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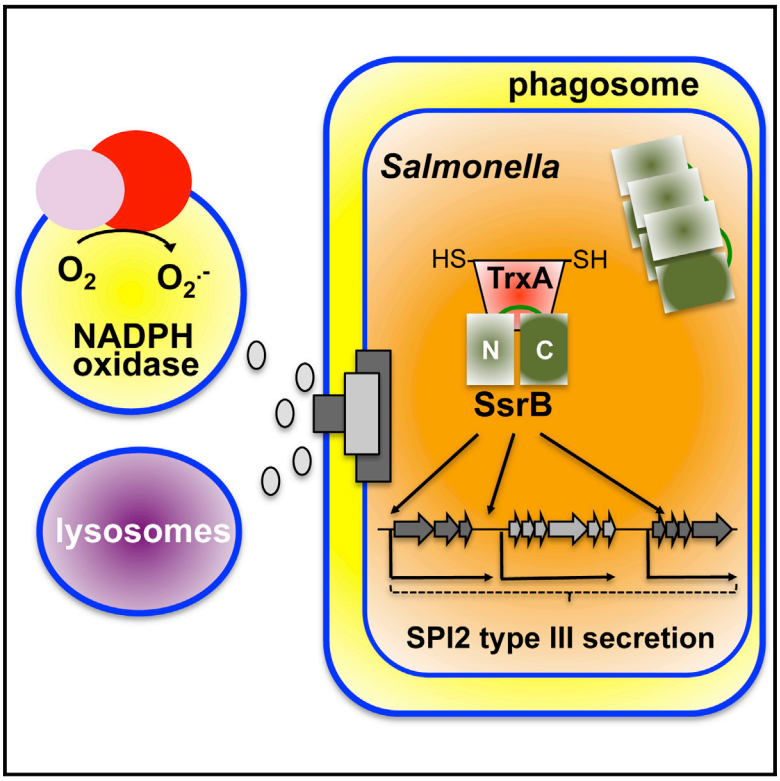
Graphical Abstract



Authors

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In Brief

Song et al. find that thioredoxin promotes expression of the SPI2 type III secretion to help *Salmonella* survive oxidative stress caused by NADPH phagocyte oxidase. Independent of its canonical thiol-disulfide oxidoreductase enzymatic activity, thioredoxin regulates SsrB post-translationally, thereby activating antioxidant defenses associated with SPI2.

Highlights

- Thioredoxin defends *Salmonella* against the NADPH phagocyte oxidase
- Thioredoxin promotes antioxidant defense by facilitating SPI2 transcription
- Thioredoxin binds to the SsrB linker, stabilizing this SPI2 response regulator
- Thioredoxin regulates SsrB independently of its CXXC catalytic motif

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Antioxidant Defense by Thioredoxin Can Occur Independently of Canonical Thiol-Disulfide Oxidoreductase Enzymatic Activity

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SUMMARY

The thiol-disulfide oxidoreductase CXXC catalytic domain of thioredoxin contributes to antioxidant defense in phylogenetically diverse organisms. We find that although the oxidoreductase activity of thioredoxin-1 protects *Salmonella enterica* serovar Typhimurium from hydrogen peroxide in vitro, it does not appear to contribute to *Salmonella's* antioxidant defenses in vivo. Nonetheless, thioredoxin-1 defends *Salmonella* from oxidative stress resulting from NADPH phagocyte oxidase macrophage expression during the innate immune response in mice. Thioredoxin-1 binds to the flexible linker, which connects the receiver and effector domains of SsrB, thereby keeping this response regulator in the soluble fraction. Thioredoxin-1, independently of thiol-disulfide exchange, activates intracellular SPI2 gene transcription required for *Salmonella* resistance to both reactive species generated by NADPH phagocyte oxidase and oxygen-independent lysosomal host defenses. These findings suggest that the horizontally acquired virulence determinant SsrB is regulated post-translationally by ancestrally present thioredoxin.

INTRODUCTION

All aerobic, and many anaerobic, organisms experience oxidative stress at some point in their lifetime. Univalent or divalent reduction of molecular oxygen in the electron transport chain or in the flavin prosthetic groups of cytosolic enzymes are sources of endogenous oxidative stress (Boveris and Chance, 1973; Husain et al., 2008; Korshunov and Imlay, 2010). Steady-state oxidative stress resulting from these metabolic processes is, nonetheless, overshadowed by the high flux of reactive oxygen species (ROS) synthesized by the multisubunit NADPH phagocyte oxidase during the respiratory burst in macrophages and neutrophils (Babior, 1999). *Salmonella enterica* are able to survive activity of this flavohemoprotein in polymorphonuclear and mononuclear phagocytes (Burton et al., 2014; Vázquez-Torres et al., 2000a). The respiratory burst produced by the NADPH phagocyte oxidase is essential to the host defense against salmonellosis, as demonstrated by the prevalence of *Salmonella* infections in chronic granulomatous disease patients bearing autosomal or X-linked mutations in cytosolic and membrane-bound components of this enzymatic complex (Mouy et al., 1989). Mice deficient in the gp91*phox* or p47*phox* subunits of the NADPH phagocyte oxidase recapitulate the hypersusceptibility of patients with chronic granulomatous disease to *Salmonella* infection (Burton et al., 2014; Mastroeni et al., 2000; van Diepen et al., 2002).

Salmonella employ multiple strategies to combat oxidative stress resulting from NADPH phagocyte oxidase activity. Periplasmic Cu-Zn superoxide dismutase SodCI, glutathione, and the ABC-type efflux pump MacAB defend this enteropathogen against cytotoxicity resulting from NADPH phagocyte oxidase (Bogomolnaya et al., 2013; De Groote et al., 1997; Song et al., 2013). In addition, the type III secretion system, encoded by the *Salmonella* pathogenicity island 2 (SPI2), reduces contact between *Salmonella* vacuoles and NADPH phagocyte oxidase-containing vesicles (Berger et al., 2010; Gallois et al., 2001; Vázquez-Torres et al., 2000b), thereby helping this bacterium maintain intracytoplasmic redox homeostasis in macrophages (van der Heijden et al., 2015). Despite the benefits associated with these antioxidant defenses, *Salmonella* suffer oxidative stress in phagocytic cells (Burton et al., 2014). Hydrogen peroxide (H_2O_2) is a critical effector of oxidative stress engendered in the respiratory burst of mononuclear phagocytes (Vázquez-Torres et al., 2000a). H_2O_2 leads to DNA double-strand breaks in a ferrous iron-dependent manner. In addition to this mode I killing, H_2O_2 oxidizes both Feα of [4Fe-4S] prosthetic groups in dehydratases and thiol groups in cysteine residues of target proteins (Imlay, 2003). Disulfide bond formation between neighboring cysteine residues is a common H_2O_2-mediated modification. Thioredoxins and cognate thioredoxin reductases help maintain thiol-disulfide redox homeostasis (Holmgren, 1989). Thioredoxin-1 increases *Salmonella* fitness in a murine model of salmonellosis, but it does not seem to protect this

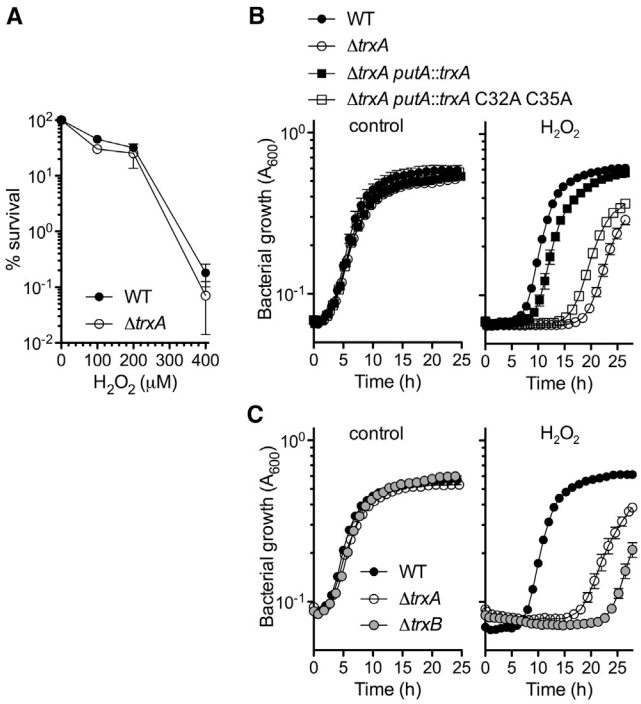

Figure 1. Thioredoxin-1-Mediated Resistance to H_2O_2

(A) The percentage survival of wild-type (WT) and Δ*trxA Salmonella* was calculated 2 hr after exposure to increasing concentrations of H_2O_2.

