Regulation of Reactive Oxygen Species Homeostasis by Peroxiredoxins and c-Myc^*\textsuperscript{S}

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Peroxiredoxins (Prxs) are highly conserved proteins found in most organisms, where they function primarily to scavenge reactive oxygen species (ROS). Loss of the most ubiquitous member of the family, Prx1, is associated with the accumulation of oxidatively damaged DNA and a tumor-prone phenotype. Prx1 interacts with the transcriptional regulatory domain of the c-Myc oncoprotein and suppresses its transforming activity. The DNA damage in tissues of prx1\textsuperscript{−/-} mice is associated in some cases with only modest increases in total ROS levels. However, these cells show dramatic increases in nuclear ROS and reduced levels of cytoplasmic ROS, which explains their mutational susceptibility. In the current work, we have investigated whether changes in other ROS scavengers might account for the observed ROS redistribution pattern in prx1\textsuperscript{−/-} cells. We show \textsuperscript{−}5-fold increases in Prx5 levels in prx1\textsuperscript{−/-} embryo fibroblasts relative to prx1\textsuperscript{+/+} cells. Moreover, Prx5 levels normalize when Prx1 expression is restored. Prx5 levels also appear to be highly dependent on c-Myc, and chromatin immunoprecipitation experiments showed differential occupancy of c-Myc and Prx1 complexes at E-box elements in the prx5 gene proximal promoter. This study represents a heretofore unreported mechanism for the c-Myc-dependent regulation of one Prx family member by another and identifies a novel means by which cells reestablish ROS homeostasis when one of these family members is compromised.

ROS such as hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}) and superoxide are created as a result of normal cellular signaling and metabolism (1), including oxidative phosphorylation (2). Other causes of increased ROS levels include exposure to various drugs and hormones (3) and the overexpression of certain oncogenes (4–6). One of the most potent oncogene inducers of ROS is c-Myc, a global basic helix-loop-helix-leucine zipper transcription factor (4). The exact mechanism by which c-Myc increases ROS has not been determined. One possibility is that changes in the regulation of the expression of various c-Myc target genes could be involved. For example, CYP2C9 (7) is a cytochrome P450 isoenzyme that is increased in the presence of c-Myc under specific cellular conditions (8). It has been estimated that approximately half of the ROS generated by c-Myc overexpression can be accounted for by induction of CYP2C9 (8). Another proposed mechanism is by the c-Myc-dependent induction of the p53 tumor suppressor, some of whose target genes encode proteins that regulate ROS (9). Other data support the idea that c-Myc influences mitochondrial biogenesis via the regulation of mitochondrial gene expression, leading to redox state changes (10).

High intracellular ROS can produce genomic instability via the oxidation and subsequent mutation of nucleotide bases and the generation of both single- and double-stranded DNA breaks (11). Genomic instability is believed to be an important, if not essential, component in the development of many malignancies (3, 12). Despite the potential for widespread genomic damage, ROS are not exclusively agents of destruction. In fact, H\textsubscript{2}O\textsubscript{2} has been implicated as an important second messenger molecule that can affect essential cellular processes, including proliferation and differentiation (13).

Given the clear importance of tightly regulating ROS levels, it is not surprising that cells have evolved intricate and redundant methods of maintaining homeostasis. The primary method for reducing ROS levels is via their degradation by enzymes such as superoxide dismutase and catalase (11). Another group of enzymes that plays a major role in ROS regulation is the peroxiredoxin (Prx) family, which consists of six related members in mammals and which maintains a high degree of conservation down to bacteria (14–17). Prxs reduce hydrogen peroxide and other peroxide substrates via conserved cysteine residues utilizing thiol-containing proteins, such as glutathione or thioredoxin, as electron donors (16–18).

The first identified member of the Prx family was Prx1, which was initially noted to be elevated in response to transformation
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by Ras (19) and was subsequently shown to be induced by pro-
iferative stimuli (19), nitric oxide (20), and oxidative stress (21).
The latter response appears to be mediated through the trans-
scription factor Nrf2, which binds to antioxidant response ele-
ments within the prx1 promoter (22–24).

