Angioplasty and stenting is the primary treatment for flow-limiting atherosclerosis; however, this strategy is limited by pathological vascular remodeling. Using a systems approach, we identified a role for the network hub gene glutathione peroxidase-1 (GPX1) in pathological remodeling following human blood vessel stenting. Constitutive deletion of Gpx1 in atherosclerotic mice recapitulated this phenotype of increased vascular smooth muscle cell (VSMC) proliferation and plaque formation. In an independent patient cohort, gene variant pair analysis identified an interaction of GPX1 with the orphan protooncogene receptor tyrosine kinase ROS1. A meta-analysis of the only genome-wide association studies of human neointima-induced in-stent stenosis confirmed the association of the ROS1 variant with pathological remodeling. Decreased GPX1 expression in atherosclerotic mice led to reductive stress via a time-dependent increase in glutathione, corresponding to phosphorylation of the ROS1 kinase activation site Y2274. Loss of GPX1 function was associated with both oxidative and reductive stress, the latter driving ROS1 activity via s-glutathiolation of critical residues of the ROS1 tyrosine phosphatase SHP-2. ROS1 inhibition with crizotinib and deglutathiolation of SHP-2 abolished GPX1-mediated increases in VSMC proliferation while leaving endothelialization intact. Our results indicate that GPX1-dependent alterations in oxido-reductive stress promote ROS1 activation and mediate vascular remodeling.

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Oxido-reductive regulation of vascular remodeling by receptor tyrosine kinase ROS1

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Angioplasty and stenting is the primary treatment for flow-limiting atherosclerosis; however, this strategy is limited by pathological vascular remodeling. Using a systems approach, we identified a role for the network hub gene glutathione peroxidase-1 (GPX1) in pathological remodeling following human blood vessel stenting. Constitutive deletion of Gpx1 in atherosclerotic mice recapitulated this phenotype of increased vascular smooth muscle cell (VSMC) proliferation and plaque formation. In an independent patient cohort, gene variant pair analysis identified an interaction of GPX1 with the orphan protooncogene receptor tyrosine kinase ROS1. A meta-analysis of the only genome-wide association studies of human neointima-induced in-stent stenosis confirmed the association of the ROS1 variant with pathological remodeling. Decreased GPX1 expression in atherosclerotic mice led to reductive stress via a time-dependent increase in glutathione, corresponding to phosphorylation of the ROS1 kinase activation site Y2274. Loss of GPX1 function was associated with both oxidative and reductive stress, the latter driving ROS1 activity via s-glutathiolation of critical residues of the ROS1 tyrosine phosphatase SHP-2. ROS1 inhibition with crizotinib and deglutathiolation of SHP-2 abolished GPX1-mediated increases in VSMC proliferation while leaving endothelialization intact. Our results indicate that GPX1-dependent alterations in oxido-reductive stress promote ROS1 activation and mediate vascular remodeling.

Introduction

Balloon angioplasty and stenting (BAS) is the most commonly performed method of revascularization for flow-limiting atherosclerosis, with over 1.5 million procedures performed annually in the United States (1). The immediate vascular injury following BAS predisposes to acute thrombosis and initiates vascular smooth muscle cell (VSMC) proliferation and neointimal hyperplasia, leading to a phenotype significantly different from that of de novo atherosclerosis (2). This reaction to vascular injury activates a homeostatic healing response that can lead to neointima-induced in-stent stenosis, the major limitation to the most widely used method for treatment of coronary and peripheral arterial disease. The use of drug-eluting stents (DESs), which target excessive VSMC proliferation and neointimal hyperplasia, has greatly reduced clinical restenosis rates (3). However, current agents used in DESs inhibit reendothelialization, potentially increasing late thrombosis risk within the stented vessel (4). Considering that over 1 million patients undergo DES implantation each year within the United States (1), there remains a pressing need to identify more specific and effective pharmacological agents targeting neointimal hyperplasia without detrimental effects on reendothelialization. Accordingly, we developed and validated a mouse model of BAS that allows for interrogation of relevant molecular pathways by high-throughput genomic approaches not readily feasible using larger animal models (5).

Glutathione peroxidase-1 (GPX1), the most abundant intracellular isoform of the GPX family of selenoprotein antioxidant enzymes, diminishes oxidant stress by utilizing reduced glutathione (GSH) to reduce hydrogen peroxide and lipid peroxides to alcohols. In conditions in which GPX1 becomes deficient, electron transfer is no longer coupled to GSH reduction, leading to increased bioavailability of ROS. In humans with coronary artery disease, GPX1 is independently associated with cardiovascular events (6). GPX1 activity is decreased in atherosclerotic plaque excised from carotid artery (7). Mice with GPX1 deficiency have endothelial dysfunction, impaired capacity for angiogenesis due to endothelial progenitor cell dysfunction (8), and significant structural vascular and cardiac abnormalities (9). Corresponding-

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When GPX1 is deficient in animal models of vascular disease, such as hypercholesterolemia (10) or diabetes (11), atherosclerosis is increased. Despite such evidence, large-scale clinical trials using nonspecific antioxidant drugs to prevent atherosclerosis (12) or in-stent stenosis (13) have not been successful. Such lack of success speaks to a need for a more advanced understanding of redox signaling mechanisms as mediators of vascular processes.