(B and C) Growth (A_{600}) of *Salmonella* in high-Mg^{2+} N salts medium in the presence of 100 μM H_2O_2. PBS was used as control. The data are the mean ± SD of five independent experiments.

See also Figure S1.

enteropathogen from H_2O_2 killing (Bjur et al., 2006). It remains unknown if thioredoxin-1 is a component of *Salmonella*'s antioxidant toolbox.

Members of the highly conserved thioredoxin family contain two catalytic cysteine residues within the canonical CXXC sequence motif. During thiol-disulfide exchange, the catalytic cysteine residues in the CXXC sequence motif become oxidized. The resulting disulfide bond is reduced by thioredoxin reductase, a flavoprotein that is powered by electrons from NADPH (Arnér and Holmgren, 2000). Thioredoxin-mediated thiol-disulfide exchange reactions control the activity of a range of biomolecules, including ribonucleotide reductase, phosphoadenosine-phosphosulfate reductase, methionine sulfoxide reductase, and arsenate reductase (Holmgren, 1989). Thioredoxin-1 has been shown to regulate *Salmonella*'s SPI2 type III secretion system (Negrea et al., 2009). How thioredoxin-1 controls SPI2 expression is currently unclear. Here we find that thioredoxin-1 promotes antioxidant defense of *Salmonella* against NADPH phagocyte oxidase-mediated oxidative stress in vivo independently of classical thiol-disulfide oxidoreductase. Specifically, we find that thioredoxin-1, independent of its canonical thiol-disulfide oxidoreductase enzymatic activity, binds to and stabilizes the SPI2 master regulator SsrB, thereby helping *Salmonella* survive the antimicrobial activity of NADPH phagocyte oxidase activated during the innate immune response in primary macrophages and mice.

RESULTS

The Thioredoxin System Protects *Salmonella* from the Bacteriostatic Activity of H_2O_2

Despite its well-documented contributions to antioxidant defense (Carmel-Harel and Storz, 2000), thioredoxin-1 has yet to be identified as an important component of the antioxidant arsenal of *Salmonella*. In agreement with previous published data (Bjur et al., 2006), our investigations showed similar susceptibility of wild-type and *trxA* mutant *Salmonella* to H_2O_2 killing (Figure 1A). Together, these investigations indicate that thioredoxin-1 does not protect *Salmonella* against the genotoxicity associated with mode I H_2O_2 killing (Imlay and Linn, 1986). Neither does thioredoxin-1 appear to defend *Salmonella* against the thiol-oxidizer diamide (Figure S1A) or superoxide-mediated cytotoxicity of the redox-cycling drug menadione (Figure S1B). Although the NADPH phagocyte oxidase predominantly kills *Salmonella* during the initial phases of the infection, bacteriostasis appears to be the dominant antimicrobial activity associated with this flavohemoprotein as the infection proceeds (Grant et al., 2008).

We therefore developed an in vitro system to test the effects of low concentrations of H_2O_2 on *Salmonella* growth. The addition of 100 μM H_2O_2 to exponentially growing *Salmonella* delayed bacterial replication to about 5 hr (Figure 1B, right panel). In contrast, 100 μM H_2O_2 extended the lag phase of Δ*trxA Salmonella* by about 15 hr. The profound H_2O_2-mediated cytotoxicity of thioredoxin-1-deficient *Salmonella* was reversed upon expression of *trxA* from the *put* intergenic region of the bacterial chromosome. The thiol-disulfide exchange reaction catalyzed by the thiolate of the attacking Cys^{32} and the thiol group of resolving Cys^{35} is responsible for thioredoxin-1 antioxidant defenses (Arnér and Holmgren, 2000). To test whether thioredoxin-1 thiol-disulfide exchange antagonizes H_2O_2 bacteriostasis, Δ*trxA Salmonella* were complemented with enzymatically inactive *trxA* C32A C35A. In contrast to the isogenic strain expressing the wild-type gene, *Salmonella* expressing the *trxA* C32A C35A allele were hypersusceptible to the bacteriostatic activity of 100 μM H_2O_2. These data indicate that the thioredoxin-1-mediated protection of *Salmonella* against the bacteriostatic activity of H_2O_2 relies on the thiol-disulfide exchange system. Further supporting this idea, a strain deficient in the *trxB*-encoded thioredoxin reductase, a flavoprotein that reduces disulfide-bonded thioredoxin-1, also was hypersusceptible to the bacteriostatic activity of 100 μM H_2O_2 (Figure 1C).

Thioredoxin-1 Protects *Salmonella* from the Antimicrobial Activity of the NADPH Phagocyte Oxidase

We used a C57BL/6 murine model of infection to begin testing the hypothesis that thioredoxin-1 protects *Salmonella* from ROS generated by the NADPH phagocyte oxidase (Vázquez-Torres et al., 2000a). C57BL/6 mice survived an oral challenge with Δ*trxA Salmonella* but succumbed to infection with an isogenic wild-type control (Figure 2A). The Δ*trxA* mutant became as virulent as wild-type controls in immunodeficient mice lacking the gp91*phox*-encoded membrane-bound subunit of the NADPH phagocyte oxidase (Figure 2B). The Δ*trxA* mutant, however, remained attenuated in inducible nitric oxide (NO) synthase

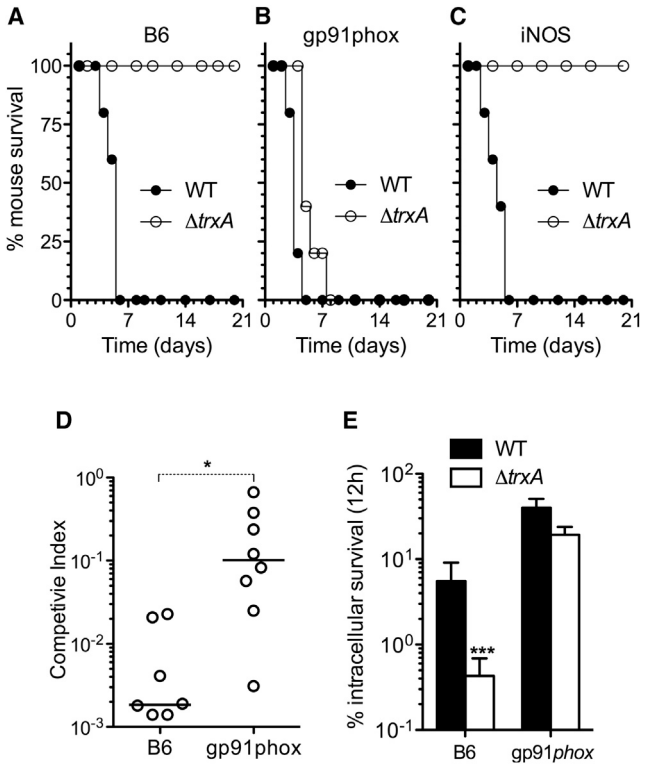

Figure 2. Thioredoxin-1 Protects *Salmonella* against the Antimicrobial Activity of the NADPH Phagocyte Oxidase

(A–C) C57BL/6 (B6) and congenic gp91*phox*- or iNOS-deficient mice were challenged orally with ~3 × 10^6 CFUs/mouse of WT or Δ*trxA Salmonella*. The survival of *Salmonella*-infected mice was scored over time. According to log-rank, Mantel-Cox survival test, Δ*trxA Salmonella* are attenuated ($p < 0.001$) in B6 and iNOS-deficient mice.

