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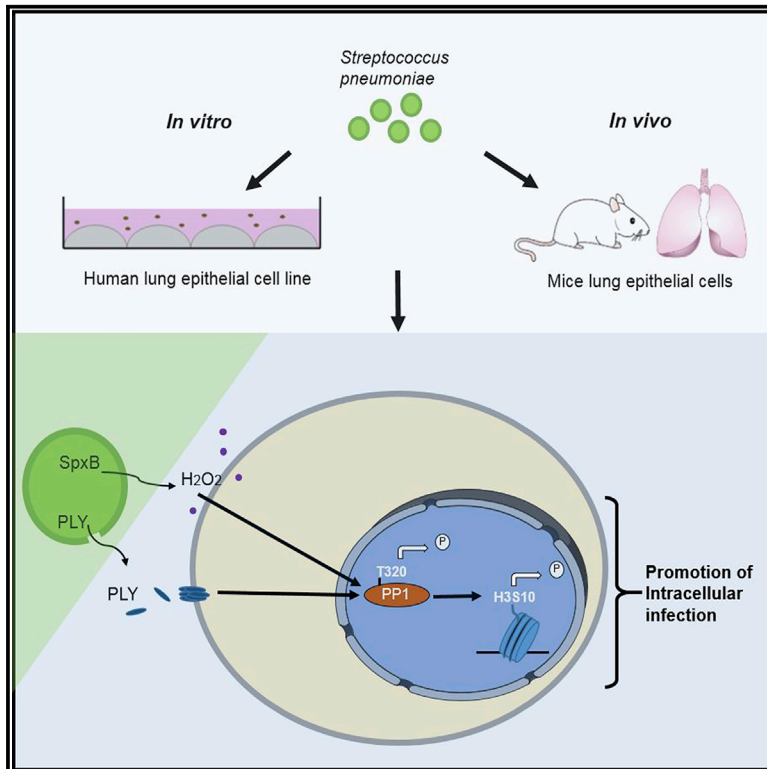


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# Cell Reports

## ***Streptococcus pneumoniae* Infection Promotes Histone H3 Dephosphorylation by Modulating Host PP1 Phosphatase**

### Graphical Abstract



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### In Brief

Dong et al. show that *Streptococcus pneumoniae* infection induces histone H3 dephosphorylation in lung epithelial cells, through two bacterial factors, Ply and SpxB. Infection-triggered activation of the host phosphatase PP1 is required for this histone modification and efficient intracellular infection.

### Highlights

- *Streptococcus pneumoniae* infection induces dephosphorylation of histone H3
- The bacterial factors Ply and SpxB contribute to H3S10 dephosphorylation
- Host PP1 mediates H3S10 dephosphorylation and is important for intracellular infection
- PP1 is activated upon *S. pneumoniae* infection through T320 dephosphorylation



# *Streptococcus pneumoniae* Infection Promotes Histone H3 Dephosphorylation by Modulating Host PP1 Phosphatase

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## SUMMARY

Pathogenic bacteria can alter host gene expression through post-translational modifications of histones. We show that a natural colonizer, *Streptococcus pneumoniae*, induces specific histone modifications, including robust dephosphorylation of histone H3 on serine 10 (H3S10), during infection of respiratory epithelial cells. The bacterial pore-forming toxin pneumolysin (PLY), along with the pyruvate oxidase SpxB responsible for H<sub>2</sub>O<sub>2</sub> production, play important roles in the induction of this modification. The combined effects of PLY and H<sub>2</sub>O<sub>2</sub> trigger host signaling that culminates in H3S10 dephosphorylation, which is mediated by the host cell phosphatase PP1. Strikingly, *S. pneumoniae* infection induces dephosphorylation and subsequent activation of PP1 catalytic activity. Colonization of PP1 catalytically deficient cells results in impaired intracellular *S. pneumoniae* survival and infection. Interestingly, PP1 activation and H3S10 dephosphorylation are not restricted to *S. pneumoniae* and appear to be general epigenomic mechanisms favoring intracellular survival of pathogenic bacteria.

## INTRODUCTION

*Streptococcus pneumoniae* is a Gram-positive, extracellular bacterium, which colonizes the human nasopharynx and respiratory tract. It is a leading cause of bacterial pneumonia and meningitis worldwide, particularly in developing nations (Wahl et al., 2018). In the complex relationship with its host, *S. pneumoniae* can act as both an adapted commensal and an invasive pathogen. On the one hand, *S. pneumoniae* may reside asymptotically in the upper respiratory tract, a phenomenon that is particularly common in children and greatly contributes to transmission. On the other hand, bacteria can cause pneumonia or breach epithelial barriers, enter the bloodstream, and eventually cause severe invasive pneumococcal diseases (IPDs), such as meningitis, and sepsis. To date, 98 serotypes of this encapsu-

lated diplococcus have been described, based on variations in their capsule polysaccharide (CPS) (Kadioglu et al., 2008). However, not all serotypes are equal in their capacity to cause IPDs; only 20–30 of them are principally associated with invasiveness diseases, such as serotypes 1, 4, and 14 (Geno et al., 2015; Mehr and Wood, 2012). In contrast, several serotypes, such as 6B, 11A, and 23F, are more likely to be carried for longer periods and are thus regarded as carriage serotypes (Geno et al., 2015). However, because of the introduction of pneumococcal conjugate vaccines and prominent capsule switching, the serotype prevalence and distribution in IPDs and carriage serotypes regularly change.

For both carriage and invasive pneumococcal strains, the establishment of colonization begins upon contact with the host respiratory epithelium. *S. pneumoniae* interacts with epithelial cells via multiple and complex processes, and this multifactorial event has been well characterized with respect to the bacterial factors involved. Surface factors, such as CbpA and ChoP, have been reported to participate in adhesion to epithelial cells (Kadioglu et al., 2008). Another major virulence factor, the pore-forming toxin pneumolysin (PLY) is proposed to be involved in invasion by breaching the epithelium (Weiser et al., 2018). However, the processes involved in pneumococcal-epithelial interactions, such as how *S. pneumoniae* modulates host-cell signaling, are understudied.

Recent studies show that pathogens reprogram host cells during infection through bacteria-triggered histone modifications, which modulate host transcription (Bierne et al., 2012). In eukaryotic cells, DNA is packaged with histones into chromatin, and the covalent post-translational modifications at the tails of the core histones function to dynamically control DNA accessibility, affect the recruitment and stabilization of transcription-associated factors, and therefore, regulate the cell's transcriptional programs (Hamon and Cossart, 2008). Bacterial pathogens have been shown to target host histones directly, through the secretion of factors targeting chromatin and named nucleomodulins, or indirectly, through modulation of host signaling cascades. However, histone modifications induced by a natural colonizer, such as *S. pneumoniae*, have not yet, to our knowledge, been explored.

During bacterial infection, histone H3 was found to be dephosphorylated after the loss of membrane integrity mediated either by the secretion of cholesterol-dependent cytolysin (CDC) toxin



or by insertion of translocon from the type III secretion system (Dortet et al., 2018; Hamon et al., 2007). H3 dephosphorylation on serine 10 (H3S10) was shown to be induced *in vitro* in epithelial cells and to correlate with the repression of inflammatory genes. Although cellular processes, such as entry into mitosis or activation by extracellular signals (EGF, cellular stress, or inflammation) have been shown to be associated with H3S10 phosphorylation, the role of this modification in these processes is not fully understood (Mahadevan et al., 1991; Saccani et al., 2002; Sawicka et al., 2014). Key kinase signaling pathways, including mitogen-activated protein kinase (MAPK) and nuclear factor  $\kappa$ B (NF- $\kappa$ B), induce the phosphorylation of histone H3 on residue 10 (H3S10) or 28 (H3S28), which are important for cross-talk with histone H3 acetylation, a canonical mark of transcriptional activation (Drobic et al., 2010; Yamamoto et al., 2003). In contrast, how H3S10 becomes dephosphorylated is less well understood. Furthermore, the molecular basis and the role of this histone modification during bacterial infection remain unknown.