In studies reported here, we identify GPX1 as a determinant of vascular remodeling and identify the orphan RTK ROS1 as a mediator of these effects. We find that loss of GPX1 leads to increased VSMC proliferation, migration, and apoptosis, in vitro and in vivo, and that this is attenuated by inhibition of ROS1 through cell-fate regulation. Further, in contrast to the oxidative stress induced by GPX1 deficiency, we find ROS1 activation coincides with reductive stress induced by GSH. We show that this reductive stress has no direct effect on ROS1, but rather that it leads to inhibition of the regulatory phosphatase SHP-2 by s-glutathiolation of 2 critical phosphatase active-site cysteine residues. Inhibition of SHP-2 prevents dephosphorylation of ROS1, resulting in sustained ROS1 kinase activation, triggering VSMC processes that manifest as neointimal hyperplasia and abnormal vascular remodeling. Further, we determined that pharmacological inhibition of ROS1 attenuates these processes without affecting reendothelialization. Finally, in humans, we show that genetic variation within ROS1 is associated with risk for abnormal vascular remodeling leading to in-stent stenosis, confirming ROS1 as a pivotal regulator of the response to vascular injury in humans and a therapeutic target with substantial clinical impact.

Results

Network analysis of human coronary neointima prioritizes the GPX1 subnetwork for investigation. We analyzed coronary vascular gene expression from 89 patients who underwent coronary atherectomy. RNA samples derived from de novo atherosclerosis (n = 55) and in-stent stenosis (n = 34) were hybridized to microarrays with genome-wide representation of coding genes (14). Independently, networks of gene-gene interactions were generated using text mining of the entire abstracted academic literature. Generated networks exhibited scale-free topology. Gene expression from atherectomy tissue was overlaid on these networks and individual networks were scored according to the average differential significance of network members within the experimental data set. While upregulated networks were highly enriched for collagen and inflammatory genes involved in the healing response, we found the network with GPX1 as its hub to be the most significantly downregulated of all gene networks (nodes, 13; cumulative D statistic for the network, 22.4; mean statistic for the network, 1.72; SAM statistic for GPX1, −3.2; Table 1). In contrast, the significance analysis of microarrays alone ranked GPX1 only 247th in a list of most differentially regulated genes.

**Constitutive deletion of GPx1 in atherosclerotic mice increases aortic smooth muscle–rich plaque.** Human coronary artery atherectomy specimens demonstrated higher GPX1 gene expression in de novo atherosclerosis than in in-stent neointima (Figure 1A). We used mice with ubiquitous homozygous deletion of Gpx1 crossed with hypercholesterolemic atherosclerotic ApoE−/− (Gpx1+/−Apoe−/−) mice as our primary model. As expected, GPX1 activity was decreased in Gpx1+/−Apoe−/− mice without differences in body weight or cholesterol (Supplemental Results, Supplemental Figure 1, and Supplemental Table 1; supplemental material available online with this article; doi:10.1172/JCI77484DS1). Atherosclerotic plaque burden in the aortic root (42%, P = 0.02; Figure 1B) and the thoracoabdominal aorta (41%, P = 0.01; Figure 1B) of Gpx1+/−Apoe−/− mice was increased compared with that in Gpx1+/−Apoe−/− controls. Plaque composition analysis revealed a significant increase in the percentage area occupied by α-actin–positive smooth muscle cells (SMCs) (42%, P = 0.02), but not macrophages, lipid, or collagen in Gpx1+/−Apoe−/− mice (Supplemental Table 2 and Supplemental Figure 2). Thus, targeted deletion of Gpx1 leads to increased VSMC-rich plaque formation in atherosclerotic mice.

**In-stent neointima is increased in mice with constitutive deletion of Gpx1.** We developed a mouse model of BAS analogous to human coronary stenting, allowing us to mimic human vascular
disease states in a manner not possible in larger animal models (5). In our model, balloon deployment of a custom stent in the descending aorta of hypercholesterolemic atherosclerotic mice was performed in a donor mouse just prior to euthanasia and the stented segment explanted. The stented segment was then secured microsurgically as a vascular graft using end-to-end anastomoses (Supplemental Figure 3, A and B). We performed BAS in Gpx1−/− Apoe−/− and control Gpx1+/+ Apoe−/− mice. In keeping with our transcriptomic findings from human coronary artery in-stent neointima (Figure 1A), we found that Gpx1 mRNA was significantly decreased in Gpx1+/+ Apoe−/− vessels that had undergone BAS compared with uninjured aorta (P = 0.05; Figure 1C). Correspondingly, we found an increase in neointima (50%, P = 0.006; Figure 1D) in Gpx1−/− Apoe−/− stented vessels compared with control Gpx1+/− Apoe−/−. There was no difference in total vessel wall area or lumen area between groups (Supplemental Figure 3C). The extent of vascular injury caused by stent deployment is an important determinant of neointimal hyperplasia (15). As stents were deployed using an identical angioplasty balloon inflated to a consistent pressure in all animals, there were no differences between groups in either stent expansion (0.82 ± 0.11 vs. 0.89 ± 0.13 mm², \( P = 0.67 \)) or mean Schwartz vessel injury score (1.4 ± 0.4 vs. 1.4 ± 0.3, \( P = 0.50 \)) (Supplemental Figure 3C). Total neointimal cell count was greater in the Gpx1−/− Apoe−/− mice; however, there was no difference in cell density when expressed per unit area between groups. Scale bar: 50 μm. Black arrowheads denote neointima.