(D) The competitive index was measured in the livers of C57BL/6 and gp91*phox*-deficient mice 48 hr after i.p. inoculation with 500 CFUs of a mixture containing equal numbers of Δ*trxA*::*km* and WT *Salmonella* (*$p < 0.5$).

(E) Survival of *Salmonella* in periodate-elicited peritoneal macrophages (***$p < 0.001$). The data are the mean ± SD of nine biological repeats done on three independent days.

(iNOS)-deficient mice unable to synthesize NO in response to this intracellular pathogen (Figure 2C).

To analyze further the role played by thioredoxin-1 on antioxidant defense, the competitive index of wild-type and Δ*trxA*::*km Salmonella* was defined in an intraperitoneal (i.p.) model of acute infection. The number of Δ*trxA*::*km Salmonella* recovered from the livers of C57BL/6 mice 48 hr after infection was 100- to 1,000-fold lower when compared to wild-type controls. The competitive disadvantage of the Δ*trxA Salmonella* was greatly diminished in gp91*phox*-deficient mice (Figure 2D). These investigations indicate that thioredoxin-1 contributes to the antioxidant defenses that protect *Salmonella* against the enzymatic activity of the NADPH phagocyte oxidase during infection but does not seem to be critical to the antinitrosative arsenal of this intracellular pathogen. In vivo, *Salmonella* are exposed to the antimicrobial activity of the NADPH phagocyte oxidase in macrophages (Burton et al., 2014). Thus, we examined the survival of Δ*trxA Salmonella* in periodate-elicited peritoneal macrophages from C57BL/6 mice known to sustain a respiratory burst in response to *Salmonella* (Vázquez-Torres et al., 2000a). *Salmonella* lacking *trxA* survived 10-fold less than wild-type controls in macrophages from C57BL/6 mice (Figure 2E). When compared to immunocompetent cells, a higher burden of wild-type *Salmonella* was recovered from gp91*phox*-deficient macrophages. Importantly, the Δ*trxA Salmonella* survived as well as wild-type *Salmonella* in gp91*phox*-deficient macrophages. These results demonstrate that thioredoxin-1 protects *Salmonella* from the oxidative stress engendered within host cell macrophages upon the assembly of a functional NADPH phagocyte oxidase.

Salmonella Virulence Is Co-dependent on Thioredoxin-1 and the SPI2 Type III Secretion System

The SPI2 type III secretion system lessens exposure of *Salmonella* to the antimicrobial activity of the NADPH phagocyte oxidase (Berger et al., 2010; Gallois et al., 2001; van der Heijden et al., 2015; Vázquez-Torres et al., 2000b). To start investigating whether the contribution of thioredoxin-1 to *Salmonella* antioxidant defenses depends on SPI2, we compared the antimicrobial activity of primary macrophages against *trxA*-deficient *Salmonella* or a mutant lacking the *spiC* gene encoding a structural component and effector of the SPI2 type III secretion system (Uchiya et al., 1999; Yu et al., 2002). Both of these mutants showed similar hypersusceptibility to the bactericidal activity associated with the respiratory burst of primary macrophages (Figure 3A). To quantify the interdependence of thioredoxin-1 and SPI2 in *Salmonella* pathogenesis, we performed competition assays between a Δ*spiC* Δ*trxA* double mutant and Δ*spiC* or Δ*trxA* single mutants in an i.p. model of acute *Salmonella* infection (Figure 3B). These investigations showed that the Δ*spiC* Δ*trxA* double mutant was recovered from C57BL/6 mice in similar numbers to Δ*trxA* or Δ*spiC* single mutant *Salmonella*, suggesting that the contributions of thioredoxin-1 and SPI2 to *Salmonella* pathogenesis are co-dependent.

In addition to antioxidant defense, the SPI2 type III secretion system contributes to the intracellular lifestyle of *Salmonella* by interfering with lysosomal trafficking and, thus, promoting intracellular replication (McGourty et al., 2012; Uchiya et al., 1999). To determine whether thioredoxin-1 adds to the defenses of *Salmonella* against ROS-independent host responses, the intracellular replication of Δ*trxA* or Δ*spiC Salmonella* were measured in two macrophage-like murine cell lines. Whereas wild-type *Salmonella* replicated about 70- to 100-fold 18 hr after infection, Δ*trxA* and Δ*spiC* mutants replicated poorly in J774 (Figures 3C and 3D) and RAW cells (Figure S2A). The intracellular growth defect of *ΔtrxA Salmonella* could be complemented in *trans* with *trxA*, but not the related *trxC*-encoded thioredoxin-2 (Figure 3C). The bacteriostatic activity exerted by J774 and RAW cells against Δ*trxA Salmonella* is unlikely to be mediated by the NADPH phagocyte oxidase, because the J744 and RAW cells used in the course of these investigations do not produce superoxide in response to *Salmonella* (Figures S2B and S2C). As the SPI2 type III secretion system promotes intracellular replication by preventing fusion of *Salmonella* phagosomes with lysosomes (McGourty et al., 2012), we measured the interactions of vacuoles containing Δ*trxA Salmonella* with mannose-6P receptor$^+$ (MPR$^+$) lysosomes. To facilitate visualization of vesicular

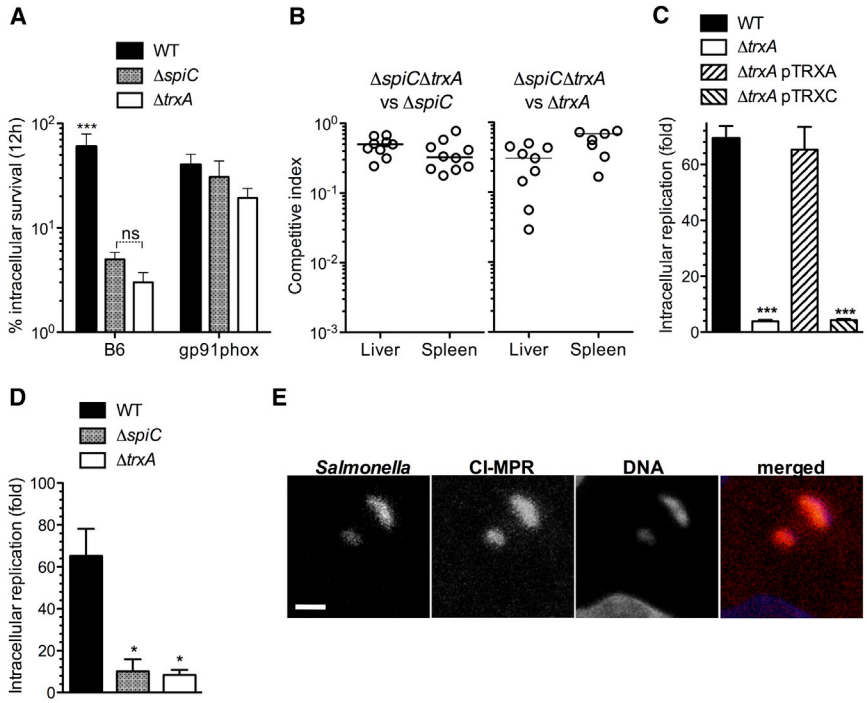

Figure 3. Co-dependence of Thioredoxin-1 and the SPI2 Type III Secretion System

(A) Survival of WT and mutant *Salmonella* in primary macrophages from C57BL/6 (B6) or gp91*phox*-deficient mice 12 hr after challenge is shown ($p < 0.001$ when compared to *spiC* or *trxA* mutant controls).