Although the primary function of Prx1 appears to be ROS scavenging, it has also been implicated in enhancing the cyto-
toxic effects of natural killer cells (25) as well as having home-
binding properties (26). There are also substantial data that
implicate Prx1 as a tumor suppressor, including its ability to
interact with and inhibit the functions of certain oncoproteins.
For example, Prx1 binds to the Src homology 3 domain of c-Abl
and inhibits its intrinsic kinase activity (27). Prx1 also directly
interacts with the transcriptional regulatory domain of c-Myc,
specifically, with a conserved 15–20-amino acid region known
as Myc Box II (28). This interaction results in reduced in vitro
transformation by c-Myc and is associated with complex
changes in the c-Myc target gene expression profile (28, 29).
Further evidence for a tumor suppressor role for Prx1 comes
from analyses of tumor prone prx1−/− mice (30). This onco-
genic susceptibility is at least partly a consequence of wide-
spread oxidative DNA damage, the precise nature of which is
highly tissue-specific (29, 30). In addition, c-Myc activity is up-
regulated as a result of its loss of interaction with Prx1, thus
making fibroblasts from prx1−/− animals susceptible to single-
hit transformation by mutant ras oncogenes.

Interestingly, oxidative DNA damage in prx1−/− tissues is
often seen in the absence of any significant changes in overall
ROS levels (29). This apparent contradiction has been explained by showing that the subcellular localization of ROS in
prx1−/− cells is primarily nuclear (29), whereas in prx1+/+ cells,
it was primarily cytoplasmic and in accord with the find-
ing the Prx1 is primarily a nuclear protein (28). Thus, although
not necessarily showing changes in overall ROS levels, cells
from prx1−/− mice appear to be prone to the development of
DNA damage as a consequence of ROS redistribution.

In this study, we tested the hypothesis that changes in the
levels of other Prx family members might account for the rela-
tively modest changes in overall ROS levels in prx1−/− cells. We
demonstrate here that prx1−/− cells up-regulate Prx5, the sec-
cond most widely distributed peroxiredoxin (14). It does not
appear that the increase in Prx5 is a result in changes in cellular
oxidative stress. Rather we offer evidence that prx5 is a direct
c-Myc target gene. We believe that the ability of Prx1 to mod-
ulate Prx5 levels is the first example of a peroxiredoxin regulat-
ing the expression of another. Furthermore, we believe that this
regulatory model may play a significant role in the ability of the
cell to regulate ROS homeostasis, particularly that mediated in
response to alterations in c-Myc.

EXPERIMENTAL PROCEDURES

Cell Culture—Day 14 prx1+/+ and prx1−/− mouse embry-
onic fibroblasts (MEFs) were previously described (29) and
were spontaneously immortalized using a 3T3-type passage
protocol (31). Cells were declared immortalized once they
reached passage 10. Cells were cultured in Dulbecco’s modified
Eagle’s essential medium supplemented with 10% fetal bovine
serum, 2 mm glutamine, and antibiotics as previously described
(32) and grown at 5% CO2 at 37 °C.

Detection of ROS—The detection of ROS was performed after
staining cells with 5-(and 6)-chloromethyl-2′,7′-dichlorodihy-
drofluorescein diacetate, acetyl ester (CM-H2-DCFDA; Invitrogen). A stock solution was reconstituted in molecular
grade DMSO (Sigma) to a concentration of 0.5 mM and stored
at −20 °C. Cells were grown to 50–75% confluence in
6-well plates. They were washed once with RPMI 1640
medium. CM-H2-DCFDA was added to RPMI 1640 medium
at a final concentration of 0.25 µM, and then 1 ml of the solution
was added to each well. Samples were incubated for 10 min at
37 °C. Cells were then washed twice with ice-cold phosphate-
buffered saline (PBS) and scraped with a policeman into 0.5 ml
of ice-cold PBS. CM-H2-DCFDA fluorescence was determined
by measuring 10,000 events/sample following excitation with a
488-nm wavelength laser and reading through a 530/30 filter.
CM-H2DCFDA has an excitation maximum of 495 nm and an
emission maximum of 529 nm. Samples were run in triplicate.