Figure 1. Increased atherosclerosis and in-stent neointima in Gpx1−/− Apoe−/− mice. (A) Transcriptional profiling of human coronary atherectomy specimens of control (AHA class I lesions), atherosclerosis (AHA class III–V lesions), or in-stent stenosis identified that Gpx1 expression was incrementally lower in atherosclerosis and in-stent stenosis. *\( P < 0.01 \) vs. control; **\( P < 0.05 \) vs. atherosclerosis. \( n = 55 \) atherosclerosis/34 in-stent stenosis. (B) Relative area of atherosclerosis in the aortic root and lesion burden in aorta were increased in Gpx1−/− Apoe−/− mice compared with Gpx1+/+ Apoe−/− mice (*\( P < 0.02, n = 8–15 \)/group). Black scale bar: 50 μm. White scale bar: 0.5 mm. (C) mRNA levels of Gpx1 in Gpx1−/− Apoe−/− mice were lower in aorta that had undergone BAS compared with uninjured aorta harvested at 28 days. *\( P = 0.05, n = 4 \)/group. (D) Gpx1−/− Apoe−/− mice 4 weeks following BAS developed significantly greater neointimal hyperplasia compared with control Gpx1+/− Apoe−/−. *\( P < 0.05 \). Although overall neointimal cell density was higher in Gpx1−/− Apoe−/− mice, there was no difference in cell density when expressed per unit area between groups. Scale bar: 50 μm. Black arrowheads denote neointima.
in regulating endothelial cell regeneration following BAS, we generated Gpx1−/− Apoe−/− and Gpx1+/+ Apoe−/− knockout mice with an additional transgene to allow endothelium-targeted LacZ expression. Endothelial cells in freshly harvested aorta from both Gpx1−/− Apoe−/− Tie2-LacZ+ and Gpx1+/+ Apoe−/− Tie2-LacZ+ control mice demonstrated β-gal staining (Figure 2, A and B, and Supplemental Figure 3D), whereas no β-gal staining was evident in animals without LacZ. Twenty-eight days following BAS, stented vessels from Gpx1−/− Apoe−/− Tie2-LacZ+ mice had over 50% reduction in endothelial coverage compared with Gpx1+/+ Apoe−/− Tie2-LacZ+ (P = 0.03; Figure 2C), suggesting that GPX1 is required for endothelial repopulation.

Increased bioavailability of ROS in Gpx1−/− Apoe−/− mice. Given the central role of GPX1 in protecting cells from ROS and the impairment in endothelialization shown above, we next investigated redox state in different layers of the aortic wall from experimental animals using dihydroethidium (DHE) oxidative fluorescence microscopy. We observed striking differences in superoxide production, in regulating endothelial cell regeneration following BAS, we generated Gpx1−/− Apoe−/− and Gpx1+/+ Apoe−/− knockout mice with an additional transgene to allow endothelium-targeted LacZ expression. Endothelial cells in freshly harvested aorta from both Gpx1−/− Apoe−/− Tie2-LacZ+ and Gpx1+/+ Apoe−/− Tie2-LacZ+ control mice demonstrated β-gal staining (Figure 2, A and B, and Supplemental Figure 3D), whereas no β-gal staining was evident in animals without LacZ. Twenty-eight days following BAS, stented vessels from Gpx1−/− Apoe−/− Tie2-LacZ+ mice had over 50% reduction in endothelial coverage compared with Gpx1+/+ Apoe−/− Tie2-LacZ+ (P = 0.03; Figure 2C), suggesting that GPX1 is required for endothelial repopulation.

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finding vessel wall fluorescence 3-fold higher in Gpx1<sup>−/−</sup> Apoe<sup>−/−</sup> mice compared with Gpx1<sup>+/+</sup> Apoe<sup>−/−</sup>. Surprisingly, superoxide production was markedly increased only in the media (73%, P < 0.01), but not the endothelium or adventitia (Figure 3A). Similarly, total vascular superoxide generation from eNOS was minimal (Figure 3B). significantly different, suggesting that the endothelial contribution to superoxide production was dependent on the normal vascular phenotype in mice deficient in Gpx1<sup>−/−</sup>. Because of the only GWAS of in-stent stenosis (CardioGene, GeneBank, deCODE, and GENDER). Participants in these studies comprise a total of 1,033 cases of in-stent stenosis and 2,669 controls. In this, the largest genotyped cohort of patients after BAS, we found an association between rs529038 and the development of in-stent stenosis (P = 0.056) to be of borderline significance.

The ROS1 variant associates with in-stent stenosis in GWAS. To replicate the association of rs529038 ROS1 with in-stent stenosis in humans, we performed an independent meta-analysis of the only GWAS of in-stent stenosis (CardioGene, GeneBank, deCODE, and GENDER). Participants in these studies comprise a total of 1,033 cases of in-stent stenosis and 2,669 controls. In this, the largest genotyped cohort of patients after BAS, we found an association between rs529038 and the development of in-stent stenosis (P = 0.056) to be of borderline significance.

ROS1 is a mediator of atherosclerosis and in-stent stenosis. To investigate the mechanistic basis of the GPX1-ROS1 interaction, we first measured basal levels of expression of ROS1 in tissues from experimental animals and in cell lines relevant to atherosclerosis and in-stent neointima and found that ROS1 is not expressed in vascular tissue and overall is present at very low levels in tissues and cells (Supplemental Figure 5A). In contrast, we found dramatic differences in ROS1 gene expression in human coronary artery tissue expression was increased in de novo atherosclerosis, but was significantly higher in in-stent neointima compared with control; Figure 4A). This pattern of expression is in contrast to that of GPX1 expression (Figure 1A), suggesting a counterregulatory role. We thus hypothesized that ROS1 would be upregulated following vascular injury. In keeping with human coronary artery gene expression (Figure 1A), we found Ros1 mRNA was increased nearly 150-fold in Gpx1<sup>−/−</sup> Apoe<sup>−/−</sup> vessels that had undergone stenting compared with uninjured aorta (P < 0.001; Figure 4B).