In this article, we reveal that *S. pneumoniae* induces histone modifications in host lung epithelial cells, both *in vitro* and *in vivo*. We identify the CDC toxin PLY as a key factor in mediating H3 dephosphorylation but also reveal the strong contribution of the pyruvate oxidase, SpxB. Both PLY and SpxB factors activate the host phosphatase PP1, which we show is responsible for H3 dephosphorylation. Interestingly, we find that infection triggers dephosphorylation of PP1 on threonine 320 (T320), which is required for its activation, and that this process is necessary to permit efficient intracellular infection. By describing the molecular basis and the role of H3S10 dephosphorylation, we illustrate a common mechanism of host alteration by bacteria relevant during both colonization and infection.

## RESULTS

### ***Streptococcus pneumoniae* Induces Dephosphorylation of Histone H3 on Serine 10 during Infection**

Histone H3 dephosphorylation on serine 10 was initially observed after treatment of epithelial cells with purified bacterial toxins, including PLY of *S. pneumoniae* (Hamon et al., 2007). To evaluate whether histone H3 phosphorylation is modulated by bacteria during infection, we exposed A549 lung epithelial cells to two different strains of *S. pneumoniae* (R6 and TIGR4) at increasing multiplicities of infection (MOIs) and assessed the phosphorylation of H3S10 by immunoblotting. As a control, the total levels of H3 and actin were also measured. Data in Figure 1A show that H3S10 is strongly dephosphorylated, and the effect is proportional to the number of bacteria used in the infection. We further validated that this effect was not restricted only to alveolar epithelial A549 cells because bronchial epithelial Beas-2B cells displayed the same reduction in phosphorylation of histone H3 at serine 10 (H3S10ph) levels (Figure S1A). H3T3, H3T11, and H3S28 were also dephosphorylated in response to bacteria; however, H3T6 was not, suggesting only specific residues are targeted (Figure S1B). Because H3S10ph has been previously associated with bacterial infection (Arbibe et al., 2007; Hamon et al., 2007; Saccani et al., 2002), we focused our study on this modification.

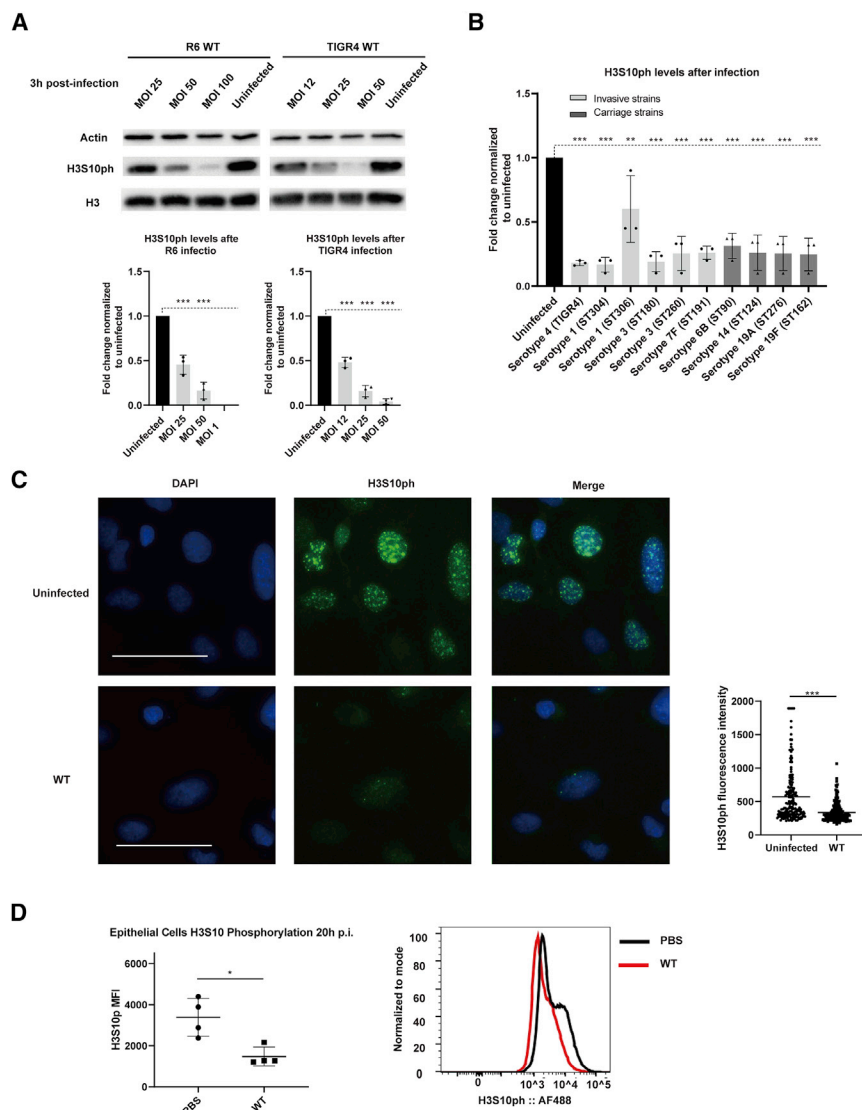
Given that H3S10 dephosphorylation was observed during infection with two different laboratory strains, we hypothesized that this effect was a shared mechanism among *S. pneumoniae* serotypes. We assessed clonal representatives of different invasive and carriage serotypes for their ability to induce H3S10 dephosphorylation. We show that all serotypes tested induced strong H3S10 dephosphorylation in A549 cells (Figure 1B). These results demonstrate that reduction of H3S10ph levels is a common feature of *S. pneumoniae* infection and is induced by clinical isolates as well as laboratory strains.

H3S10ph has been associated with the mitotic phase of the cell cycle (Sawicka and Seiser, 2012); therefore, we investigated whether the observed loss reflected changes in the cell cycle induced by infection. Cells were synchronized with thymidine, infected upon release, and the proportion of cells in each stage of the cell cycle was measured using propidium iodine staining. Fluorescence-activated cell sorting (FACS) analysis showed no significant difference in the percentage of cells in each stage between uninfected and infected cells (Figure S1C), strongly suggesting that infection does not affect the cell cycle. Furthermore, immunofluorescence experiments were performed to determine H3S10ph levels in non-mitotic cells. Nuclear staining with DAPI clearly distinguishes cells in interphase versus mitosis and allows direct visualization of interphase cells, confirming that many cells which do not have condensed chromosomes stain positive for H3S10ph (Figure 1C). Quantification of fluorescence intensity upon infection with *S. pneumoniae* shows a significant reduction compared with uninfected cells (Figure 1C). These data further support the finding that infection-induced dephosphorylation occurs in interphase cells, independent of the cell cycle.

We next determined whether H3S10 dephosphorylation occurred during *in vivo* infection. We performed intranasal inoculation of mice with *S. pneumoniae* and used antibodies against cell lineage markers to specific cell types isolated from collected mouse lungs (Figure S1D). The levels of H3S10 phosphorylation in epithelial cells after 24 h of infection were further evaluated by FACS analysis. In comparing infected to uninfected cells, a shift in the fluorescence level of a large proportion of epithelial cells was observed (Figure 1D). Interestingly, dephosphorylation was mainly detected in epithelial cells, and other monitored cells did not display significant changes in this histone mark (Figure S1E). These data further support that H3 dephosphorylation occurs in terminally differentiated cells, independent of the cell cycle. Therefore, *S. pneumoniae* induces specific H3S10 dephosphorylation in epithelial cells during infection.