(B) Competitive index of Δ*spiC* Δ*trxA*::*km*, Δ*trxA*, and Δ*spiC Salmonella*. The competitive index in livers and spleens was estimated 7 days after C57BL/6 mice ($n = 7–10$) were challenged i.p. with $\sim 10^5$ CFUs of an equal mixture of the indicated strains.

(C and D) Intracellular growth of Δ*trxA* (C) and Δ*spiC* (D) *Salmonella* in J774 cells. The effect of *trxA*- or *trxC*-expressing pTRXA or pTRXC plasmids on the intracellular growth of Δ*trxA Salmonella* also was evaluated. The data are the mean ± SD of three independent experiments (*$p < 0.05$).

(E) Confocal microscopy of GFP-expressing Δ*trxA Salmonella* and CI-MPR$^+$ lysosomes in HeLa cells. In the merged panel, *Salmonella*, lysosomes, and cell host nucleus are seen in green, red, and blue, respectively. White scale bar, 1 μm.

See also Figure S2 and Movies S1, S2, and S3.

trafficking, these investigations used HeLa cells (McGourty et al., 2012). *Salmonella* lacking *trxA* colocalized with MPR$^+$ lysosomes (Figure 3E; Movie S1). A *spiC*-deficient strain, but not the wild-type isogenic control, also colocalized with MPR$^+$ lysosomes (Movies S2 and S3). Collectively, these findings indicate that thioredoxin not only boosts *Salmonella* antioxidant defenses but also seems to help this intracellular pathogen avoid terminal stages of the degradative pathway. In addition, our investigations suggest that the thioredoxin-mediated resistance of *Salmonella* to oxygen-dependent and -independent innate responses relies on SPI2.

Thioredoxin Optimizes Intracellular SPI2 Expression

To get insights into a possible relationship between SPI2 and thioredoxin-1, we compared the intracellular SPI2 expression supported by wild-type and Δ*trxA* mutant *Salmonella*. The absence of *trxA* resulted in decreased intracellular expression of both the SPI2 effector *sifA* (Figure 4A) and the structural SPI2 gene *ssaG* (Figure 4B). These findings suggest that thioredoxin-1 affects proper SPI2 function.

SsrB Is a Substrate of Both Thioredoxin-1 Thiol-Disulfide Oxidoreductase-Dependent and -Independent Activities

The SsrB response regulator, which controls overall SPI2 transcription, has a redox-active cysteine at position 203 that is susceptible to oxidation (Husain et al., 2010). We therefore tested the possibility that SsrB Cys^{203} could be a substrate of thioredoxin-1. To identify possible interactions between TrxA and SsrB, we performed tandem affinity purification using a C-terminal fusion of thioredoxin-1 with a calmodulin-binding peptide followed by tobacco etch virus protease cleavage site and Protein A. Tandem affinity purification showed that the TrxA C35A variant, which is unable to resolve mixed disulfides, and to a lesser extent TrxA C32A interact with SsrB in stationary phase *Salmonella* (Figure 5A). These investigations raise the interesting possibility that thioredoxin-1 may regulate SPI2 transcription through its interactions with the response regulator SsrB. The interaction of full-length SsrB and thioredoxin-1 was further examined in vitro with recombinant proteins (Figure S3). Utilization of GST-SsrB as bait, but not GST, showed a direct interaction between SsrB and TrxA (Figure 5B).

Cys^{203} in the dimerization domain of SsrB undergoes S-nitrosation after exposure of *Salmonella* to reactive nitrogen species (Husain et al., 2010). Therefore, we investigated whether oxidized SsrB serves as substrate of thioredoxin-1 thiol-disulfide oxidoreductase activity. A fragment containing the C-terminal domain of SsrB dimerized upon exposure to 250 μM H_2O_2 (Figure 5C). The addition of 25 μM TrxA, but not the TrxA C32A C35A variant, resolved the oxidized SsrB homodimer, demonstrating that the disulfide bond formed between SsrB Cys^{203} and SsrB Cys$^{203'}$ is a substrate of thioredoxin-1 thiol-disulfide oxidoreductase activity. The biological relevance of the interaction of disulfide-bonded SsrB and thioredoxin-1 remains unknown. The thiol-disulfide oxidoreductase-dependent and -independent interactions of thioredoxin-1 with full-length SsrB were studied further with recombinant proteins in vitro (Figure 5D). These studies showed interactions of SsrB with both TrxA and TrxA C32A C35A (Figure 5D), suggesting that thioredoxin-1 can bind to SsrB independently of its CXXC catalytic motif. To gain more insights into the binding of TrxA and SsrB, we constructed a bacterial two-hybrid system that reconstitutes the enzymatic activity of adenylate cyclase through the interactions of T18-SsrB and T25-TrxA fusions. The bacterial two-hybrid system confirmed direct binding between SsrB and TrxA (Figure 5E).

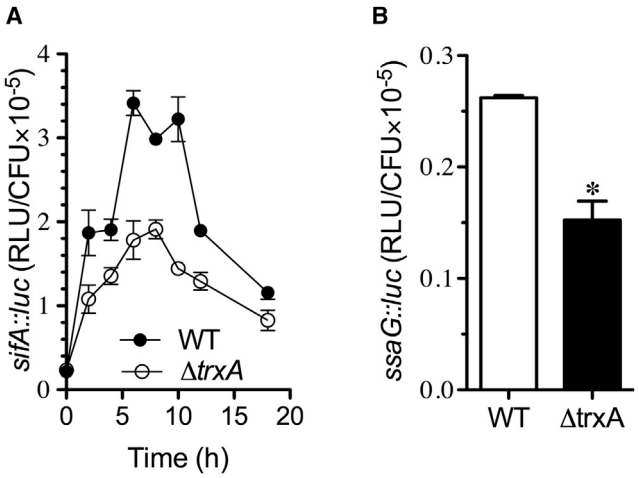

Figure 4. Intracellular SPI2 Gene Expression in *ΔtrxA Salmonella*

(A and B) Intracellular transcription of *sifA-luc* (A) and *ssaG-luc* (B) genes as measured by luciferase activity in J774 cells infected with WT or Δ*trxA* mutant *Salmonella*. The data are from three independent experiments (*$p < 0.05$).

As seen above with recombinant proteins, TrxA C32A C35A associated with SsrB as efficiently as wild-type TrxA, confirming that catalytic cysteine residues are dispensable for binding of thioredoxin-1 to SsrB.

We measured the abundance of SsrB protein in stationary phase wild-type or Δ*trxA Salmonella*. In the absence of *trxA*, the intracellular concentration of SsrB protein in the cytoplasmic soluble fraction was dramatically diminished (Figure 5F), even though the amount of *ssrB* mRNA was similar in wild-type and Δ*trxA Salmonella* (Figure 5G). Complementation of Δ*trxA Salmonella* with either wild-type *trxA* or the *trxA C32A C35A* allele supported normal SsrB expression (Figure 5F). When the amount of SsrB was measured in whole-cell lysates, no differences were found between wild-type and Δ*trxA Salmonella*, indicating that thioredoxin-1 helps maintain SsrB in the soluble fraction. To gain more insights into this possibility, recombinant full-length SsrB protein was incubated in vitro with or without equimolar amounts of thioredoxin-1. SsrB remained soluble for at least 5 days when incubated with thioredoxin-1 (Figure 5H); in the absence of thioredoxin-1, full-length SsrB became insoluble.