Given that we previously demonstrated reduced reendothelialization, but increased medial layer superoxide generation, in Gpx1<sup>−/−</sup> Apoe<sup>−/−</sup> vessels, we next aimed to determine the vascular cell type in which this putative interaction was constitutively active. We first measured mRNA levels of ROS1 in human coro-
nary artery endothelial cells grown under both serum-fed proliferative and serum-starved nonproliferative conditions. We found that ROS1 mRNA levels were not detectable in endothelium under either condition (Supplemental Figure 5B). Conversely, in VSMCs, we found that ROS1 was expressed at low levels in serum-fed proliferative cells, but was dramatically upregulated as these cells differentiated into a serum-starved contractile phenotype (5-fold, P < 0.05 vs. serum-fed), furthered by confluence (15-fold, P < 0.05 vs. serum-fed), and then decreased to baseline following passage. Addition of siRNA specific for ROS1 (siROS1) to serum-starved VSMCs successfully decreased mRNA levels to very low levels (P < 0.05 vs. serum-fed; Figure 4C).

ROS1 regulates VSMC migration, proliferation, and survival. We hypothesized that ROS1 might directly affect vascular remodeling via proliferation, migration, and survival of VSMCs. We derived primary aortic VSMCs from Gpx1<sup>+/−</sup> Apoe<sup>−/−</sup> mice following balloon angioplasty and found significantly higher proliferation than in Gpx1<sup>+/−</sup> Apoe<sup>−/−</sup> controls (Figure 4D). Interestingly, siROS1, the broad-spectrum RTK antagonist genistein, and the more specific ALK-MET-ROS1 inhibitor crizotinib significantly decreased proliferation in Gpx1<sup>+/−</sup> Apoe<sup>−/−</sup>, but not Gpx1<sup>+/−</sup> Apoe<sup>−/−</sup> cells (Figure 4D), indicating a dependency on perturbation of the network hub. When siROS1 and genistein or siROS1 and crizotinib were used in combination, there was no further reduction in proliferation (Figure 4D), suggesting these agents share a common pathway. To clarify that tyrosine kinase inhibition rather than oxidative quenching was the mechanism, we used a structural analogue of genistein, which has no effect on tyrosine kinase inhibition, but similar antioxidant effects. Daidzein did not inhibit proliferation in Gpx1<sup>+/−</sup> Apoe<sup>−/−</sup> VSMCs (Figure 4D). Furthermore, addition of siROS1 to daidzein-treated cells showed a reduction in proliferation similar to that in siROS1 alone (Figure 4D), further clarifying that the principal mode of action was through RTK inhibition. Inhibition of proliferation by crizotinib indicated this RTK was likely ROS1.

Migration is an important parallel process to proliferation in VSMCs. We found that migration was regulated in a manner similar to that of proliferation (Supplemental Figure 5C). Apoptosis was lower in Gpx1<sup>+/−</sup> Apoe<sup>−/−</sup> VSMCs at baseline compared with Gpx1<sup>+/−</sup> Apoe<sup>−/−</sup> (Supplemental Figure 5D). siROS1, genistein, or crizotinib with or without siROS1 in Gpx1<sup>+/−</sup> Apoe<sup>−/−</sup> VSMCs decreased apoptosis to Gpx1<sup>+/−</sup> Apoe<sup>−/−</sup> levels (Supplemental Figure 5D). These findings confirm that the proliferative and antiapoptotic functions of ROS1 as a protooncogene are active in VSMCs.

Reductive stress from intracellular accumulation of GSH activates ROS1. To explore more directly the mechanism of action linking GPX1 to ROS1, we assessed the ratio of reduced (GSH) to oxidized GSH (GSSG) (GPX oxidizes GSH to GSSG). We found GSH levels to be mildly elevated in Gpx1<sup>+/−</sup> Apoe<sup>−/−</sup> aortas compared with Gpx1<sup>+/−</sup> Apoe<sup>−/−</sup> at baseline (Figure 5A). Although we could not detect ROS1 phosphorylation in Gpx1<sup>+/−</sup> Apoe<sup>−/−</sup> mice, phosphorylated ROS1 protein was constitutively present in Gpx1<sup>+/−</sup> Apoe<sup>−/−</sup> animals (Figure 5A). When we performed balloon angioplasty in Gpx1<sup>+/−</sup> Apoe<sup>−/−</sup> mice, we found a time-dependent increase in GSH reaching nearly 10-fold higher than baseline (Figure 5B) and a corresponding increase in GSH/GSSG ratio (Figure 5C) compared with that of uninjured con-
animals that had undergone angioplasty, additionally administered GSH in vitro. Proliferation in Gpx1+/− Apoe−/− VSMCs treated with high concentrations of GSH monoethyl ester could be inhibited by siROS1, suggesting a phenotype switch toward Gpx1+/− Apoe−/− VSMCs (Figure 7). The addition of GSH to Gpx1+/− Apoe−/− cells did not affect proliferation patterns, suggesting preexisting saturation of intracellular GSH in these cells. Having found a physiological upregulation of the deglutathiolating enzyme GRX1 (Figure 6B) and complementing the effects of GSH augmentation, adenoviral overexpression of GRX1 (AdGRX1) decreased proliferation to levels similar to those in siROS1, while siRNA directed toward GRX1 (siGRX1) led to increased proliferation in VSMCs from both groups of mice (Figure 7). These findings not only support GSH reductive stress as a mediator of ROS1 activation, but also suggest protein s-glutathiolation as a potential mechanism.