### ***S. Pneumoniae* Toxin PLY Is Important for H3S10 Dephosphorylation**

The cholesterol-dependent cytolysin of *S. pneumoniae*, PLY, was previously shown to induce H3S10 dephosphorylation when treating epithelial cells with purified toxin (Hamon et al., 2007). We thus hypothesized that PLY was the main factor responsible for this modification during infection. Chromosomal deletion mutants of PLY were generated in both R6 and TIGR4 strains of *S. pneumoniae* and were tested for their ability to dephosphorylate H3. Immunoblotting experiments show that H3 dephosphorylation was only partially blocked with the  $\Delta$ ply



**Figure 1. *Streptococcus pneumoniae* Induces Specific Dephosphorylation of Histone H3 on Serine 10 Independently of the Cell Cycle**

(A) Phosphorylation levels of histone H3S10, as detected by immunoblotting in uninfected A549 cells and cells infected with *S. pneumoniae* strain R6 or TIGR4, at the indicated multiplicities of infection (MOIs). Quantification of H3S10ph immunoblots were normalized first to total actin levels and then to the uninfected sample. Error bars represent SD of three independent experiments. Statistical significance was calculated by the one-way ANOVA method (Dunnett's post hoc test, uninfected as control group). \*\*\* $p < 0.001$ .

(B) Quantification of levels of H3S10 phosphorylation detected by immunoblotting in uninfected A549 cells and cells infected with TIGR4 or clinical *S. pneumoniae* strains (from carriage or invasiveness); serotypes and MLST types at MOI 25 are indicated. Error bars represent SD of three independent experiments. Statistical significance was calculated using one-way ANOVA method (Dunnett's post hoc test, uninfected as the control group). \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .

(C) H3S10ph was detected by immunofluorescence in Beas-2B cells under uninfected and 1-h TIGR4 (MOI = 25)-infected conditions. Size bars represent 50  $\mu\text{m}$ . On the right, the quantification of nuclear fluorescence intensity integrates independent duplicate experiments ( $n \geq 200$  cells per condition). Statistical significance was calculated using a Student's *t* test. \*\*\* $p < 0.001$ .

(D) H3S10ph levels of lung epithelial cells from mice treated with PBS or infected with *S. pneumoniae* TIGR4 ( $5 \times 10^6$  colony-forming units [CFUs]) for 20 h were analyzed by FACS. Mean fluorescence intensity (MFI) is represented with error bars indicating SD.  $n = 4$  mice per condition. Statistical significance was calculated using a Student's *t* test. \* $p < 0.05$ .

See also Figure S1.

mutant as compared with wild-type (WT) infection (Figure 2A). Because PLY does not have a signal sequence for secretion, it is thought that the toxin is released upon lysis of bacteria in a manner dependent upon the autolysin LytA. We, therefore, generated a *lytA*-deletion mutant, which releases less PLY than WT bacteria and induces a similar dephosphorylation of H3 as a  $\Delta ply$  mutant (Figures S2A and SB). Therefore, H3 dephosphorylation is in part mediated by PLY, which is released upon LytA-dependent lysis of a subpopulation of bacteria.

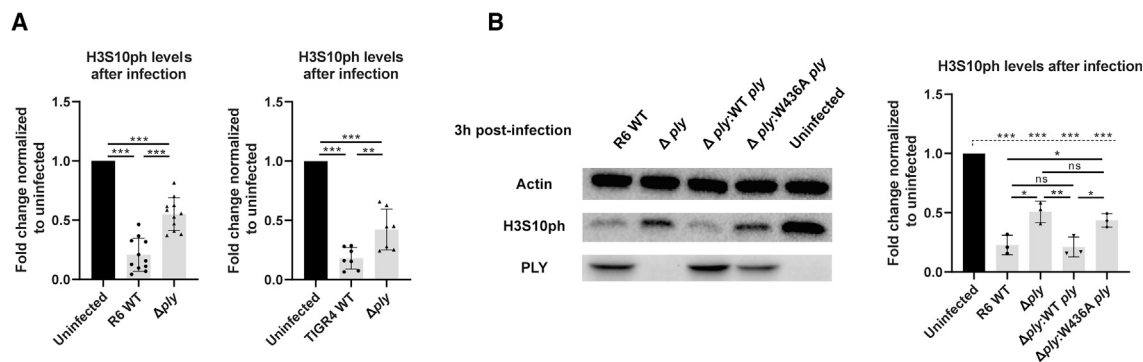
To understand how PLY was inducing histone modifications, we complemented the  $\Delta ply$  mutant with a PLY bearing a point mutation, W436A, rendering it non-hemolytic (Malet et al., 2017). We compared the level of H3S10ph of the mutant to that of a strain complemented with WT PLY. The complemented WT induced the same level of H3S10 dephosphorylation as WT bacteria, whereas the W436A mutant phenocopied the  $\Delta ply$  mutant (Figure 2B). Therefore, PLY contributes to dephosphorylation of H3 in a pore formation-

dependent manner. Given that the level of H3S10ph was clearly not restored to uninfected levels upon infection with the  $\Delta ply$  mutant, other additional contributing bacterial factors are probably also responsible for inducing this histone modification.

### H<sub>2</sub>O<sub>2</sub> Generated by SpxB Is Required for *S. pneumoniae*-Mediated H3S10 Dephosphorylation

Reports have shown that a stress response, such as oxidative stress, can lead to H3 dephosphorylation (Jeong et al., 2013; Shimada et al., 2010). Interestingly, *S. pneumoniae* expresses a pyruvate oxidase that converts pyruvate to acetyl-phosphate and generates H<sub>2</sub>O<sub>2</sub> as a byproduct. The produced H<sub>2</sub>O<sub>2</sub>, which is freely diffusible across membranes, can be detected in the supernatant of infected cells, and a deletion of the SpxB oxidase results in a complete block of H<sub>2</sub>O<sub>2</sub> production (Figure S3A). To determine whether SpxB was contributing to H3 dephosphorylation, we infected cells with a deletion mutant. In both strains





**Figure 2. *S. pneumoniae* Toxin PLY Is Important for H3S10 Dephosphorylation**

(A) Quantification of H3S10ph in uninfected cells, cells infected with WT *S. pneumoniae* strains R6 (MOI = 50) and TIGR4 (MOI = 25) and their respective  $\Delta$ ply mutant strains, for 3 h.

(B) Representative immunoblots of cells infected with WT *S. pneumoniae* (R6 strain), a  $\Delta$ ply mutant, a  $\Delta$ ply mutant complemented with WT ply, or a ply point mutant without pore-forming activity (W436A) at MOI 50 for 3 h.

All quantifications in graphs show the means  $\pm$  SD of at least three independent experiments. Statistical significance was calculated by one-way ANOVA method (Turkey post hoc test when compared within infection groups, as indicated with solid line, or Dunnett's post hoc test when compared with the uninfected control, as indicated with dotted line). \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .

See also Figure S2.

R6 and Tigr4, H3 dephosphorylation was prevented upon infection with a  $\Delta$ spxB mutant. In fact, H3S10ph levels were comparable to the uninfected level upon infection with  $\Delta$ spxB or a double  $\Delta$ ply $\Delta$ spxB mutant (Figure 3A). Additionally, we performed immunofluorescence experiments with the double  $\Delta$ ply $\Delta$ spxB mutant, and quantification of H3S10ph fluorescence intensity showed that the double mutant did not induce dephosphorylation (Figure 3B).

