogen peroxide (150 μM) for 2 h. Cells were then recovered in complete medium for 24 h. Untreated cells ($-H_2O_2$) were used as control. Caveolin-1 and HO1 expression were determined by immunoblotting analysis using antibody probes specific for caveolin-1 and HO1. Immunoblotting with anti–β-actin IgGs was done as a control.

antibiotics (penicillin and streptomycin), and 10% fetal bovine serum (FBS). NIH 3T3 cells were grown in DMEM supplemented with glutamine, antibiotics (penicillin and streptomycin), and 10% donor bovine calf serum. Human colon carcinoma cells HCT116 (a generous gift from Bert Vogelstein, Johns Hopkins University, Baltimore, MD) were grown in McCoy's 5a medium containing glutamine, antibiotics, and 10% FBS. Oxidative stress was induced by subcytotoxic levels of hydrogen peroxide (150 μM for NIH 3T3 and HCT116 cells; 450 μM for WI-38 cells) for 2 h. Cells were then recovered in normal medium for different periods of time (see *Results* for details).

GST fusion protein pull-down assay

The GST–caveolin-1 (GST–Cav-1) fusion protein constructs were transformed into *Escherichia coli* (BL21 strain; Novagen, Gibbstown, NJ). After induction of expression through addition of 5 mM isopropyl-β-D-galactoside (Sigma-Aldrich), GST–Cav-1 constructs were affinity purified on glutathione–agarose beads, using the detergent Sarcosyl for initial solubilization. GST–Cav-1 and GST alone (bound to glutathione–agarose beads) were washed three times with TNET buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100) containing protease inhibitors. SDS–PAGE followed by Coomassie staining was used to determine the concentration of GST–Cav-1 per 100 μl of packed bead volume. Precleared cell lysates were diluted in buffer A (10 mM Tris, pH 8.0, 0.1% Tween 20) and added to ~100 μl of equalized bead volume for overnight incubation at 4°C. After binding, the beads were extensively washed with phosphate-buffered saline (PBS; six times). Finally, the beads were resuspended in 3× sample buffer and subjected to SDS–PAGE.

Direct binding assays

WT-Nrf2 and Φ→A-Nrf2 were cloned into the pET-22b(+) vector (from Novagen) to generate WT-Nrf2-His and Φ→A-Nrf2-His. WT-Nrf2-His and Φ→A-Nrf2-His were affinity purified from bacterial cell lysates using nickel-nitriloacetic acid agarose beads. Affinity-purified WT-Nrf2-His and Φ→A-Nrf2-His were then incubated with affinity-purified GST alone or GST fused to residues 82–101 (Cav-1(82–101)–GST) immobilized on glutathione-agarose beads. The beads were then subjected to immunoblotting analysis with anti–hexahistidine IgGs to detect Nrf2 binding.

Immunoblotting

Cells were collected in boiling sample buffer. Cellular proteins were resolved by SDS–PAGE (12.5% acrylamide) and transferred to BA83 nitrocellulose membranes (Schleicher & Schuell, Keene, NH). Blots were incubated for 2 h in TBST (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.2% Tween 20) containing 2% powdered skim milk and 1% bovine serum albumin. After three washes with TBST, membranes were incubated for 2 h with the primary antibody and for 1 h with horseradish peroxidase–conjugated goat anti–rabbit/mouse IgG. Bound antibodies were detected using an ECL detection kit (Pierce).

Preparation of caveolae-enriched membrane fractions

Cells were scraped into 2 ml of 2-(*N*-morpholino)ethanesulfonic acid (MES)-buffered saline containing 1% (vol/vol) Triton X-100. Homogenization was carried out with 10 strokes of a loose-fitting Dounce homogenizer. The homogenate was adjusted to 40% sucrose by the addition of 2 ml of 80% sucrose prepared in MES-buffered saline and placed at the bottom of an ultracentrifuge tube. A 5–30% linear sucrose gradient was formed above the homogenate and centrifuged at 45,000 rpm for 16–20 h in a SW60 rotor (Beckman Coulter, Fullerton, CA). A light-scattering band confined to the 15–20% sucrose region was observed that contained endogenous caveolin-1 but excluded most of other cellular proteins. From the top of each gradient, 375-μl gradient fractions were collected to yield a total of 11 fractions. Fractions 4–6, representing caveolar membranes, and 9–11, representing noncaveolar membranes, were pooled together. An equal amount of protein from each of the two groups was separated by SDS–PAGE and subjected to immunoblot analysis.