Fluorescence Microscopy—Cells were grown to 50–75% con-
fluence on 9-mm glass coverslips. These were first washed with
ice-cold PBS and fixed in 4% paraformaldehyde (Sigma) for 10
min at room temperature. Cells were then washed twice with
ice-cold PBS, and then permeabilized with PBS containing 0.1%
Triton X (Sigma) at room temperature for 5 min. Following a
final PBS wash, 5% fetal bovine serum in PBS was added for 20
min at room temperature to block nonspecific binding. All sub-
sequent antibody incubations were performed in PBS plus 3% fetal bovine serum. Anti-Prx5 antibody (Santa Cruz Biotech-
nology, Inc., Santa Cruz, CA) was added to each coverslip at a
concentration of 5 µg/ml, and cells were incubated at room
temperature for 1 h. Following three washes in PBS, fluorescein
isothiocyanate-conjugated goat anti-mouse antibody (3 µg/ml)
(Santa Cruz Biotechnology) was added, and cells were incu-
bated at room temperature for 45 min. Following three addi-
tional washes in PBS, coverslips were counterstained with 1×
4′,6-diamidino-2-phenylindole and then mounted onto micro-
scope slides and stored in the dark at room temperature for
24 h.

To observe cells stained with CM-H2-DCFDA by fluores-
cence microscopy, coverslips were prepared as above and then
stained with a 2.5 µm solution of CM-H2-DCFDA. Each cover-
slip was then fixed in 4% paraformaldehyde for 10 min at room
temperature before being viewed by standard fluorescence
microscopy.

Western Analysis—Whole cell lysates were prepared by
scraping cells grown to 75% confluence in a 6-well plate into
SDS sample buffer (65 mm Tris-HCl pH 8.0, 2.3% SDS, 1% di-
thiothreitol, 10% glycerol, 0.01% bromphenol blue). Samples
were sonicated with a microtip probe for 10 s and then boiled
for 4 min prior to loading onto a 15% polyacrylamide gel. Pro-
tein electrophoresis and transfer to a polyvinylidene difluo-
ride membrane were performed in a Bio-Rad Mini-PROTEAN unit
according to the included instructions (Bio-Rad). The blot was
blocked in 5% nonfat dried milk for 1 h at room temperature
and then washed three times for 10 min/wash in PBS with 0.1%
Tween (PBS-T). Primary antibodies were suspended in 5% non-
fat dried milk at empirically determined concentrations: anti-
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Prx1 (1:10,000; Biomol International, Plymouth Meeting, PA), anti-Prx2 (1:1000; Santa Cruz Biotechnology), anti-Prx3 (1:1000; Santa Cruz Biotechnology), anti-Prx4 (1:200; Abcam, Cambridge, MA), anti-Prx5 (1:1500; Santa Cruz Biotechnology), anti-Prx6 (1:2000, Abcam), anti-c-Myc (9E10; 1:1000; Santa Cruz Biotechnology), and β-tubulin (1:500; Santa Cruz Biotechnology). Blots were incubated with primary antibodies overnight at 4 °C and then washed with PBS-T at room temperature. The appropriate horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology) was diluted to a concentration of 1:20,000 in 5% nonfat dried milk in PBS-T and added to the blots for 1 h at room temperature. Following multiple washes with PBS-T, the blots were visualized using a standard chemiluminescence protocol (Thermo Scientific, Waltham, MA).

**Recombinant DNA Methods**—Retroviral vectors encoding short hairpin RNA (shRNAs) designed against both the murine prx5 and c-myc genes were purchased commercially (Origene, Rockford, IL). These constructs were packaged in Phoenix-A cells by transient transfection using the SuperFect reagent according to the recommendations of the supplier (Qiagen, Chatsworth, CA). Retroviral supernatants were isolated 48–72 h later, filtered, and used to infect MEFs in the presence of 8 μg/ml Sequabrene (Sigma). Constructs containing irrelevant sequences and the empty retroviral vector were used as negative controls.

**Quantitative Real Time PCR**—RNAs were prepared according to the protocol provided with the RNeasy Mini Kit (Qiagen) and stored at −20 °C. RNA was used for RT-PCR according to the protocol provided with the Quantitect SYBR Green RT-PCR kit (Qiagen). The sequences for the primer pairs were obtained from the Primer Bank (available on the World Wide Web) and commercially synthesized (IDT, Coralville, IA). Specific primer sequences are listed in supplemental Table 1. Samples were run on the Roche Light Cycler according to manufacturer’s specifications. Amplification of a PCR product of the predicted size was verified by electrophoresis of an aliquot of the final reaction on a 4% NuSieve agarose gel (Cambrex, Rockford, IL).