Migration of VSMCs was regulated in a manner similar to proliferation (Supplemental Figure 6A). In cell apoptosis assays, addition of GSH to Gpx1+/− Apoe−/− VSMCs led to a decrease in apoptosis that could be partially reversed by the addition of siROS1 or AdGRX1 (Supplemental Figure 6B), suggesting that GSH sensitizes Gpx1+/− Apoe−/− VSMCs to ROS1 activation by s-glutathiolation, producing a phenotype similar to that of Gpx1+/− Apoe−/− cells (Supplemental Figure 6B).

s-Glutathiolation of critical SHP-2 cysteines inhibits ROS1 deactivation. We next sought to determine the mechanism of ROS1 as a redox sensor in VSMCs. Disulfide bonds in proteins are formed by oxidation of the thiol groups of cysteine residues. Specifically, GSH, which is composed of glutamate-cysteine-glycine, can react with oxidatively modified cysteine on other proteins to form a GSH conjugate: s-glutathiolation. To investigate the role of s-glutathiolation on ROS1 function, HEK293T cells were transfected with myc-tagged ROS1 plasmid. s-Glutathiolation was induced by treating cells with the oxidizing agent diamide and incubating cells with biotinylated GSH-ethyl-ester (Figure 8A). s-Glutathiolation could be inhibited by the reducing agent DTT, indicating the need for oxidative modification of Cys for s-glutathiolation to occur (Figure 8A). IP for ROS1 following s-glutathiolation did not identify ROS1 as a glutathiolated protein (Figure 8B); however, other GSS-protein conjugates between the molecular weights 55 and 90 kDa were detected (Figure 8B). To identify these proteins, we performed MS peptide mass fingerprinting of the IP and screened proteins at 55–90 kDa (Supplemental Table 4). Among the potential candidates, we identified the 68-kDa protein tyrosine phosphatases SHP-1 and SHP-2, which we and others have

We performed further in vitro studies examining proliferation, migration, and survival in primary aortic VSMCs from experimental controls. We also found a corresponding time-dependent increase in phosphorylation of ROS1 at Y2274 (Figure 5D), a phosphorylation site known to activate the kinase (21), which was in line with the time course of neointima development following vascular injury (5). Taken together, these findings suggest GSH reductive stress as a mediator of ROS1 phosphorylation and activation.

We next aimed to determine the cause of increased GSH levels in Gpx1+/− Apoe−/− mice, particularly following vascular injury. We measured expression levels of key enzymes in the GSH metabolic pathway, including those that synthesize GSH and those that utilize GSH as a substrate. We found significant increases in expression levels of the enzymes involved in GSH synthesis. GCS-heavy subunit and GCS-light subunit as well as GSH synthetase expression levels of the enzymes involved in GSH synthesis. GCS—light subunit as well as GSH synthetase increased in both Gpx1+/− Apoe−/− vessels that had undergone balloon angioplasty (Figure 6A) and human neointima from instant stenosis (Figure 6B). These findings are in keeping with the inflammation, oxidative stress, and cell proliferation that occur as a response to vascular injury known to promote the synthesis of GSH through these enzymes (22). When we measured expression levels of enzymes involved in GSH utilization, we found a significant increase in the deglutathiolating enzyme glutaredoxin 1 (GRX1) in Gpx1+/− Apoe−/− mice (Figure 6C).

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Figure 5. GPX1 deletion–dependent reductive stress activates ROS1. (A) ROS1 activity, assessed semiquantitatively, expressed as phosphorylated ROS1, was detectable in aortic lysates of Gpx1+/− Apoe−/− mice. *P < 0.05 versus Gpx1+/− Apoe−/− P-ROS1; †P < 0.05 versus Gpx1+/− Apoe−/− GSH. (B and C) Deficiency of GPX1 in Gpx1+/− Apoe−/− mice that underwent balloon angioplasty led to a time-dependent increase in intracellular GSH levels and GSH/GSSG ratios with a (D) corresponding increase in ROS1 phosphorylation indicative of increasing ROS1 activity. *P < 0.05 compared with same time point; **P < 0.05 across time points. n = 3–6/group.
previously shown regulate ROS1 function (23, 24). When s-glutathiolation was induced in HEK293T cells transfected with myc-tagged SHP-1 or SHP-2 plasmid, we noted s-glutathiolation of SHP-2 (Figure 8C) following IP.

To confirm our findings and determine the Cys sites of s-glutathiolation on SHP-2, we performed liquid chromatograph–electron spray ionization–mass spectrometry/mass spectrometry (LC-ESI-MS/MS). To maintain the physical relationship between ROS1 and SHP-2, we induced s-glutathiolation in HEK 293T cells overexpressing myc–ROS1 instead of myc–SHP-2. MS/MS confirmed s-glutathiolation of SHP-2, specifically the catalytic Cys 463 (Figure 8D) and backdoor Cys 333 (Figure 8E). Interestingly, unlike s-glutathiolation of Cys 463, which could be reversed by the addition of the potent reducing agent DTT (total ion chromatogram [TIC]; 6.0 × 10^6), s-glutathiolation of Cys 333 could not be reduced using even 0.1 M DTT (TIC 6.0 × 10^4 → 1.3 × 10^4 with DTT), suggesting an irreversible ionic interaction. Computational protein structure modeling identified s-glutathiolation of the catalytic Cys 463 as physically blocking the phosphatase active site of the enzyme (Figure 8F).

To test the functional significance of s-glutathiolation on critical cysteines in SHP-2, we performed site-directed mutagenesis of catalytic Cys 463 and the 2 backdoor cysteines, Cys 333 and Cys 367, which protect catalytic Cys 463 from irreversible oxidation (25). When Cys 367 and Cys 333 are oxidized, their physical distance favors the generation of a disulfide bridge between them, thus protecting the catalytic domain Cys 463 from oxidation. In conditions in which 1 backdoor cysteine is irreversibly oxidized or in a mixed disulfide with another protein or molecule, it is then possible for the free backdoor cysteine to interact directly with the catalytic cysteine, rendering the phosphatase inactive (26). When phosphatase activity of SHP-2 mutants was assessed, with or without the SH2 domain activator bisphosphoryl tyrosine-based activation motif (BTAM), we found that mutation of the C367S was not different from that of wild-type SHP-2. However, the C333S mutant had less than 10% phosphatase activity, and C463S was completely inactive (Figure 9A).