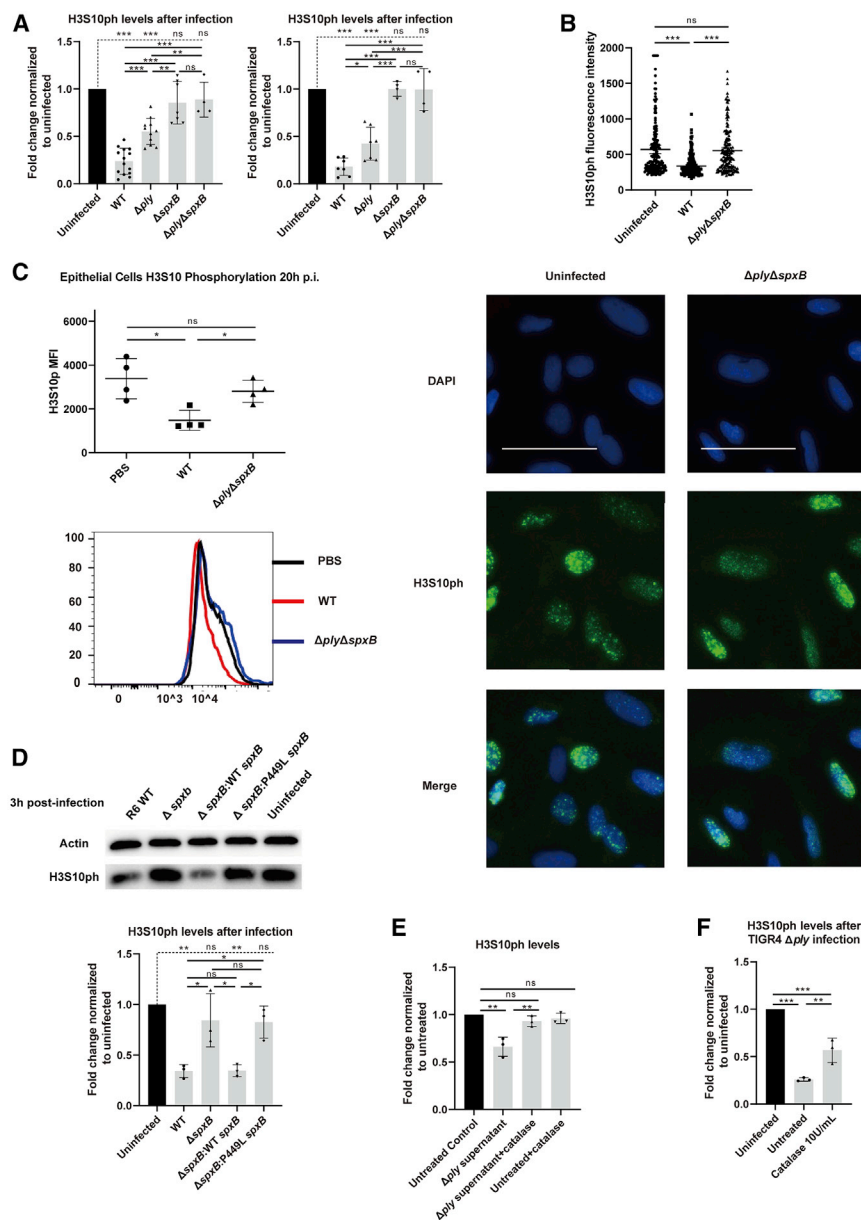
To determine whether *S. pneumoniae*-mediated H3 dephosphorylation requires the catalytic activity of SpxB and  $H_2O_2$ , the byproduct of catalysis, we generated a  $\Delta$ spxB mutant complemented with WT spxB and/or a catalytically inactive spxB (spxB P449L) (Ramos-Montañez et al., 2008). Both strains were used in infection, and the levels of H3S10ph were determined by immunoblotting. Although  $\Delta$ spxB:spxB was able to restore H3 dephosphorylation to WT levels, the catalytically inactive complement,  $\Delta$ spxB:spxBP449L, did not (Figure 3D). To further show that it is the byproduct of SpxB catalysis,  $H_2O_2$ , rather than another metabolic intermediate, which is important for H3 dephosphorylation, we performed experiments in the presence of catalase. Catalase is an enzyme that converts  $H_2O_2$  to water and oxygen and thereby neutralizes the downstream effects of  $H_2O_2$ . Cells infected with  $\Delta$ ply TIGR4 or treated with infection supernatant displayed significant H3 dephosphorylation (Figures 3E and 3F). However, upon catalase treatment, H3 dephosphorylation was significantly impaired, reinforcing the finding that  $H_2O_2$  produced by SpxB is the main driver for H3 dephosphorylation. In fact,  $H_2O_2$  alone induces H3 dephosphorylation in A549 cells in a dose-dependent manner (Figure S3B). Therefore,  $H_2O_2$  generated by SpxB is a factor contributing to H3 dephosphorylation in addition to the pore-forming toxin PLY.

*In vivo* experiments were performed to determine whether SpxB and PLY were necessary for H3 dephosphorylation in lung epithelial cells. Although WT infection led to a signifi-

cant shift in phosphorylation H3 levels, the double  $\Delta$ ply $\Delta$ spxB mutant did not. In fact, H3S10 dephosphorylation did not occur when inoculating mice intranasally with the  $\Delta$ ply $\Delta$ spxB mutant (Figure 3C). Therefore, both PLY and SpxB are required to induce H3 dephosphorylation upon infection in both *in vitro* and *in vivo* epithelial cells. We noticed that a single  $\Delta$ spxB mutant did not induce greater H3S10 dephosphorylation than a double  $\Delta$ ply $\Delta$ spxB mutant. This could be explained by the finding that a mutant in SpxB is diminished in PLY release and pore-formation on epithelial cells (Bryant et al., 2016).

### PP1 Is the Host Phosphatase Mediating H3S10 Dephosphorylation

Given that neither PLY nor SpxB directly target H3S10 for modification, we searched for a host phosphatase that could mediate the observed dephosphorylation. We first used chemical inhibitors that target known phosphatases: okadaic acid, a potent and selective inhibitor of protein phosphatase 1 (PP1) and 2A (PP2A), with a greater potency for 2A, and tautomycin, which specifically inhibits PP1. Cells were pretreated with the two inhibitors and subsequently infected with either R6 or TIGR4 strains of *S. pneumoniae*. Detection of phosphorylated H3 levels by immunoblotting show that okadaic acid had no effect, whereas tautomycin blocked infection-induced dephosphorylation (Figures 4A and S4A). These results were further validated by RNA interference using small interfering RNA (siRNA) targeting all isoforms of PP1 ( $\alpha$ ,  $\beta$ , and  $\gamma$ ). The knockdown efficiency siRNA clearly showed that the level of all isoforms was equally decreased (Figure S4B). Under those conditions, H3S10 dephosphorylation was fully blocked upon infection, similar to the results obtained with tautomycin (Figure 4B). Consistent with PP1 being the main phosphatase targeting H3, siRNA of PP1 also fully blocked  $H_2O_2$  and purified PLY-mediated H3 dephosphorylation



**Figure 3. H<sub>2</sub>O<sub>2</sub> Generated by SpxB Is Required for *S. pneumoniae*-Mediated H3S10 Dephosphorylation**

(A) Quantification of H3S10ph in uninfected cells, cells infected with WT *S. pneumoniae* strains R6 (MOI = 50) and TIGR4 (MOI = 25), a  $\Delta spxB$  mutant, a  $\Delta ply \Delta spxB$  double mutant for 3 h.

(B) H3S10ph was detected by immunofluorescence in Beas-2B cells under uninfected and 1h (MOI = 25) infected conditions. On the right, the quantification of nuclear fluorescence intensity integrates independent duplicate experiments ( $n \geq 200$  cells per condition). Size bars represent 50  $\mu m$ .

(C) H3S10ph levels of lung epithelial cells from mice treated with PBS, WT *S. pneumoniae* TIGR4 ( $5 \times 10^6$  CFU), or a  $\Delta ply \Delta spxB$  double mutant ( $5 \times 10^6$  CFU) for 20 h were analyzed by FACS. Mean fluorescence intensity (MFI) is represented with error bars indicating SD,  $n = 4$  mice per condition.

(D) Immunoblot images are shown on the left and quantifications on the right. The left image is shown for representative immunoblots of cells infected with WT *S. pneumoniae* (R6 strain), a  $\Delta spxB$  mutant, a  $\Delta spxB$  mutant complemented with WT *spxB*, or a *spxB* point mutant without catalytic activity (P449L) at MOI = 50 for 3 h.

(E) Cells were incubated 2h with the filter-sterilized supernatant from infection wells, or with 15 min catalase pretreated filter-sterilized supernatant, or 15 min catalase pretreated cell culture medium, respectively.

(F) Quantification of H3S10ph in uninfected cells, cells infected with TIGR4  $\Delta ply$  mutant (with or without catalase during infection) at MOI = 25 for 3 h.