**Chromatin Immunoprecipitation (ChIP)**—MEFs were grown to 90% confluence in 15-cm plates and then exposed to 1% formaldehyde for 10 min at room temperature. Glycine was added to a final concentration of 0.125 M, and after 5 min, the samples were washed twice and harvested in cold PBS with protease inhibitors (Roche Applied Science). Cells were collected by centrifugation for 4 min at 2,000 × g and suspended in lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris, pH 8.1). ChIP assays were performed by following the Upstate Biotechnology ChIP assay kit protocol (Millipore, Billerica, MA) with slight modifications, as previously described (33). Briefly, the cell lysate was sonicated to yield 100–1,000-bp genomic DNA fragments with a Bioruptor (Diagenode, Liège, Belgium). The lysate (2 ml) was precleared with 75 μl of a 50% slurry of protein A-agarose that contained 32 μg of sonicated salmon sperm DNA, 80 μg of bovine serum albumin, and 160 μg of recombinant protein A-agarose suspended in 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, and 0.05% sodium azide for 30 min at 4 °C with agitation. After centrifugation at 1,000 × g for 2 min, aliquots of the supernatant were incubated with an antibody directed against c-Myc or with normal rabbit IgG (Santa Cruz) overnight with agitation at 4 °C. Immunocomplexes were recovered by incubation with a 50% slurry of salmon sperm DNA/protein A-agarose, in the buffer described above, for 1 h at 4 °C. The beads were washed for 5 min each with low salt, high salt, and LiCl immune complex buffer and twice with TE buffer (10 mM Tris, pH 8.0, 0.5 mM EDTA). The chromatin complexes were eluted by adding freshly prepared elution buffer (1% SDS, 0.1 M NaHCO3) with constant rotation at room temperature for 15 min, followed by centrifugation and collection of the supernatant. The process was repeated, and the two eluates were combined. The cross-linking was reversed by adding NaCl to a final concentration of 500 mM and heating at 65 °C for 4 h. After incubation with 20 mg/ml proteinase K for 1 h, the DNA was purified using a PCR purification column (Qiagen), and target genes were quantified by RT-PCR using the purified DNA. Standard curves were constructed using 2-fold serial dilutions of the DNA extracted from the IgG treatment (2.5 μl) as a reference input. The protein content was normalized using the Pierce BCA protein assay (Thermo Scientific) before immunoprecipitation, and the quantity of the amplicons was expressed as a percentage of the total reference input (33). The primer sequences for the PCRs are shown in supplemental Table 2.

**Statistical Analysis**—The calculation of the total cellular ROS was derived by first establishing a consistent histogram for wild type MEFs stained with CM-H2-DCFDA. A threshold of the top 5% of the histogram was established, and the total fluorescence of that area was set as the denominator. Histograms for the other samples were overlaid, and the total fluorescence that exceeded the previously established control threshold was the numerator. Experiments were performed at least five times for all samples and expressed as relative mean values ± S.E.

The transcript expression level for the RT-PCR experiment was calculated as a ratio of each experimental sample to the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase. Samples were run in triplicate and are expressed as a relative mean ± S.E.

The values for the ChIP experiments were calculated by subtracting the percentage of the lysates input obtained from precipitations with control IgG antibody from the values obtained when using an antibody directed against c-Myc or Prx1. Experiments were performed at least three times for all samples and expressed as relative mean values ± S.E. p values were calculated by a two-tailed t test using Microsoft Excel.

**RESULTS**

**ROS are Modestly Increased and Redistributed in prx1−/− MEFs**—Primary cells derived from various prx1−/− mouse tissues do not always display significant increases in ROS despite showing an increased amount of oxidative DNA damage (29). Spontaneously immortalized MEFs from prx1−/− and prx1+/+ mice were established in culture and stained with CM-H2DCFDA to measure total cellular ROS under logarithmic growth conditions. When ROS in these cells were measured by flow cytometry, a modest shift in the histogram could be appreciated (Fig. 1A). Quantification of the differences showed an approximate 2-fold increase in ROS in prx1−/− cells when com-
pared with \( \text{prx}^{+/+} \) cells (Fig. 1B), which was consistent with previous studies in primary cells (29).