To investigate in more detail the region of SsrB that interacts with thioredoxin-1, we used the bacterial two-hybrid described above and pull-downs of recombinant proteins. As shown in Figures 5D and 5E, thioredoxin-1 bound to full-length SsrB (Figures 5I and 5J). Neither the receiver N-terminal domain of SsrB (i.e., $SsrB_N$) nor the effector C-terminal domain (i.e., $SsrB_C$) bound to thioredoxin-1. A fragment of SsrB encompassing the N-terminal receiver domain and the flexible linker (i.e., $SsrB_{NL}$) appeared to bind to thioredoxin-1 as effectively as full-length SsrB. A fragment of SsrB containing the linker and the C-terminal effector domain ($SsrB_{CL}$) also bound to thioredoxin-1, although with seemingly less affinity than full-length SsrB or the $SsrB_{NL}$ fragment. These investigations indicate that thioredoxin-1 recognizes the flexible linker region of SsrB in the context of, in this order, receiver and effector domains.

Collectively, our investigations indicate that thioredoxin-1 can interact post-translationally with the SsrB response regulator through both thiol-disulfide oxidoreductase-dependent and -independent functions.

Thioredoxin-1, Independently of Thiol-Disulfide Oxidoreductase Activity, Promotes SPI2 Expression, Resistance to the NADPH Phagocyte Oxidase, and *Salmonella* Virulence

Our investigations have shown that (1) most of the contribution of thioredoxin to *Salmonella* pathogenesis is co-dependent on SPI2 function, and (2) thioredoxin-1 binds to the SsrB response regulator in both thiol-disulfide oxidoreductase-dependent and -independent manners. The following experiments examined the relative contribution of thioredoxin-1 thiol-disulfide oxidoreductase-dependent and -independent activities to SPI2 function and *Salmonella* pathogenesis. Toward this end, Δ*trxA Salmonella* was complemented with wild-type *trxA* or a *trxA* C32A C35A variant. Both wild-type or *trxA* C32A C35A supported growth of *Salmonella* in J774 cells (Figure 6A), demonstrating that the thioredoxin-1 thiol-disulfide oxidoreductase activity is largely irrelevant for intracellular replication of *Salmonella* in these cells that do not sustain a respiratory burst. In support of this notion, Δ*trxB Salmonella* lacking thioredoxin reductase replicated as efficiently as wild-type *Salmonella* in J774 cells (Figure 6B). Moreover, wild-type *Salmonella* and controls expressing the *trxA* C32A C35A variant activated similar levels of *sifA* transcription in J774 macrophage-like cells (Figure 6C). These investigations indicate that thioredoxin-1 does not depend on its well-characterized thiol-disulfide oxidoreductase to support SPI2 expression needed for the intracellular growth of *Salmonella*. These findings are consistent with previous published work that showed a role for TrxA in regulation of SPI2 function (Negrea et al., 2009).

We also tested the survival of *Salmonella* expressing wild-type *trxA* or the *trxA* C32A C35A variant in primary macrophages producing large amounts of ROS through the enzymatic activity of the NADPH phagocyte oxidase (Figure S2D). Expression of either wild-type *trxA* or the *trxA* C32A C35A variant restored the survival of Δ*trxA Salmonella* in periodate-elicited macrophages from C57BL/6 mice (Figure 6D). We find it remarkable that the canonical oxidoreductase activity of thioredoxin-1 seems to be dispensable against the cytotoxicity of ROS generated by the NADPH phagocyte oxidase in this population of primary macrophages. Lastly, we monitored the virulence of *Salmonella* expressing the *trxA C32A C35A* variant in a murine model of infection dominated by the innate response of the NADPH phagocyte oxidase. *Salmonella* expressing either of these two alleles killed C57BL/6 mice with similar kinetics to wild-type bacteria ($p = 0.4$). Together, these findings suggest that most contributions of thioredoxin-1 to *Salmonella* pathogenesis are independent of its thiol-disulfide oxidoreductase enzymatic activity. Accordingly, Δ*trxB Salmonella* appear to be fully virulent in this model of experimental salmonellosis (Figure 6F).

DISCUSSION

Members of the thioredoxin family contribute to the antioxidant defenses of phylogenetically diverse organisms including

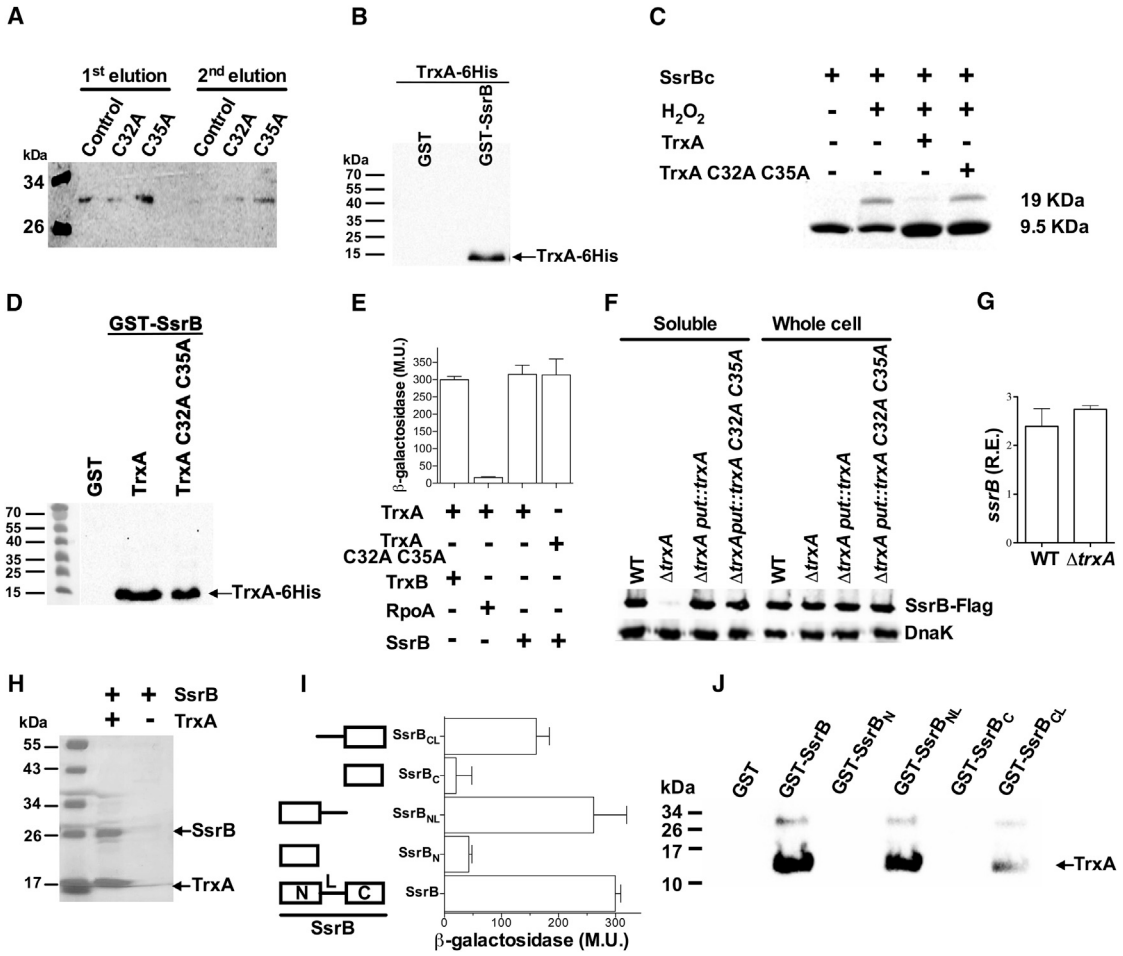

Figure 5. SsrB Is a Substrate of Thioredoxin-1 Thiol-Disulfide Oxidoreductase-Dependent and -Independent Activities

(A) Western blotting shows SsrB-3XFLAG after lysates of *Salmonella* containing pTRXATAP plasmids were purified sequentially with IgG (first elution) and calmodulin (second elution).