Coimmunoprecipitation

Cells were washed twice with PBS and lysed for 30 min at 4°C in a buffer containing 10 mM Tris, pH 8.0, 0.15 M NaCl, 5 mM EDTA, 1% Triton X-100, and 60 mM octyl glucoside. Samples were precleared for 1 h at 4°C using protein A–Sepharose (20 μl; slurry, 1:1) and

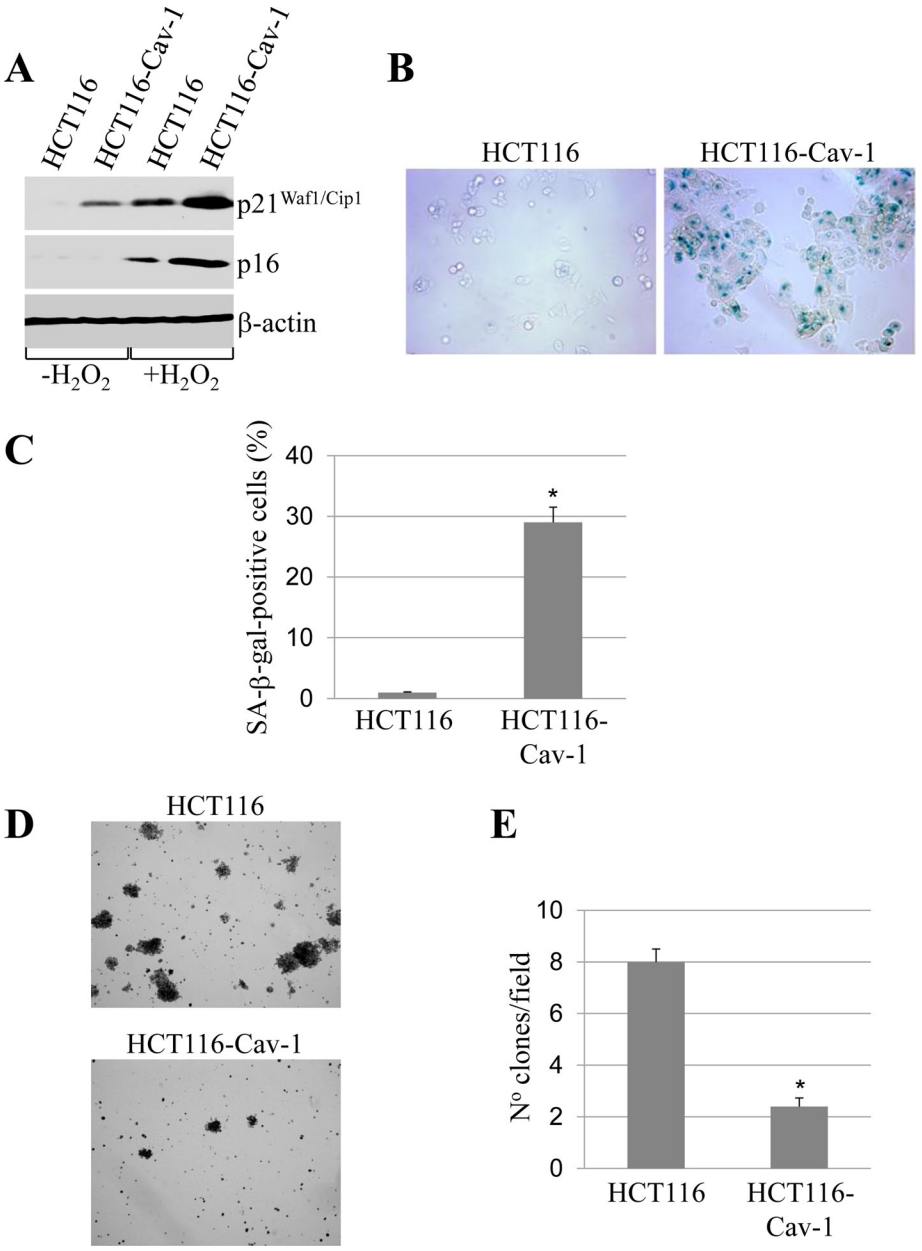

FIGURE 7: Overexpression of caveolin-1 in HCT116 cells promotes SIPS and inhibits growth in soft agar. HCT116 cells were stably transfected with pCAGGS–caveolin-1 to overexpress caveolin-1 (HCT116-Cav-1). HCT116 and HCT116-Cav-1 were treated with sublethal doses of hydrogen peroxide (150 μM) for 2 h. Untreated cells (–H_2O_2) were used as control. (A–C) Cells were recovered in complete medium for 5 d. (A) The expression level of $p21^{Waf1/Cip1}$ and p16 was determined by immunoblotting analysis using specific antibody probes. Immunoblotting with anti–β-actin IgGs was done as a control. (B, C) Cells were subjected to senescence-associated β-galactosidase activity staining. Representative images are shown in B, and a quantitative analysis is shown in C. Values in C represent means ± SEM; $n = 6$; *$p < 0.001$. (D, E) Cells were recovered in complete medium for 48 h. Cells were then collected and cultured in soft agar for 7 d. A representative image is shown in D, and quantification of growth in soft agar is shown in E. Values in E represent means ± SEM; $n = 80$; *$p < 0.001$.

subjected to overnight immunoprecipitation at 4°C using the intended antibody and protein A–Sepharose (30 μl; slurry, 1:1). After three washes with the immunoprecipitation buffer, samples were separated by SDS–PAGE (12.5% acrylamide) and transferred to nitrocellulose. Then the blots were probed with the intended antibody. Experiments were performed three independent times, and representative images are shown.