The same cells were next grown on glass coverslips, incubated with CM-H\(_2\)-DCFDA, and examined individually by fluorescence microscopy. \( \text{prx}^{+/+} \) cells showed a strong cytoplasmic signal but virtually no nuclear signal (Fig. 1C). This finding is not surprising, given the fact that Prx1 is primarily a nuclear protein and would be expected to be highly efficient at scavenging nuclear ROS (14, 28). In contrast, \( \text{prx}^{-/-} \) MEFs showed almost exclusively nuclear CM-H\(_2\)-DCFDA staining, indicating that the bulk of ROS is concentrated in that compartment (Fig. 1C). Again, this finding is consistent with the previous results observed with primary cells from these same animals (29). We also noted that \( \text{prx}^{-/-} \) cells showed little of the cytoplasmic...
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staining that was seen in prx1+/+ cells. We have shown these changes in ROS distribution to be specific to the loss of Prx1, because prx1−/− cells that were reconstituted with lentivirally expressed Prx1 (Fig. 1D) reestablished the CM-H2-DCFDA staining pattern of prx1+/+ cells; specifically, they have reduced total levels of ROS when compared with prx1−/− cells, and the bulk ROS now again resides almost exclusively in the cytoplasm (Fig. 1E). Given these findings, we hypothesized that another ROS scavenger might be active in the cytoplasm of prx1−/− cells, which show distinct patterns of expression and localization (14). We examined the expression of these different Prx isoforms in whole cell lysates from prx1+/+ and prx1−/− MEFs. As seen in Fig. 2A, Prx2, -3, -4, and -6 were expressed at nearly equivalent levels in both prx1+/+ and prx1−/− MEFs (less than 2-fold differences). In contrast, Prx5 levels were 4–6-fold higher in prx1−/− cells. Similar and statistically significant results were obtained when prx5 transcripts were quantified by quantitative real time PCR (Fig. 2B). When Prx1 expression was restored in prx1−/− MEFs via lentiviral transduction, a dramatic normalization of Prx5 levels was seen that closely matched those seen in prx1+/+ cells (Fig. 2C).

Prx5 has been previously identified as both a cytosolic and mitochondrial protein (14). We therefore used immunofluorescence to localize Prx5 in both prx1+/+ and prx1−/− MEFs. In the former case (Fig. 2D), as well as in prx1−/− cells in which Prx1 expression was restored (data not shown), Prx5 was difficult to detect but appeared to be primarily nuclear in its distribution. In contrast, strong staining for Prx5 was seen in prx1−/− MEFs (Fig. 2D). Although staining appeared to be most intense and diffuse in the cytoplasm, there was some residual nuclear signal. Furthermore, some cells displayed punctate staining within the cytoplasm that may represent mitochondrial localization (Fig. 2D, inset). Since Prx5 resides in the cytoplasm of prx1−/− MEFs, we conclude that the up-regulation and possible nucleus-to-cytoplasm redistribution of this protein probably explains the differential pattern of ROS localization between prx1+/+ and prx1−/− cells as well as the modest differences in ROS levels.

Prx5 Helps to Maintain ROS Homeostasis in MEFs—To determine whether Prx5 has any influence on ROS levels in prx1−/− MEFs, we used stable expression of an shRNA to reduce Prx5 (Fig. 3A). Next, we measured total ROS in these cells by flow cytometry and were able to demonstrate a statistically significant increase in ROS of ~3-fold (Fig. 3B). Furthermore, when these cells were stained with CM-H2-DCFDA and examined by fluorescence microscopy, they demonstrated a much more intense staining of both the nucleus and cytoplasm (Fig. 3C). From these data, we conclude that Prx5 contributes significantly to the maintenance of ROS levels in the absence of prx1−/−. It also appears that Prx1 specifically regulates ROS levels in the nucleus, whereas Prx5 specifically controls ROS levels in the cytoplasm and mitochondria.