(B) Detection of the purified TrxA-6His proteins bound to the full-length of GST-SsrB by anti-6His immunoblot analysis after pull-down. The GST protein was used as control. Molecular markers are indicated.

(C) A recombinant C-terminal domain of SsrB separated by PAGE was visualized by Coomassie brilliant blue staining. Where indicated, 25 μM of the C-terminal domain of SsrB was oxidized with 250 μM H_2O_2. Some of the specimens exposed to H_2O_2 were treated with 25 μM recombinant TrxA or TrxA C32A C35A. The sizes of SsrB monomers and dimers are indicated on the right.

(D) TrxA-6His and TrxA C32A C35A-6His proteins were visualized by western blot using an anti-6His antibody after the pull-down with recombinant GST-SsrB.

(E) Interactions between WT or TrxA C32A C35A with full-length SsrB were studied in a bacterial two-hybrid system that reconstitutes the T18 and T25 domains of adenylate cyclase. Thioredoxin reductase (TrxB) and the RpoA α-subunit of the RNA polymerase were included as positive and negative controls, respectively. The activity of reconstituted adenylate cyclase is expressed in Miller units (M.U.).

(F) Abundance of TrxA in soluble and whole-cell cytoplasmic extracts of stationary phase *Salmonella* was determined by western blotting.

(G) The amount of *ssrB* mRNA was quantified in overnight cultures of WT and Δ*trxA Salmonella*. The data are expressed relative to the *rpoD* housekeeping gene.

(H) Purified SsrB-6His was incubated with or without recombinant TrxA-6His at 24°C for 5 days. The proteins were resolved in SDS-PAGE and visualized by Coomassie blue staining.

(I and J) Binding of SsrB fragments and TrxA was studied in bacterial two-hybrid (I) and protein-protein reconstituted (J) systems. TrxA proteins recovered in the pull-downs were visualized after western blotting of specimens separated by SDS-PAGE. The SsrB fragments containing N- and/or C-terminal domains ± linker region L are shown on the left side of (I).

Data in (A)–(D), (F), (H), and (J) are representative of two to four blots run on independent days. The data in (E), (G), and (I) are from four to eight independent experiments collected in 2–3 days. See also Figure S3.

bacteria and humans (Carmel-Harel and Storz, 2000). By directing electrons from NADPH and thioredoxin reductase to disulfide bonds in target proteins, thioredoxins maintain thiol redox homeostasis. Regulation of SPI2 expression by thioredoxin-1 is critical to *Salmonella* pathogenesis (Bjur et al., 2006; Negrea et al., 2009), but the molecular mechanism by which thioredoxin-1 promotes SPI2 expression and *Salmonella* virulence is incompletely understood. The classical oxidoreductase activity of thioredoxin-1 promotes antioxidant defenses of *Salmonella* in vitro, but it appears to be largely dispensable in vivo.

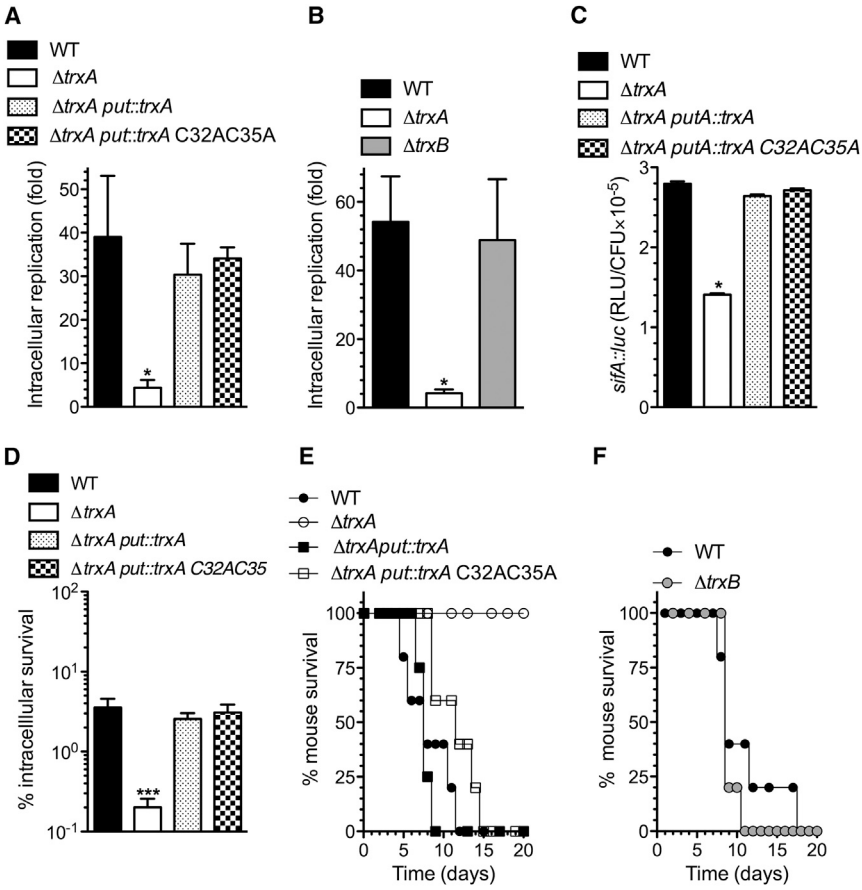

Figure 6. Contribution of Thioredoxin-1 Thiol-Disulfide Oxidoreductase-Dependent and -Independent Activities to SPI2 Function and *Salmonella* Pathogenesis

(A–D) Replication of *trxA* variants (A) and Δ*trxB* (B) *Salmonella* in J774 cells. WT *Salmonella* were used as controls. (C) Activity of the *sifA::luc* chromosomal reporter in J774 cells was measured by luciferase luminescence in *Salmonella* expressing the indicated *trxA* variants. (D) Survival of Δ*trxA* *Salmonella* in periodate-elicited macrophages from C57BL/6 mice is shown (***$p < 0.001$).

(E and F) Survival of C57BL/6 mice challenged orally with ~3 × 10^6 CFUs/mouse of Δ*trxA* (E) or Δ*trxB* (F) *Salmonella*. Data are from three to six biological replicates.

Nonetheless, thioredoxin-1, independent of canonical thiol-disulfide oxidoreductase, protects *Salmonella* against the NADPH phagocyte oxidase. Thioredoxin-1 binds to SsrB, thereby stimulating intracellular SPI2 expression, facilitating growth in professional phagocytes, and ultimately protecting *Salmonella* against the oxidative stress emanating from the enzymatic activity of the NADPH phagocyte oxidase in primary macrophages and a murine model of acute systemic infection (Figure 7).