To test the idea that inactivation of ROS1 could modulate vascular remodeling in vivo, we administered genistein...
or crizotinib to a cohort of mice. We found that the increase in atherosclerotic burden in the aortic root (Figure 1B) of Gpx1+/+ Apoe−/− mice was abolished with the addition of genistein, but not crizotinib (Figure 10A), implying the need for vascular injury to activate ROS1. Accordingly, the increase in in-stent neointima in stented vessels (Figure 1D) in Gpx1+/+ Apoe−/− mice was abolished with the addition of genistein and crizotinib (Figure 10B) to levels observed with the mTOR inhibitor rapamycin. AdGRX1 decreased in-stent neointima to levels similar to those of crizotinib in Gpx1+/+ Apoe−/−, but not in Gpx1+/+ Apoe−/− vessels (Figure 10B), suggesting deglutathiolation of SHP-2 as the mechanism. The increase in the percentage of plaque area occupied by α-actin–positive SMCs was also abolished in crizotinib-treated Gpx1+/+ Apoe−/− mice, confirming the effect on VSMCs (Supplemental Table 2). Although overall neointimal cell number was higher in Gpx1+/+ Apoe−/− mice, there was no difference in cell density when expressed per unit area between groups. However addition of genistein, crizotinib, or rapamycin significantly decreased cell density per unit area in both experimental groups, but AdGRX1 treatment only decreased cell density in Gpx1+/+ Apoe−/− mice (Figure 10B), implicating an effect of both ROS1 inhibition and quiescent uninjured cells, but dramatically upregulated and activated following cell injury, leading to a proliferative antiapoptotic phenotype in VSMCs, but not endothelium. Intracellular accumulation of GSH as a result of GPX1 deficiency led to a time-dependent, dose-dependent increase in ROS1 activity. While in vivo ROS1 was upregulated following BAS, pharmacological inhibition of ROS1 abolished this increase and the greater atherosclerosis and ISS previously demonstrated in Gpx1+/+ Apoe−/− mice. Together, these findings provide evidence that GPX1 is a critical regulator of the response to vascular injury and that VSMC ROS1 is a major determinant of this effect.

Systems approaches can be powerful enablers of hypothesis generation in biology. Network models exhibiting scale-free topology identify disproportionately important roles for highly connected hubs. Hub genes revealed in this way represent attractive targets for biological and therapeutic exploration (29). Here, we took advantage of the availability of human tissue derived from coronary atherectomy to build blood vessel–specific network models identifying GPX1 as a hypothetical mediator of disease. While powerful, such large scale unbiased approaches are optimal as tools of hypothesis generation. The incorporation of a priori knowledge in our analysis helps strengthen network connections for which prior literature exists while incorporating novel associations found in the tissue of interest. Thus, network approaches represent a starting point, and further elucidation of biological mechanisms is critical to confirming the significance of the identified associations.

Consistent with such systems interactions, vascular cellular homeostasis depends on a balanced redox environment. Simplistic concepts of “oxidative stress” have failed to deliver therapeuti-
predicted a physical obstruction of the phosphatase site of SHP-2 by s-glutathiolation, which was corroborated by our site-directed mutagenesis experiments showing a loss of SHP-2 function with perturbation of the backdoor Cys 333, but not Cys 367. These 2 backdoor cysteines protect the catalytic Cys 463 from oxidative modification (25). When Cys 367 and Cys 333 are oxidized, their physical distance favors the generation of a disulfide bridge between the two of them, protecting the catalytic domain Cys 463 from oxidation (25). In conditions in which one backdoor cysteine is irreversibly oxidized or in a mixed disulfide with another protein or molecule, it is then possible for the free backdoor cysteine to interact directly with the catalytic cysteine, rendering the phosphatase inactive (26). Our findings that s-glutathiolation of Cys 463 was reversible, while that of Cys 333 was not, support this mechanism and suggest that irreversible oxidation of Cys 333 leads the catalytic Cys 463 to be prone to s-glutathiolation.

Several features of ROS1 make it an attractive candidate for potential therapeutic intervention in vascular remodeling. ROS1 encodes a type I integral membrane protein with RTK activity (20). The ectodomain of ROS1 has high-sequence homology to the extracellular matrix protein fibronectin present in cell surface and extracellular matrix proteins, such as cell-adhesion molecules (20). The distinctive structure of the cell-binding ectodomain with intracellular kinase domain suggests a unique capability of ROS1 to transmit cell adhesion to intracellular signaling. As cell adhesion is critical to the pathogenesis of vascular remodeling, the structural properties of ROS1 make it attractive as a candidate for a role in such processes. From a clinical standpoint, ROS1 has been described predominantly in the setting of chromosomal translocations creating gene fusions in highly malignant cancers (33–38). ROS1 is an attractive therapeutic target, not only because it drives proliferation, but also because of its preferential expression. In humans, ROS1 expression is undetectable in most tissues except heart, skeletal muscle (39), and lung (20). At a cellular level, we show that ROS1 is expressed in VSMCs, but not endothelium. Current DES have reduced the problem of in-stent stenosis by targeting all proliferating cells using chemotherapeutic agents, but the beneficial reductions in neointima formation are accompanied by impaired endothelial regeneration and vascular healing that has created a host of new concerns, including stent thrombosis (40), neointimal atherosclerosis (41), and localized hypersensitivity reactions leading to peri-strut cavity and aneurysm formation (28). Our findings that crizotinib inhibited in-stent stenosis without affecting reendothelialization could thus have a major clinical impact.