Oxidative Stress Does Not Regulate Prx5 Expression—We next investigated the mechanism(s) underlying the up-regulation of Prx5 in prx1−/− cells. Changes in environmental oxidative stress have been shown to influence prx1 gene expression (22). This occurs at the transcriptional levels and is mediated through a DNA sequence known as the antioxidant response element. Since the Prx5 gene promoter has been shown to have multiple antioxidant response elements (34, 35), we asked whether the increased Prx5 in prx1−/− cells could be explained by changes in intracellular ROS levels. prx1−/− MEFs were therefore grown in the presence of increasing amounts of a strong antioxidant (N-acetylcysteine) or a strong oxidant (H2O2) and then analyzed by immunoblotting for Prx5. As shown in Fig. 4, no change in Prx5 expression was seen under either of these conditions. The same experiment performed...
with prx1+/+ cells again showed no changes in Prx5 expression; however, Prx1 protein levels did increase in these cells following exposure to H2O2 (data not shown).

**c-Myc Expression Paradoxically Reduces ROS Levels in prx1+/− MEFS**—Substantial data indicate that the enforced expression of c-Myc is associated with increased levels of ROS in mammalian cells (4). This activity requires the MBII domain of c-Myc (data not shown), although the exact mechanism by which this occurs remains unclear. In order to determine how c-Myc influenced ROS levels in the absence of Prx1, we used lentivirus-mediated transduction to enforce the expression of c-Myc in prx1+/− MEFS (Fig. 5A). The result was an unexpected yet reproducible decrease in total ROS levels (Fig. 5B).

Next, we examined Prx5 levels in these c-Myc-prx1+/− cells, because 1) c-Myc and Prx1 can influence ROS in opposite directions, 2) Prx1 and c-Myc physically interact, 3) depletion of Prx1 leads to increased Prx5, and 4) the prx5 gene is an unverified c-Myc target (36). Prx5 protein levels in these cells were found to be highly elevated (Fig. 5A).

In order to better define the relationship between c-Myc and Prx5 and the subsequent influence on ROS levels, we manipulated c-Myc levels in the prx1+/− cells. As shown in Fig. 5C, we used shRNA to deplete c-Myc levels in prx1+/− MEFS. The result was a decrease in Prx5 protein levels (Fig. 5C) and a concomitant profound increase in ROS levels compared with cells expressing control shRNA (Fig. 5D). From these experiments, we concluded that c-Myc increases Prx5 levels in the absence of Prx1. As a result, there is an overall decrease in ROS in these cells, as we had previously demonstrated (Fig. 3B). We conclude that the paradoxical decrease in ROS in c-Myc-overexpressing prx1+/− MEFS stems from the increased expression of Prx5, which leads to a more efficient scavenging of ROS.

**Differential prx5 Promoter Occupancy by c-Myc and Prx1 in prx1+/+ and prx1−/− Cells**—The murine prx5 promoter contains four consensus E-box elements upstream of the transcrip-
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FIGURE 5. c-Myc expression reduces ROS levels in prx1−/− MEFs. A, prx1−/− MEFs were transduced with a human c-Myc expression vector or the empty control vector. Stable, pooled clones were then immunoblotted for c-Myc or Prx5. β-Tubulin was used as a loading control. B, MEFs from the previous experiment were stained with CM-H2-DCFDA and analyzed by flow cytometry. The histogram shows two independently isolated pools of clones for each cell line and is representative of several typical experiments. Note that the significantly reduced levels of ROS in c-Myc-overexpressing prx1−/− cells. C, prx1−/− MEFs were transduced with a c-myc shRNA-encoding retrovirus. Stable clones were then pooled and immunoblotted for c-Myc and Prx5 expression. β-Tubulin was used as a loading control. Note that reduction of endogenous c-Myc reduces expression of Prx5. Also note that the exposure time of the c-Myc immunoblot was significantly longer than that depicted in A in order to detect endogenous c-Myc protein. D, MEFs from C were assayed for total ROS by flow cytometry as previously described. Strains are as follows. a, prx1−/−; b, prx1−/− + control shRNA; c, prx1−/− + c-myc shRNA. The inset shows quantification of the histograms as previously described. *, p value was significant when comparing prx1−/− cells expressing control shRNA with those expressing c-myc shRNA (p = 0.03).