Error bars in the quantifications represent SD of at least three independent experiments. Statistical significance was calculated by one-way ANOVA method (Turkey post hoc test when compared within infection groups, as indicated with the solid line, or Dunnett's post hoc test when compared with uninfected control, as indicated with the dotted line). \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ . See also Figure S3.

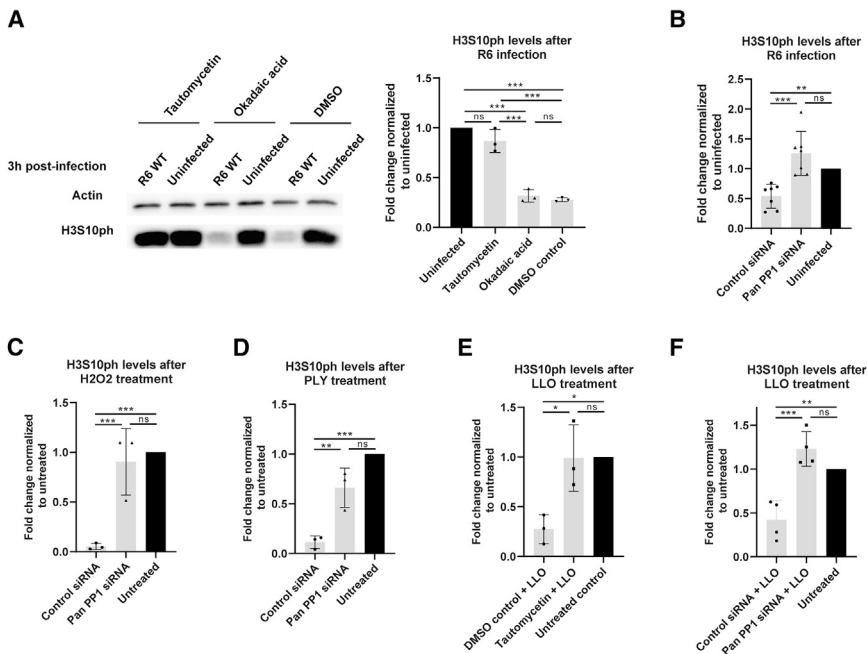
(Figures 4B–4D). Together these results show that PP1 is the phosphatase involved in dephosphorylating H3 and that both PLY and SpxB mediate this effect through the same host pathway.

Previous studies have demonstrated that other toxins of the CDC family, such as listeriolysin O (LLO) from *Listeria monocytogenes*, also induced dephosphorylation of H3S10, but they did not identify the mechanism at play (Hamon et al., 2007). Therefore, we tested whether PP1 was also required for LLO-induced H3 dephosphorylation. Cells were treated with purified LLO, and H3 dephosphorylation was observed in HeLa cells (Figures 4E and 4F). However, upon pretreating cells with tautomycin or by silencing the expression PP1 with siRNA, H3 dephosphorylation was blocked (Figures 4E

and 4F). Therefore, PP1 is modulated by at least two bacterial toxins mediating H3S10 dephosphorylation.

### Bacterial Infection Induces Dephosphorylation of PP1

In its resting state, PP1 is phosphorylated on T320, and dephosphorylation of this residue correlates with PP1 activation. Therefore, we determined whether PP1 was being activated by infection through dephosphorylation of T320 (Hou et al., 2013). First, lysates from infected and non-infected cells were probed by immunoblotting for the total levels of each PP1 isoform (Figure 5A). Importantly, the total level of PP1 was unaltered by infection, regardless of the mutant used. In contrast, the level of phosphorylated PP1 was significantly decreased upon infection with WT *S. pneumoniae* (TIGR4). Interestingly, PP1



**Figure 4. PP1 Is the Host Phosphatase Mediating H3S10 Dephosphorylation**

(A) Representative immunoblot images are shown on the left and quantifications on the right. A549 cells pretreated with the PP1 inhibitor Tautomycin, the PP2A inhibitor okadaic acid, or DMSO as the control and infected with R6 (MOI = 50) for 3 h. (B) A549 cells are transfected with PP1 siRNA or control siRNA before 3 h R6 (MOI = 50) infection. (C) A549 cells are transfected with PP1 siRNA or control siRNA before PLY treatment for 30 min. (D) A549 cells are transfected with PP1 siRNA or control siRNA before H<sub>2</sub>O<sub>2</sub> treatment for 1 h. (E) HeLa cells are pretreated with PP1 inhibitor Tautomycin before LLO treatment for 20 min. (F) HeLa cells are transfected with PP1 siRNA or control siRNA before LLO treatment for 20 min. All quantification graphs are of at least three independent experiments. All quantifications in graphs show the means  $\pm$  SD, and statistical significance was calculated by one-way ANOVA method (Turkey post hoc test). \*\**p* < 0.01, \*\*\**p* < 0.001.

See also Figure S4.

dephosphorylation was partially rescued upon infection with a  $\Delta$ ply mutant and as fully prevented upon infection with a  $\Delta$ spxB or the double  $\Delta$ ply $\Delta$ spxB mutant. Therefore, the levels of phosphorylated PP1 fully correlated with the levels of phosphorylated H3S10 (Figure 5B), strongly suggesting that activation of PP1 by dephosphorylation led to dephosphorylation of H3.

We confirmed this finding by immunofluorescence by evaluating the level of phosphorylated PP1 in A549 cells. Interestingly, PP1 became dephosphorylated specifically in the nucleus of cells infected with WT bacteria (TIGR4), where histone H3 dephosphorylation occurs (Figure 5C). In contrast, the levels of nuclear phosphorylated PP1 did not change upon infection with the double  $\Delta$ ply $\Delta$ spxB mutant. Because tautomycin blocked infection-induced H3 dephosphorylation, we measured the corresponding levels of phosphorylated PP1. Tautomycin fully blocked infection-induced PP1 dephosphorylation and appeared to induce phosphorylation (Figures 5C and S5A). These results show that the effect of PLY and SpxB converge on PP1, which likely auto-dephosphorylates itself.

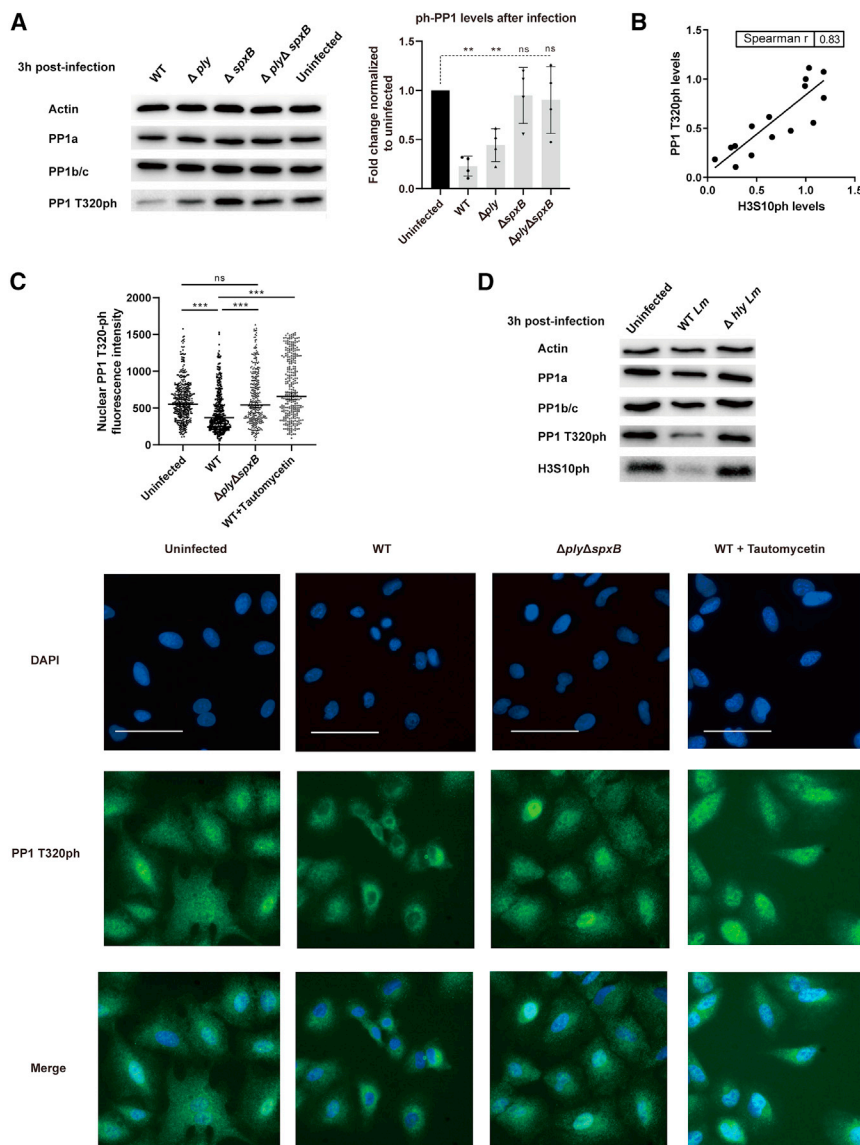
Our data show that other toxins in the CDC family, such as LLO, mediate H3 dephosphorylation through PP1. We, therefore, wanted to determine whether infection with *Listeria monocytogenes*, which produces LLO, also induced PP1 T320 dephosphorylation. To address this point, we infected HeLa cells with either WT *L. monocytogenes* or a mutant lacking the LLO toxin ( $\Delta$ hly). The representative immunoblots in Figure 5D show that the WT bacteria induced dephosphorylation in both H3 and PP1. However, infection with the  $\Delta$ hly strain did not induce dephosphorylation. Therefore, dephosphorylation of PP1, leading to H3 dephosphorylation, is a common mechanism activated by bacteria that produce cholesterol-dependent cytolysins and is essential for H3 dephosphorylation.