Reactive oxygen and nitrogen species generated by the enzymatic activity of NADPH phagocyte oxidase and iNOS flavohemoproteins are critical components of the anti-*Salmonella* arsenal of human and murine macrophages (Stevanin et al., 2002; Vázquez-Torres et al., 2000a). We find that thioredoxin-1-deficient *Salmonella* become virulent in gp91*phox*-deficient macrophages and mice lacking the membrane-bound subunit of the NADPH phagocyte oxidase but remain attenuated in iNOS-deficient mice. This indicates that thioredoxin-1 contributes to the antioxidant defenses that protect this intracellular pathogen against the respiratory burst of professional phagocytic cells but appears to be dispensable for the antinitrosative defenses of *Salmonella*. It should be noted that the recovery of fitness of Δ*trxA* *Salmonella* in gp91*phox*-deficient mice is substantial but not complete, suggesting that the thioredoxin-dependent regulation of SsrB protects *Salmonella* against oxygen-independent host defenses as well. According to this idea, Δ*trxA* *Salmonella* fail to grow intracellularly in J774 cells unable to sustain a productive respiratory burst. Halting the final steps of the degradative pathway (Uchiya et al., 1999) and intersection of vesicles from the trans-Golgi network (Kuhle et al., 2006; Salcedo and Holden, 2003) are additional mechanisms by which the thioredoxin-1-dependent regulation of SsrB function may contribute to *Salmonella* virulence. In fact, the lack of fusion of *Salmonella*-containing vesicles with MPR^+ lysosomes is dependent on thioredoxin-1.

The enzymatic activity of periplasmic Cu-Zn superoxide dismutase and the MacAB multidrug efflux pump protect *Salmonella* extracytoplasmic molecular targets from the oxidative stress of macrophages (Bogomolnaya et al., 2013; De Groote et al., 1997). In addition, the concerted action of glutathione, catalases, and hydroperoxidases boost the antioxidant defenses of intracellular *Salmonella* (Hébrard et al., 2009; Song et al., 2013). Our investigations have identified thioredoxin-1 as an additional component of the antioxidant toolbox of intracellular *Salmonella* in macrophages and mice. In contrast to its previously described roles in antioxidant defense of prokaryotic and eukaryotic organisms (Carmel-Harel and Storz, 2000), the protection afforded by thioredoxin-1 against the NADPH phagocyte oxidase occurs independently of its canonical thiol-disulfide oxidoreductase enzymatic activity. By controlling the expression of SPI2, a type III secretion system that reduces contact of phagosomes with incoming NADPH phagocyte oxidase-containing vesicles (Berger et al., 2010; Gallois et al., 2001; Suvarnapunya and Stein, 2005; van der Heijden et al., 2015; Vázquez-Torres et al., 2000b, 2001), thioredoxin protects *Salmonella* from the oxidative stress generated during the innate immune response in macrophages.

ROS generated at the early stages of the *Salmonella* infection are bactericidal (Grant et al., 2008). Our investigations, as well as previously published work (Bjur et al., 2006), indicate that thiol-disulfide exchange reactions of thioredoxin-1 do not protect *Salmonella* against the microbicidal activity of authentic H_2O_2. Failure of thioredoxin-1 to protect against the bactericidal

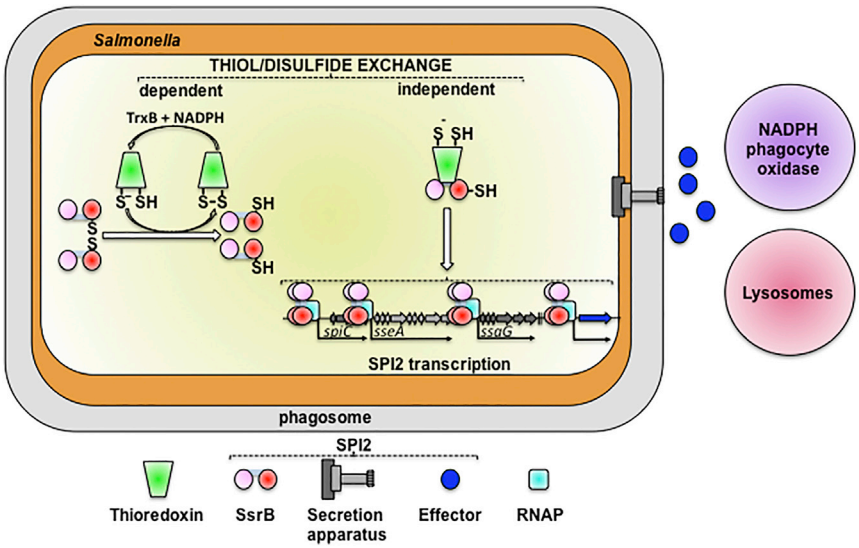

Figure 7. Model for Post-translational Regulation of SsrB by Thioredoxin-1 Thiol-Disulfide Oxidoreductase-Dependent and -Independent Activities

Oxidation of SsrB results in reversible disulfide bond formation between Cys^{203} and $Cys^{203'}$ in the homodimer. The disulfide bond in the SsrB homodimer is attacked by the thiolate ($-S^-$) of thioredoxin-1 Cys^{32}; the resulting mixed disulfide is resolved by thioredoxin-1 Cys^{35} (-SH). Disulfide-bonded thioredoxin-1 is repaired by the enzymatic activity of thioredoxin reductase TrxB, using NADPH as reducing power. In addition to serving as a substrate of thiol-disulfide oxidoreductase activity, thioredoxin-1 binds to SsrB independently of its thiol-disulfide oxidoreductase activity, resulting in stabilization of SsrB. By doing so, thioredoxin-1 aids with the activation of the SPI2 type III secretion system, thus lessening the cytotoxicity of the NADPH phagocyte oxidase in the innate response of macrophages while minimizing interactions of *Salmonella*-containing vacuoles with lysosomes. Receiver (pink) and effector (red) domains of SsrB, thioredoxin-1 (green), SPI2 apparatus (gray), SPI2 effectors (blue), and RNA polymerase (RNAP, cyan) are shown.

activity of H_2O_2 can be explained if we consider that this ROS kills bacteria such as *Salmonella* by Fenton-mediated chemistry, in which ferrous iron reduces H_2O_2 to generate highly genotoxic hydroxyl radicals (Imlay and Linn, 1988). Nonetheless, the thiol-disulfide oxidoreductase activity of thioredoxin-1 ameliorates the bacteriostatic effects of H_2O_2 in vitro. The importance of classical thioredoxin-1-thioredoxin reductase in resistance of *Salmonella* to the antimicrobial activity of the NADPH phagocyte oxidase remains uncertain, as indicated by the fact that strains lacking thioredoxin reductase or expressing the TrxA C32A C35A variant survive normally the respiratory burst of primary macrophages. It seems unlikely that H_2O_2 produced in the respiratory burst of macrophages does not oxidize cysteine residues in *Salmonella* proteins. Glutathione, glutathione peroxidase, and glutaredoxins may maintain thiol homeostasis in the absence of the thioredoxin/thioredoxin reductase system.

Thioredoxin-1 participates in the post-translational regulation of SsrB. Biochemical and genetic lines of evidence have shown that thioredoxin-1 interacts with SsrB independently of its thiol-disulfide oxidoreductase. In analogy to the binding of *E. coli*'s thioredoxin to a flexible loop in the thumb of gene 5 protein DNA polymerase of bacteriophage T7 (Doublié et al., 1998), thioredoxin-1 seems to interact with the linker region joining receiver and effector domains of SsrB. Based on crystal structures of response regulators (Buckler et al., 2002; Menon and Wang, 2011), the linker region of SsrB is likely to be disorganized. The initial interaction of SsrB with the unorganized linker region seems to promote interactions with globular parts of the receiver and effector domains, keeping SsrB in the soluble fraction. The associations between thioredoxin-1 and SsrB might be generalizable to other response regulators, as suggested by the pull-down of RcsB and OmpR with thioredoxin-1 in a proteomic screen done in *E. coli* (Kumar et al., 2004). Future work will need to test if the associations discovered here between thioredoxin-1 and SsrB apply to other response regulators. Thioredoxin-1 also can bind to disulfide-bonded SsrB in vitro through its thiol-disulfide oxidoreductase catalytic domain. The biological relevance of this interaction, however, remains unknown.