FIGURE 6. Differential prx5 promoter occupancy by c-Myc and Prx1 in prx1+/+ and prx1−/− MEFs. A, diagrammatic representation of the murine prx5 gene promoter showing the location of two perfect consensus E-box elements (CACGTG) (−1047 (site D) and −310 (site F) relative to the start of transcription) and two alternate elements CATGTG (−1319 (site C) and −606 (site E)) oriented in the opposite direction of the D and F sites. The arrows indicate the approximate positions of primers used to amplify each site (see supplemental Table 2 for the sequences of all primers). Sites A and B are non-E-box-containing sites that were used in control quantitative PCR amplifications. B, ChIP of the prx5 promoter in both prx1+/+ and prx1−/− cells was performed with anti-c-Myc antibodies to identify sites of c-Myc occupancy. *, p value was significant (p < 0.05) when comparing signals from prx1−/− cells and prx1+/+ cells. The horizontal line represents an arbitrary value below which the signal is believed to represent background. C, ChIP of the prx5 promoter was performed and displayed as described for B except that an anti-Prx1 antibody was used to identify sites of Prx1 occupancy.
DISCUSSION

Intracellular ROS are a two-edged sword with both beneficial and deleterious consequences. On the one hand, ROS participate in normal cell signaling pathways (13, 16, 37–39). For example, H$_2$O$_2$ oxidizes cysteine residues on protein-tyrosine phosphatases, which inhibits their function and leads to the up-regulation of multiple tyrosine kinase-dependent signaling pathways in response to growth factors (40–44). In fact, these pathways may be further potentiated by the ability of H$_2$O$_2$ to directly oxidize the kinases themselves and further enhance their signaling potential (13, 45). These reactions are reversible, which makes this a modifiable signal. On the other hand, H$_2$O$_2$ and other ROS can damage numerous macromolecules. Most notably, the oxidation of DNA can lead to significant mutations that have been implicated in the development of cancer (46, 47). Interestingly, it has been proposed that both the second messenger properties of ROS as well as their mutagenic effects may contribute additively to the development of cancer by driving cells into mitosis before genomic damage can be adequately repaired (17).

Given the significant and sometimes opposing roles that ROS play within the cell, it is not surprising that intricate and redundant controls over these processes have evolved. The best studied means for regulating ROS levels is via the Prxs. Each of the six mammalian Prxs has a specific tissue and subcellular distribution (14, 17, 18). The most abundant and ubiquitous member of the Prxs, prx1, is expressed at high levels in virtually every tissue, primarily as a nuclear ROS scavenger. Indeed, prx1 null mice are prone to the accumulation of oxidative DNA damage, Heinz body-positive hemolytic anemia resulting from the precipitation of oxidized hemoglobin within erythrocytes, and a variety of cancers (29, 30).

Given the substantial amount of DNA damage in prx1 null mice, it was initially surprising that only modest increases in total ROS were observed (29). These findings were confirmed in the current report with prx1 null MEFs that show less than 2-fold increases in total ROS compared with their wild-type counterparts (Fig. 1, A and B). This finding is directly attributable to a shift in ROS from the cytoplasm to the nucleus in prx1 null cells, thus explaining the greater degree of genomic oxidative damage without the need to substantially increase overall cellular ROS. A more subtle finding was that the cytoplasmic ROS signal was concurrently decreased in these cells (Fig. 1C). This suggested that another cytoplasmic ROS scavenger might be compensating for the loss of Prx1.

Examination of other Prx family members showed that only Prx5 was elevated in prx1 null cells and that Prx5 could be normalized upon the reexpression of Prx1 (Fig. 2). Prx5, the most recently discovered Prx family member, is a 17-kDa protein that is considered an “atypical” Prx; it exists primarily in monomeric form rather than the dimeric forms typical of other Prxs (18, 48, 49). Despite its unique structure, it has been shown to possess the same ability to reduce ROS as the other Prxs (49). There has been dispute within the literature over the localization of Prx5. Depending on the cell type and the method of detection, it has previously been reported to be found in the mitochondria, peroxisome, cytosol, and occasionally the nucleus (50). In this report, we have localized Prx5 primarily to the nucleus in cells wild-type for Prx1 (Fig. 2D). However, in prx1 null cells, there is not only increased expression of Prx5 but possibly a redistribution of the protein from the nucleus to the cytoplasm. We believe this increased expression of Prx5 in the cytoplasm explains well the nuclear localization, or “redistribution,” of ROS in prx1 null MEFs as well as the near total lack of cytoplasmic ROS.