Surprisingly, although infection activates PP1, which is a pleiotropic enzyme, the general phosphorylation levels of the host do not seem to be altered. Indeed, we observed no significant change in the total levels of phosphorylated serine and threonine in total cell lysates upon infection (Figure S5B). Furthermore, we tested whether activated PP1 dephosphorylated non-histone substrates, such as AKT, a kinase known to be regulated by PP1 (Thayyullathil et al., 2011; Xiao et al., 2010). AKT phosphorylation at S473 was not altered during *S. pneumoniae* infection (Figure S5B). Therefore, PP1 activation by infection seems to have some degree of substrate specificity. However, we cannot rule out that other proteins besides histone H3 are dephosphorylated during infection.

### H3S10 Dephosphorylation Correlates with Transcriptional Repression of Inflammatory Genes but Is Not Required

Beyond correlation with the cell cycle, H3 phosphorylation has been associated with transcriptional activation of inflammatory genes downstream of lipopolysaccharide (LPS) stimulation (Jofsefowicz et al., 2016). We, therefore, hypothesized that the role of H3 dephosphorylation could be to downregulate inflammatory genes during infection. To test this, we measured the expression of a panel of 26 pro-inflammatory genes after infection with WT *S. pneumoniae*, which induces H3 dephosphorylation, and a double  $\Delta$ ply $\Delta$ spxB mutant, which does not. We focused on genes that were differentially regulated between WT and the  $\Delta$ ply $\Delta$ spxB mutant and found seven: *CCL2*, *CCL4*, *CCL5*, *CXCL1*, *TNF*, *CSF2*, and *IL1A*. Strikingly, although infection in WT bacteria did not significantly change the level of gene expression, the double mutant increased gene expression compared with uninfected cells (Figure 6). In contrast, expression levels of a control gene, *HPRT1*, were not altered. These results suggest that *S.*





**Figure 5. Bacterial Infection Induces Dephosphorylation of PP1**

(A) A549 cells were infected for 3 h with WT and the indicated mutants of *S. pneumoniae* strain TIGR4 (MOI = 25). A representative immunoblot (left) and a quantification (right) of four independent experiments are shown. Anti-phospho-PP1a (Thr320) antibody (Cell Signaling, 2581s) recognizes all PP1 isoforms (Shimada et al., 2010). The PP1 T320ph levels are normalized to PP1a and to the uninfected control condition. *S. pneumoniae*. Error bars represent SD, and statistical significance was calculated by one-way ANOVA method (Dunnett's post hoc test, uninfected as control group). \*\*p < 0.01.

(B) The correlation of H3S10ph levels and PP1 T320ph levels of infected cells from four independent experiments is calculated by the nonparametric Spearman's correlation coefficient method (C) Immunofluorescence of PP1 T320ph in A549 cells under uninfected, 3-h WT TIGR4 infection, 3-h  $\Delta ply\Delta spxB$  double mutant infection, and 3-h WT infection at MOI = 25 with 3-h Tautomycin pretreatment conditions. Size bars represent 50  $\mu m$ . Quantification of nuclear PP1 T320ph fluorescence intensity from at least two independent experiments, for more than 300 cells counted for each condition.

(D) HeLa cells were infected for 3 h with the WT *Listeria* (EGD strain) and its *hly* mutant at MOI = 50. A representative immunoblot of three independent experiments is shown. See also Figure S5.

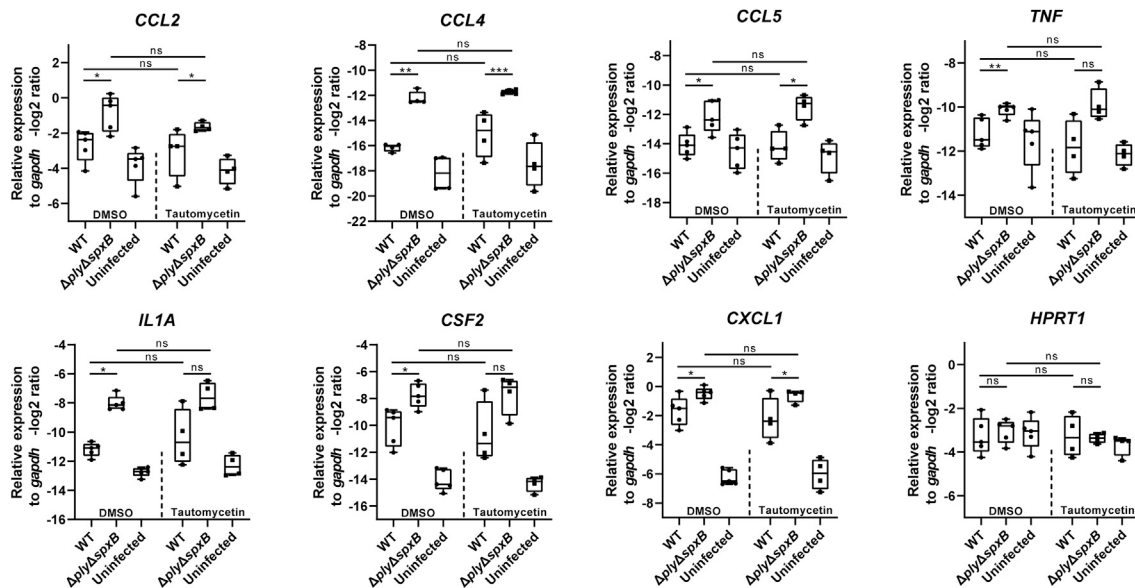
### Blocking H3S10 Dephosphorylation through PP1 Inhibition Impairs Efficient Intracellular Infection

To determine whether PP1-mediated dephosphorylation was necessary for bacterial infection, we assessed the effect of blocking PP1 catalytic activity on infection with the chemical inhibitor tautomycin. *In vitro* *S. pneumoniae* mainly

*pneumoniae* actively suppressed inflammatory gene transcription in epithelial cells in a manner that is dependent on PLY and SpxB.