Immunofluorescence microscopy

Cells grown on glass coverslips were washed three times with PBS w/Ca^{2+}/Mg^{2+} and fixed for 30 min at room temperature with 2% paraformaldehyde in PBS w/Ca^{2+}/Mg^{2+}. Fixed cells were rinsed with PBS w/Ca^{2+}/$Mg^{2+}$and permeabilized with 0.1% Triton X-100 and 0.2% bovine serum albumin for 10 min. Then cells were treated with 25 mM NH_4Cl in PBS w/Ca^{2+}/Mg^{2+} for 10 min at room temperature to quench free aldehyde groups. Cells were rinsed with PBS w/Ca^{2+}/Mg^{2+} and incubated with the primary antibody (diluted in PBS with 0.1% Triton X-100 and 0.2% bovine serum albumin) for 2 h at room temperature. After three washes with PBS w/Ca^{2+}/Mg^{2+} (10 min each), cells were incubated with the secondary antibody for 1 h at room temperature: lissamine rhodamine B sulfonyl chloride–conjugated goat anti-rabbit antibody (5 μg/ml) and fluorescein isothiocyanate–conjugated goat anti-mouse antibody (5 μg/ ml). Finally, cells were washed three times with PBS w/Ca^{2+}/Mg^{2+} (10 min each wash), and slides were mounted with slow-Fade antifade reagent (Molecular Probes, Eugene, OR) and observed using a Zeiss Confocal Microscope (LSM 5 Pascal; Carl Zeiss, Jena, Germany).

RNA isolation and RT-PCR

Cells were collected and total RNA was isolated using the RNeasy Mini Kit from Qiagen (Valencia, CA). Equal amounts of RNA were treated with RNase-free DNase and subjected to reverse transcription using the Advantage RT-for-PCR kit from Clontech (Mountain View, CA), according to the manufacturer's recommendations. PCR was then performed in the exponential linear zone of amplification for each gene studied. The Nrf2-specific primers used were forward, CATTCTGAAAGGCTGGTTTGA, and reverse, TTTCTTCCATCCTTCCAGGAT. A sequence corresponding to LR32 was also amplified as an internal control.

Subcellular fractionation

The subcellular protein fractionation kit from Pierce was used for the isolation of membrane, cytosolic, and nuclear fractions, according to the manufacturer's recommendations. The quality of the isolation of the membrane, cytosolic, and nuclear fractions was confirmed by immunoblotting analysis using antibody probes specific for caveolin-1, α-tubulin, and lamin B, respectively.

siRNA treatment

Knockdown of caveolin-1 expression was achieved by transfection of cells with siRNA duplexes using Lipofectamine 2000 (Life Technologies, Carlsbad, CA). The target sequence was

5′-AACCAGAAGGGACACACAG-3′. Scrambled siRNA was used as a negative control.

ARE-luciferase reporter assay

Cells were seeded in 60-mm dishes at 270,000 cells/dish. The next day, cells were transiently transfected, using a modified calcium–phosphate precipitation method, with 2 μg of a luciferase reporter construct carrying an ARE sequence, 1 μg of a β-galactosidase–expressing construct, and 2 μg of the indicated expression vector. In caveolin-1–knockdown experiments, cells were transfected with caveolin-1 siRNA 24 h before transfection with the ARE-luciferase construct. At 24 h posttransfection, cells were washed twice and incubated in complete medium at 37°C for an additional 48 h. Cells were then lysed in 500 μl of extraction buffer; 200 μl was used to measure luciferase activity and 150 μl was used to measure β-galactosidase activity. Three independent experiments were performed for each condition.

Acid β-galactosidase staining

Cells were subjected to acid β-galactosidase staining using the Senescence-Associated β-galactosidase Staining Kit (Cell Signaling Technology, Beverly, MA), according to the manufacturer's recommendations. Briefly, cells were washed twice with PBS and fixed with the fixative solution for 15 min. Then cells were washed twice with PBS and incubated overnight at 37°C with the staining solution. Cells were then examined for the development of blue color. Cells were photographed at low magnification (10×) using a BX50WI optical light microscope (Olympus, Tokyo, Japan).

Growth in soft agar

Parental and caveolin-1–overexpressing HCT116 cells (5×10^4) were suspended in 3 ml of McCoy's 5a medium containing 10% FBS and 0.33% SeaPlaque low–melting temperature agarose. These cells were plated over a 2-ml layer of solidified McCoy's 5a medium containing 10% FBS and 0.5% agarose and allowed to settle to the interface between these layers at 37°C. After 20 min, the plates were allowed to harden at room temperature for 30 min before returning to 37°C. After 10 d, colonies were photographed under low magnification (5×). The colonies in 80 randomly chosen fields from two independent plates were counted.

ACKNOWLEDGMENTS

This work was supported by grants from the National Institute on Aging (R01-AG030636; to F.G.), the American Heart Association (12SDG8800012), and the Competitive Medical Research Fund of the University of Pittsburgh Medical Center (UPMC) Health System (to D.V.).

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