We have found that when the compensatory increase of Prx5 in prx1 null MEFs is prevented, there is a dramatic increase in total cellular ROS in both the cytoplasm and the nucleus (Fig. 3, B and C). This lends further support to the notion that Prx5 helps to maintain ROS homeostasis within the cell and may be a first line response to situations in which the Prx1 is otherwise compromised. As previously suggested, this property is not only likely to be important for protection against the cellular damage that ROS can cause, but may be a fine-tuning mechanism for levels and localization of ROS, such as H$_2$O$_2$, that serve as second messenger molecules.

Since the regulation of Prx5 appears to be at the transcriptional level, there are several possible mechanisms that might account for its increase in prx1 null cells. One possibility is that the increased oxidative stress in prx1 null cells, albeit minimal, might lead directly to the increase in Prx5 expression via ROS intermediates. Interestingly, although the Prx5 promoter contains several oxidative response elements (22, 35), Prx5 levels do not appear to be particularly responsive to ROS levels (Fig. 4). Similar findings were reported in a previous study where Prx5 was concluded to be constitutively expressed (35).

Another possibility to explain Prx5 regulation is via the interaction of its promoter with Prx1. Although Prx1 lacks a DNA binding motif, it does interact with a number of DNA binding proteins, including the androgen receptor (51), c-Abl (27), and c-Myc (28), and indirectly alters the function of the bound transcription factor. In the case of c-Myc, the association with Prx1 changes the expression of some c-Myc target genes, although the pattern of this change is complex and involves both increases and decreases in the target gene repertoire (28, 29). Among the putative direct c-Myc targets is prx5 (36), which suggests that Prx1 could regulate Prx5 through its interaction with c-Myc. In this study, we demonstrate that the prx5 promoter contains multiple E-box elements to which c-Myc is capable of binding. Regardless of the status of Prx1, c-Myc is able to bind avidly to the three proximal E-boxes, which include two optimal binding sequences. We believe c-Myc-mediated transcriptional activation at these sites to be a possible mechanism for the increased levels of Prx5 seen in the absence of Prx1. Moreover, this may explain why c-Myc-overexpressing prx1 null MEFs actually show a decrease in ROS production (Fig. 5B) rather than the predicted increase. Our studies indicate that the ability of c-Myc to increase Prx5 leads to levels of the protein that are higher than those actually needed to counter the elevated ROS generated by c-Myc. Additional evidence for this model was provided by experiments in which endogenous c-Myc levels in prx1 null MEFs were reduced by shRNA leading to reduced amounts of Prx5 (Fig. 5C) and...
higher ROS (Fig. 5D). Collectively, this strengthens the conclusion that c-Myc is able to increase Prx5 in the absence of Prx1 by direct transcriptional activation. However, when Prx1 is present, it serves to dampen this up-regulation. Prx1 only appears to bind significantly to E-box “C”, suggesting that occupation of this more distal binding site may negatively influence the more proximal transcriptional activity. Since Prx1 does not occupy any of the other examined sites and c-Myc does not appear to have significant binding to E-box “C” in prx1−/− cells, the unopposed binding of c-Myc to sites D–F may be the primary mechanism for enhanced Prx5 expression. Experiments are currently under way to better understand the precise mechanism for this regulation and the relevance of c-Myc and Prx1 binding at these various sites.

In summary, we have demonstrated a novel feedback pathway for ROS regulation (Fig. 7). The primary ROS scavenger is Prx1, whose key role is to protect the integrity of the genome by virtue of its nuclear predominance. However, a separate function of Prx1 appears to be its ability to interact with other effector proteins, such as c-Myc. In this particular case, Prx1 reduces the ability of c-Myc to activate transcription and presumably to limit its induction of ROS. However, if Prx1 levels are lowered, c-Myc is now able to increase the transcription of Prx5, a promiscuous ROS scavenger that is normally maintained at low levels. High Prx5 levels can reduce ROS levels, although this occurs predominantly in the cytoplasm. Although this is not an optimal method for preventing genomic instability, it may serve as an effective means for regulating the second messenger functions of ROS and perhaps minimize the leakage of ROS from the cytoplasm to the nucleus.

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REFERENCES
Regulation of ROS by Peroxiredoxins and c-Myc