To determine whether *S. pneumoniae*-mediated gene suppression occurred through H3 dephosphorylation, we repeated RT-PCR analyses in the presence of the PP1 inhibitor tautomycin. Indeed, because tautomycin fully blocks H3 dephosphorylation, differential gene expression between WT and the  $\Delta ply\Delta spxB$  mutant would also be blocked in the presence of this inhibitor. However, treatment with tautomycin did not change the transcriptional repression observed upon infection with WT bacteria for the genes tested (Figure 6). These results suggest that although H3 dephosphorylation is correlated with the repression of inflammatory genes, it is not required for it. We cannot exclude that H3 dephosphorylation could be mediating transcriptional repression of other genes, which we have not yet identified.

remains adhered to the outside of epithelial cells (Novick et al., 2017; Weight et al., 2019). In some instances, however, it is able to invade and survive temporarily (approximately 24 h) inside epithelial cells, a mechanism that could be important for the "microinvasion" observed *in vivo* (Weight et al., 2019; Weiser et al., 2018). We first measured the effect of PP1 on bacterial adherence. We compared the number of bacteria recovered from cells treated or not treated with tautomycin, the PP1 inhibitor. Results in Figure 7A show that there is no significant difference between the two conditions. Similar results were obtained in evaluating the number of  $\Delta ply\Delta spxB$  mutant bacteria in the presence or absence of tautomycin (Figure 7A). We also evaluated the number of intracellular bacteria at 4 and 6 h after infection. Strikingly, upon inhibition of PP1 a slight, but significant decrease in the number of recovered intracellular bacteria was observed at both time points (Figure 7B). These results would suggest that PP1 is important for intracellular bacterial



**Figure 6. H3S10 Dephosphorylation Correlates with Transcriptional Repression of Inflammatory Genes but Is Not Required**

Untreated cells or Tautomycin-pretreated cells are infected by WT TIGR4 *S. pneumoniae* and its  $\Delta ply \Delta spxB$  double mutant at MOI = 25. RNA was extracted and qRT-PCR was performed to analyze the relative expression of the indicated inflammatory genes and the housekeeping gene *hprt1*. The gene expression levels are shown as  $-\log_2$  relative to the control gene *gapdh*; the results are shown as means and SD of four independent experiments. For statistical calculation,  $-\log_2$ -formed data are first converted to the linear form by the  $2^{-\Delta CT}$  calculation, and then tested with a Student's t test. \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001.

survival. We further tested the effect of PP1 inhibition on infection with the double  $\Delta ply \Delta spxB$  mutant. This mutant does not modify PP1, and no H3 dephosphorylation occurs. Interestingly, tautomycin has no effect on intracellular survival of the  $\Delta ply \Delta spxB$  mutant bacteria, indicating that this inhibitor is specifically blocking PP1 activation to restrict infection (Figure 7B).

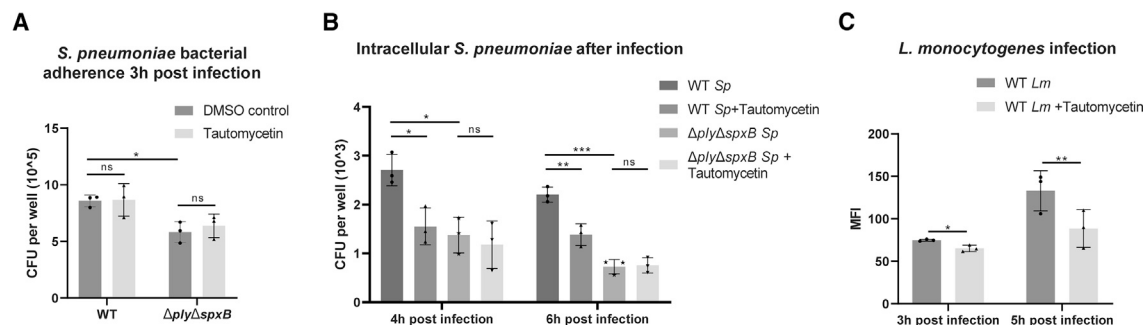
Because *L. monocytogenes*, a facultative intracellular pathogen, also activates PP1 to dephosphorylate H3, we assessed the effect of PP1 inhibition on its intracellular survival. Cells were infected with *L. monocytogenes* expressing GFP, and the fluorescence intensity of infected cells was determined by FACS analysis. Interestingly, tautomycin-treated cells were less infected than non-treated cells, further supporting a role for PP1 in intracellular bacterial survival (Figure 7C). Therefore, bacteria triggered PP1 activation and probably the downstream H3 dephosphorylation, contributing to optimal intracellular infection of at least two unrelated bacteria.

## DISCUSSION

We report here that histone H3 dephosphorylation is a common modification induced not only by pathogenic bacteria but also by a naturally colonizing bacterium, the pneumococcus. In fact, the bacterial factors involved in inducing this modification, PLY and SpxB, are common to all *S. pneumoniae* serotypes. Importantly, we demonstrate with *in vivo* samples that epithelial cells, which are the first line of defense to bacteria entering an organism, are the main cell type affected by this modification. Furthermore, the ability of bacteria to induce active PP1, and thereby H3 dephosphorylation, provides an advantage and is necessary for a productive infection.

The role of epithelial cells in a pneumococcal infection has often been overshadowed by the study of “professional” immune cells. However, more than being solely barrier cells, epithelial cells have a pivotal role in dictating pulmonary innate immune responses upon infection (Quinton and Mizgerd, 2015). In fact, selectively inhibiting canonical pathways, such as NF- $\kappa$ B signaling in epithelial cells, results in an impairment in neutrophil recruitment and poor activation of innate immune responses (Poynter et al., 2003). Furthermore, the epithelial barrier is responsible for the production of antibacterial components, such as defensins, chemokines, and toxic metabolic byproducts (Quinton and Mizgerd, 2015). Very recently, *S. pneumoniae* at mucosal surfaces was shown to shape epithelial transcriptomic response, including innate signaling and regulatory pathways and in inflammatory mediators, cellular metabolism, and stress response genes (Weight et al., 2019). Because histone modifications have an important role in regulating gene expression, their modification by bacteria has the potential to significantly alter host responses. A careful study of epithelial cell subtypes would define whether H3 dephosphorylation is found in epithelial progenitor cells or only in specialized and terminally differentiated cells. Such studies would be important to determine whether cells experiencing H3 dephosphorylation are turned over or are maintained beyond the presence of bacteria. Our findings that pneumococci modify host histones through active post-translational modification of a host phosphatase, which gives a growth advantage to bacteria, have far-reaching implications for both virulent and colonizing serotypes.

Cell intoxication by CDCs, including PLY, results in characteristic responses, which are the hallmarks of membrane damage, including ion fluxes, loss of cytoplasm, and host-cell death



**Figure 7. H3S10 Dephosphorylation Is Necessary for Efficient Bacterial Infection**

(A and B) WT *S. pneumoniae* (R6) and its  $\Delta ply \Delta spxB$  double mutant were incubated with A549 epithelial cells at MOI = 50 and assayed for either bacterial adherence (A) or intracellular bacteria (B). The results are means  $\pm$  SD from three independent experiments and statistical significance was calculated by the one-way ANOVA method (Turkey post hoc test). \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .

(C) Caco2 cells were infected with *L. monocytogenes* expressing GFP for the indicated times. Intracellular bacteria were detected by FACS analysis, and the mean fluorescence of intensity (MFI) was calculated. Results are means  $\pm$  SD over three independent experiments. In each experiment, the ratio between the inhibitor group and the untreated control group was calculated, and the statistical significance was analyzed by the Student's *t* test method. \* $p < 0.05$ , \*\* $p < 0.01$ .

(Cassidy and O'Riordan, 2013). However, at sublethal doses, membrane repair and cell signaling are also important features. Under the conditions used in this study, we did not observe a significant amount of cytotoxicity to host cells, as determined by normal cellular morphology (Figure 1) and unaltered cell cycles upon infection (Figure S1). In agreement with these findings, *in vivo* infections with TIGR4 did not induce noticeable epithelial barrier damage and an influx of inflammatory cells. Therefore, H3 dephosphorylation is most likely not linked to *S. pneumoniae*-induced cell death.