In conclusion, we have shown that GPX1 critically modulates neointimal hyperplasia following vascular injury mediated by activation of ROS1. Our study identifies GSH-dependent activation of ROS1 as a critical mediator of SMC proliferation and migration, both major determinants of vessel remodeling following vascular injury. The identification of a redox mechanism with specific effects in vascular disease pathogenesis highlights the need for targeted rather than broad-spectrum therapies.

Methods
For detailed experimental procedures, see Supplemental Experimental Procedures.
Animals. The 18- to 22-week-old male and female mice were maintained in temperature-controlled (20°C to 22°C) cages with a 12-hour light/12-hour dark cycle. Sterile water and standard chow diet were available ad libitum. Mice with ubiquitous homozygous deletion of Gpx1 (Gpx1–/–) crossed with ApoE-null (Gpx1–/– ApoE–/–) or Gpx1+/+ ApoE–/– controls were used for experiments. For endothelial survival and regeneration experiments, these mice were further crossbred with transgenic Tie2-LacZ mice expressing β-gal localized to the nucleus of endothelial cells (42), producing experimental Gpx1+/– ApoE–/– Tie2-LacZ– and Gpx1+/+ ApoE–/– Tie2-LacZ+ mice. All experiments were performed blind. Following genotyping and prior to experiments, animals were placed in cages labeled A and B. Subsequently, all experiments were performed by a group by investigators not involved in the genotyping or blinding. Experiments were unblinded after completion of data analysis.

Gene expression analysis. RNA preparation, hybridization to microarray, data processing, and data analysis for microarray performed on human coronary artery atherectomy specimens or mouse aorta (n = 30) were performed as described previously (14). All original microarray data were deposited in the NCBI’s Gene Expression Omnibus (GDS1597).

Network analysis. To determine molecular targets to investigate restenosis, we performed comprehensive histological and gene network analysis of human in-stent stenosis as described previously (14) using Cytoscape (43).

Tissue GSH and cellular GPX1 activity. Tissue concentrations of GSH (total, reduced, and oxidized) were measured in aortic homogenates after deproteinization with metaphosphoric acid in an enzymatic recycling method using GSH reductase, as described (44).

BAS procedure. BAS was performed in 18- to 22-week-old mice as described previously (5). In order to be able to track endothelial cells by in situ hybridization for the Y chromosome, stent grafts were constructed using female donors and male recipient mice.

Gene transfer. Freshly harvested vessels were incubated in a total of 1 × 1011 viral particles of AdGRX1 or AdGFP in 300 μl for 30 minutes at 37°C prior to implantation.
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**Figure 10. Increased in-stent stenosis in Gpx1−/− Apoe−/− mice is reversed by ROS1 inhibition.** (A) Relative area of atherosclerosis in the aortic root was significantly increased in Gpx1−/− Apoe−/− mice, but addition of genistein to inhibit ROS1 abolished this increase, while crizotinib did not. Scale bar: 50 μm. (B) Gpx1−/− Apoe−/− mice 4 weeks following BAS developed significantly greater neointimal hyperplasia and in-stent stenosis compared with control Gpx1+/+ Apoe−/−. Addition of genistein or crizotinib decreased neointimal hyperplasia to levels observed with the mTOR inhibitor rapamycin. AdGRX1 decreased neointimal hyperplasia to levels similar to those seen with crizotinib in Gpx1−/− Apoe−/− vessels, but not Gpx1+/+ Apoe−/−; suggesting deglutathiolation of SHP-2 as the mechanism. Scale bar: 50 μm. Black arrowheads denote neointima. Although overall neointimal cell density was higher in Gpx1−/− Apoe−/− mice, there was no difference in cell density when expressed per unit area between groups; however, addition of genistein, crizotinib, or rapamycin significantly decreased cell density per unit area in both experimental groups. Treatment with AdGRX1 reduced cell density only in Gpx1−/− Apoe−/− mice. *P < 0.05 compared with control; †P < 0.05 compared with AdGFP. n = 8–15/group. (C) Assessment of reendothelialization revealed that the ROS1 inhibitor crizotinib did not impair endothelial regeneration 4 weeks following BAS, while rapamycin did in both Gpx1−/− Apoe−/− Tie2-LacZ+ mice and control Gpx1+/+ Apoe−/− Tie2-LacZ+ mice. *P < 0.05 versus crizotinib. n = 6–10 mice/group. Gray arrowheads denote neointima. Black arrowheads denote LacZ-positive endothelial cells. Scale bar: 50 μm.

**Tissue preparation, histology, and lesion quantification.** Mice were euthanized 28 days following the stent procedure. Aorta and stented vessels were excised, fixed in paraformaldehyde overnight, dehydrated in graded ethanol solution, and paraffin embedded or removed fresh and snap-frozen in either Trizol, OCT, or maintained fresh. Histomorphometric analysis of aortic atherosclerosis lesion area and stented vessel processing and analysis were performed as previously described (5). Immunohistochemistry was performed as described previously (45).

**En face X-gal staining.** The procedure for en face preparation was similar to that described previously (45).

**Oxidative fluorescent microtopography.** Superoxide was detected in the layers of the vessel wall using fluorescent probe DHE as described (45).

Lucigenin and luminol-enhanced chemiluminescence. Total aortic superoxide was measured by lucigenin-enhanced chemiluminescence, as described previously (45).