In summary, by regulating SPI2 expression, thioredoxin-1 antagonizes a variety of oxygen-dependent and -independent host defenses. Our work indicates that the horizontally acquired virulence determinant SsrB is post-translationally regulated by the ancestral protein thioredoxin-1. Because thioredoxins are ubiquitous in the bacterial kingdom, the interactions established between thioredoxin and SsrB in ancestral bacteria may have been conserved after lateral gene transfer of the SPI2 pathogenicity island into the *Salmonella* lineage.

EXPERIMENTAL PROCEDURES

Bacterial Strains

Tables S1 and S2 list the *Salmonella* strains as well as plasmids and primers used in this study. The Supplemental Experimental Procedures describe the construction of the strains of *Salmonella enterica* serovar Typhimurium used here.

Mouse Virulence

The 6- to 8-week-old C57BL/6 and congenic gp91*phox*- (Pollock et al., 1995) or iNOS-deficient (MacMicking et al., 1995) mice bred in our animal facility, according to UC Denver Animal Care and Use Committee guidelines, were used for live/dead and competition assays, as described in the Supplemental Experimental Procedures.

H_2O_2 Killing

Salmonella grown for 20 hr in Luria-Bertani (LB) broth at 37°C in a shaker incubator were diluted in PBS to a final concentration of 5×10^5 colony-forming units (CFUs)/ml. The bacteria were challenged at 37°C with increasing concentrations of H_2O_2, menadione, or diamide for 2 hr. The cultures were then serially diluted in PBS and spotted on LB agar plates. The number of bacteria capable of forming a colony was quantified after overnight culture. The percentage of surviving bacteria was calculated according to the formula (CFUs from treated sample/CFUs from untreated sample) × 100.

Bacterial Growth in Response to H_2O_2

Salmonella grown in LB broth for 18 hr were inoculated into high-Mg^{2+} N salts medium (5 mM KCl, 7.5 mM $(NH_4)SO_4$, 0.5 mM K_2SO_4, 1 mM KH_2PO_4, 38 mM glycerol, 0.1% casamino acids supplemented with 10 mM $MgCl_2$, and 100 mM Tris-HCl [pH 7.6]). *Salmonella* were then grown for 4 hr at 37°C in a shaking incubator until the cultures reached A_{600} of 0.5. Bacteria were adjusted to a final concentration of 5×10^6 CFUs/ml and challenged with 100 μM H_2O_2. Bacterial growth was measured as A_{600} every 15 min for 25 hr in a Bioscreen-C Growth analyzer (Oy Growth Curves).

Intracellular Survival

J774 cells and primary macrophages were infected at MOIs of 10 and 2, respectively, with *Salmonella* grown for 20 hr in LB broth at 37°C in a shaker incubator. The intracellular number of bacteria was determined on LB agar plates after lysing host cells with 0.25% deoxycholate.

Immunofluorescence Microscopy

HeLa cells were infected with late-log-phase *Salmonella* expressing the P_{rpsM}-GFP construct. Specimens collected 8 hr after infection were fixed with 3.7% paraformaldehyde and stained with anti-CI MPR antibody (Developmenal Studies Hybridoma Bank) followed by an anti-mouse IgG antibody conjugated with Texas Red (Rockland). Colocalization of GFP-expressing *Salmonella* with MPR^+ lysosomes was visualized on a Leica TCS SP8 confocal laser-scanning microscope.

Transcriptional Analysis

Intracellular SPI2 expression by *sifA-luc*- or expression of *ssaG-luc*-expressing *Salmonella* in J774 was determined by recording luminescence as previously described (Gerlach et al., 2007) and explained in detail in the Supplemental Experimental Procedures.

Tandem Affinity Purification

Protein partners of thioredoxin-1 were identified by tandem affinity purification using a *trxA* construct in plasmid pFA6a-CTAP (Tasto et al., 2001) that allows for sequential purification of calmodulin-binding peptide and Protein A. This procedure is described in the Supplemental Experimental Procedures.

Binding of Thioredoxin-1 and SsrB

Recombinant TrxA-6His, TrxA C32A C35A-6His, and GST-SsrB proteins were purified as described in the Supplemental Experimental Procedures. Binding of recombinant TrxA-6His or TrxA C32A C35A-6His to GST-SsrB was analyzed as described previously (Henard et al., 2014). Briefly, 1 nmol GST-SsrB protein was incubated with 200 μl Glutathione-Sepharose 4B beads (BioWorld) at 4°C for 2 hr, washed with 20-bed volume of 50 mM Tris-HCl (pH 7.5), and then incubated with 2 nmol TrxA-6His or TrxA C32A C35A-6His proteins at 4°C for 2 hr with agitation. After washing with 30 mM NaCl buffer, samples were eluted with 500 mM NaCl, precipitated with 10% 2,2,2-trichloroacetic acid (TCA), and loaded into a 15% SDS-PAGE gel to detect the TrxA-6His or TrxA C32A C35A-6His proteins by immunoblot analysis. Electro-blotted proteins were treated with a 1/1,000 dilution of anti-6His antibody (Rockland), followed by a 1/10,000 dilution of goat anti-rabbit IgG (Pierce) conjugated with horseradish peroxidase. TrxA-6His-tagged proteins were visualized using an enhanced chemiluminescence (ECL) prime western blotting detection reagent (GE Healthcare). Purified GST protein was used as a negative control.

Thioredoxin-1 Thiol-Disulfide Oxidoreductase Activity

Full-length TrxA and a C-terminal SsrB fragment encompassing residues 137–212 expressed as GST fusions from pGEX6P1 (GE Healthcare) were purified as described previously for DksA (Henard et al., 2014). Where indicated, 25 μM recombinant SsrB was treated with 250 μM H_2O_2 for 1 hr at 37°C. Selected samples were treated with 25 μM TrxA for 30 min. The specimens were mixed with 3× Red loading buffer (New England Biolabs) in the absence of reducing agents. The samples were loaded into 4%–20% SDS-PAGE gels (Bio-Rad) and electrophoresed at 125 V on ice. Proteins were visualized by Coomassie blue staining.

SsrB Stability by TrxA

Recombinant TrxA-6His and SsrB-6His proteins were purified as described in the Supplemental Experimental Procedures. To determine the SsrB stability with and without TrxA-6His protein, 100 pmol of the purified SsrB-6His proteins in 50 mM Tris-HCl (pH 7.5) were incubated at room temperature with and without 100 pmol TrixA-6His proteins. After 5 days, samples were loaded onto a 12% SDS-PAGE gel to assess the TrxA-6His or SsrB-6His proteins by Coomassie brilliant blue staining.

Bacterial Two-Hybrid System

The *trxA* or *trxA C32A C35A* genes were cloned into the pUT18 vector of a bacterial two-hybrid system to produce fusions to the N terminus of the T18 subunit of adenlyate cyclase (Euromedox). The resulting plasmids were electroplated into *E. coli* expressing pKNT25-*ssrB* encoding SsrB fused to the N terminus of the T25 subunit of adenlyate cyclase. Binding of TrxA to SsrB was measured by following β-galactosidase in overnight cultures using o-nitrophenol-β-galactoside. The results are expressed in Miller units.

Statistical Analysis

One-way ANOVA followed by a Bonferroni post-test helped determine statistical significance for multiple comparisons. Data were considered statistically significant when $p < 0.05$.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, three figures, two tables, and three movies and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2016.02.066.

AUTHOR CONTRIBUTIONS

Conceptualization and Writing – Original Draft, M.S., J.-S.K., and A.V.-T.; Investigation, M.S., J.-S.K., L.L., M.H., and A.V.-T.; Funding Acquisition, Supervision, and Writing – Review & Editing, A.V.-T.

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