**Electron paramagnetic resonance spectroscopy.** Electron paramagnetic resonance (EPR) spectroscopy was used to quantify vascular NO production as described previously (45).

**Cell culture.** Human coronary artery SMCs were propagated in SmGM-2 growth medium containing 5% FBS. To induce growth arrest and the expression of differentiation genes, SMCs were serum starved in basal medium (SmBM) for 72 hours, according to conventional protocols (45). In vitro ROS1 inhibition studies were completed by adding 40 μM genistein to the cell-culture media. In some experiments, cells were treated with 0.5 mM GSH monoethyl ester (Sigma-Aldrich). For overexpression of Grx1, cells were incubated with adenovirus overexpressing Grx1 (25 MOI) at a concentration of 4.5 × 10^10 pfu/ml.

**Proliferation and motility assays.** Proliferation and migration experiments were performed as described (46).

**Survival assay.** Survival assays were performed using caspase 3/7 assays as described previously (46).

**Quantitative real-time PCR.** Total RNA was extracted from cells or snap-frozen tissue and used for quantitative real-time PCR as described previously (47).

**Immunoprecipitation and immunoblotting.** Human coronary artery SMCs (HCASMCs) and aorta/stented vessel lysates (n ≥ 5 per group) were used for immunoprecipitation and immunoblotting performed using a previously described method (45).

**Biotinylated GSH ester and detection of s-glutathiolated proteins.** Protein s-glutathiolation was detected in tissues and in cells as described previously (48).

**MS.** Samples were digested under reducing and nonreducing conditions using trypsin. Nano LC-MS was employed using in-house methods.
Figure 11. Model of ROS1 activation by reductive stress. In VSMCs, vascular injury induces activation of ROS1 through either a yet-unidentified ligand (ligand X) or endogenous phosphorylation. (A) Concomitant vascular injury also leads to decreased transcription of GPX1 (i), which leads to a decrease in GPX1 enzymatic activity during a time of increased GSH synthesis, resulting in increased intracellular GSH (ii). Under oxidative conditions, high GSH leads to s-glutathiolation of proteins, including the ROS1 regulatory protein tyrosine phosphatase SHP-2 (iii). (B) s-Glutathiolation deactivates SHP-2, which in turn leads to sustained phosphorylation and activation of ROS1. Sustained phosphorylation of ROS1 amplifies its ability to activate kinases involved in proliferation, migration, and apoptosis through downstream target phosphorylation (iv).

pulled or packed C18 reverse-phase analytical columns. The mass spectrometer was an LTQ Orbitrap Velos set to perform collision-induced dissociation, higher-energy C trap dissociation, or electron-transfer dissociation on multiple charged cations.

**Modeling of s-glutathiolation by protein-ligand flexible docking.** To visualize s-glutathiolation of critical SHP-2 cysteines, structural docking was performed on the binding site of interest with GSH using Swiss Dock (49) and the docking poses were refined using GROMACS (50). A final selection of glutathiolated protein-ligand–binding modes was done by scoring refined docking poses with a knowledge-based scoring function (51), which fulfilled the criterion for s-glutathiolation.

**Lipid and lipoprotein analysis.** Total plasma cholesterol and triglyceride concentrations were measured using enzymatic assay (Roche) on a Cobas Mira Plus automated analyzer (Roche).

Single nucleotide variant information gain analysis. Attribute selection analysis was performed in the Weka (Java) environment (52), using all SNVs as attributes and using an information gain evaluator conditioned on the presence or absence of in-stent restenosis with Ranker as the attribute search method.

**SNV interaction analysis.** SNV interaction analysis was performed using the Polymorphism Interaction Analysis (PIA) tool (v 2.0, available at http://www3.cancer.gov/intra/lhc/PIA2-distribution.zip) (53), which utilizes several different methods, including percentage correct, sensitivity plus specificity, PPV plus NPV, risk ratio, odds ratio, Gini index, and absolute probability difference, to evaluate for interactions between SNP combinations in case-control genotyping association studies. A 2-SNP interaction model was used for the analysis.

**Linear regression model.** Fitting of a generalized linear regression model was done in R (http://www.r-project.org/), conditioning the presence or absence of in-stent stenosis on a score consisting of the number of at-risk alleles for both GPX1 and ROS1, with a minimum of 0 and a maximum of 3 (no member of the cohort had 4 at-risk alleles).

**GWAS meta-analysis.** A meta-analysis was performed combining 4 separate cohorts (Cardiogene, GeneBank, deCODE, and GENDER) of cases and controls comparing patients after BAS who were found to have in-stent stenosis with those without in-stent stenosis. Cases of in-stent stenosis were clinically adjudicated, and patients were genotyped according to the protocols of each individual study. Statistical analysis was performed using inverse variance fixed effects meta-analysis, implemented in METAL (54). Genomic control correction was applied at the level of each cohort and then again at the meta-analysis level (double genomic control correction).

**Statistics.** Data are presented as mean ± SEM. Data were subjected to the Kolmogorov-Smirnov test to determine distribution. Groups were compared using the Mann-Whitney U test for nonparametric data or 2-tailed Student’s t test for normally distributed data. When comparing multiple groups, data were analyzed by ANOVA with Newman-Keuls post-hoc test for parametric data or Kruskal-Wallis test with Dunn’s post-hoc test for nonparametric data. A P value of less than 0.05 was considered significant.

**Study approval.** All animal studies were approved by the Stanford University or Columbia University Administrative Panel on Laboratory Animal Care (protocol 22922 and AAAE9200) and conform to NIH guidelines (Guide for the Care and Use of Laboratory Animals. NIH publication no. 85-23. Revised 1996). All human studies were performed with written informed consent and with the approval of the Stanford University Institutional Review Board.

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