H3S10 phosphorylation is the most common histone modification linked with bacterial infection. The activation of both NF- $\kappa$ B and MAPKs, which are the major host-cell response pathways to infection, leads to H3S10 phosphorylation and associated histone acetylation, resulting in transcriptional activation of target genes (Drobic et al., 2010; Yamamoto et al., 2003). Several bacterial pathogens have developed strategies to prevent H3S10 phosphorylation by disrupting pro-inflammatory kinase signaling pathways, such as lethal toxin (LT) from *Bacillus anthracis* and type III secretion-system effector OspF from *Shigella flexneri*. Both inactivate MAPKs during infection and subsequently abrogate histone H3S10 phosphorylation, which correlates with repressing inflammatory genes (Arbibe et al., 2007; Raymond et al., 2009). However, bacteria-producing CDC toxins are the only ones, to our knowledge, to induce dephosphorylation, rather than block phosphorylation, which implies that the basal levels of modified histones are changed. In addition to CDC toxins, we show that production of H<sub>2</sub>O<sub>2</sub> by *S. pneumoniae* amplifies the observed dephosphorylation. Interestingly, SpxB was shown to affect the level of PLY release and epithelial cell-pore formation, indicating that the two proteins share a close link, even though the mechanism is unknown (Bryant et al., 2016). These observations could explain the phenotype of the  $\Delta spxB$  mutant, which displays levels of H3 phosphorylation similar to the double  $\Delta spxB \Delta ply$  mutant and to uninfected cells. The production of H<sub>2</sub>O<sub>2</sub>, a freely diffusible molecule, as a metabolic byproduct has been reported for several other bacterial species, including both opportunistic

pathogens, such as streptococci, probiotic or commensal enterococci, and lactobacilli (Hertzberger et al., 2014). Therefore, all H<sub>2</sub>O<sub>2</sub>-producing bacteria have the potential to induce H3 dephosphorylation and alter host-cell responses.

Previous attempts to link H3 dephosphorylation with a cellular effect resulted in correlative studies. We previously correlated that modification with a loss of expression of a subset of host genes (Hamon et al., 2007). However, to establish a cause-and-effect link, inhibiting H3 dephosphorylation is necessary and is only possible through inhibition of the enzyme responsible for the modification. We report here that the chemical inhibitor tautomycin blocks H3 dephosphorylation, whereas previous attempts to identify such an inhibitor failed (Hamon et al., 2007). This important step allows us to determine that, during infection with *S. pneumoniae*, PP1-mediated H3S10 dephosphorylation is not necessary for the difference in expression of inflammatory genes between WT and mutant strains. Because H3 phosphorylation is tightly linked to transcriptional regulation (Mahadevan et al., 1991; Sacconi et al., 2002; Sawicka et al., 2014), we hypothesize that *S. pneumoniae* infection may alter the expression of unidentified genes controlled by basal H3S10 phosphorylation in epithelial cells. Further studies would be necessary to identify the genes involved.

The protein phosphatase PP1 is a major protein serine/threonine phosphatase in eukaryotic cells (Peti et al., 2013). PP1 has been shown to target phosphorylated H3S10 mainly after mitosis, when H3S10 become dephosphorylated (Wu et al., 2009). The activity of PP1 is regulated through phosphorylation of T320, which is an inhibitory modification probably masking the PP1 active site and blocking PP1 substrates accessibility (Hou et al., 2013). A previous report suggested that ultraviolet irradiation induced dephosphorylation of PP1, leading to its activation and triggering subsequent histone H3T11 dephosphorylation (Shimada et al., 2010). Our data highlight that bacterial infection is able to activate PP1 through dephosphorylation, a mechanism used for its own benefit. Notably, the pathways regulated by the PP1-H3 dephosphorylation axis are conserved between at least two Gram-positive bacteria, *L. monocytogenes*

and *S. pneumoniae*, and are required for efficient intracellular infection in both models. Given the pleiotropic role of PP1, we cannot fully rule out that activation of PP1 during infection dephosphorylates other factors besides histone H3. This would imply that the role of PP1 in promoting intracellular infection could involve other hosts signaling pathways. However, our data show that general dephosphorylation upon infection does not occur and that tautomycin-mediated impairment of infection only occurs with WT bacteria, not mutant strains. Although the effect of PP1-H3 dephosphorylation on bacterial infection is slight, our findings do validate a concept that targeting host chromatin-modifying enzymes alters bacterial infection. Therefore, our study characterizes and extends the knowledge on a conserved mechanism of host subversion aimed at downplaying host response to invading bacteria.

## STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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  - Transfection of siRNA
  - Immunofluorescence
  - Western blot analyses
  - RNA extraction and Quantitative PCR analyses
- QUANTIFICATION AND STATISTICAL ANALYSIS
- DATA AND CODE AVAILABILITY

## SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at <https://doi.org/10.1016/j.celrep.2020.02.116>.

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## AUTHOR CONTRIBUTIONS

Conceptualization, W.D. and M.A.H.; Methodology, W.D. and M.A.H.; Investigation, W.D., O.R., C.C., M.C., and M.J.G.E.; Writing – Original Draft, W.D. and M.A.H.; Writing – Review & Editing, W.D., O.R., C.C., M.C., M.J.G.E., and M.A.H.; Funding Acquisition, M.A.H.; Supervision, M.A.H.; Project Administration, M.A.H.

## DECLARATION OF INTERESTS

The authors declare no competing interests.

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## STAR★METHODS

### KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
<b>Antibodies</b>		
anti-actin	Sigma	Cat# A5441; RRID: AB_476744
anti-H3S10ph	Millipore	Cat# MC463; RRID: AB_1163440
anti-H3	AbCam	Cat# ab1791; RRID: AB_302613
anti-H3T3ph	AbCam	Cat# ab78531; RRID: AB_1566301
anti-H3T6ph	AbCam	Cat# ab222768; RRID: AB_2820248
anti-H3T11ph	AbCam	Cat# ab5168; RRID: AB_304759
anti-H3S28ph	AbCam	Cat# ab5169; RRID: AB_304760
anti-PP1a	Santa Cruz	Cat# sc-7482; RRID: AB_628177
anti-phospho-PP1a (Thr320)	Cell Signaling	Cat# 2581S; RRID: AB_330823
anti-PP1b	Millipore	Cat# 07-1217; RRID: AB_11214026
goat anti-rabbit AF647	Invitrogen	Cat# A27040; RRID: AB_2536101
anti-AKT	Cell Signaling	Cat# 2920S; RRID: AB_1147620
anti-phospho-AKT-S473	Cell Signaling	Cat# 9271S; RRID: AB_329825
anti-phospho-(Ser/Thr) Phe	Cell Signaling	Cat# 9631S; RRID: AB_330308
anti-CD326	Miltenyi Biotec	Cat# 130-117-863; RRID: AB_2728051
anti-CD31	Miltenyi Biotec	Cat# 130-111-540; RRID: AB_2657296
anti-CD45	Miltenyi Biotec	Cat# 130-110-797; RRID: AB_2658218
<b>Bacterial and Virus Strains</b>		
<i>L. monocytogenes</i> EGD	Prof. P. Cossart	N/A
<i>L. monocytogenes</i> EGD expressing GFP	Prof. P. Cossart	N/A
<i>S. pneumoniae</i> strains, See <a href="#">Table S2</a>	Multiple Institutions	N/A
<b>Chemicals, Peptides, and Recombinant Proteins</b>		
Tautomycin	Tocris Bioscience	119757-73-2
Okadaic acid	Sigma	N0636
Hydrogen peroxide	Sigma	7722-84-1
PLY	Dr T. Mitchell	N/A
PLY W436A	Dr T. Mitchell	N/A
LLO	Prof. Cossart	N/A
<b>Critical Commercial Assays</b>		
Pierce Peroxide Assay Reagent	ThermoFisher	23281
iScript cDNA Synthesis Kit	Bio-Rad	1708890
<b>Experimental Models: Cell Lines</b>		
A549	ATCC	CCL-185
BEAS-2B	ATCC	CRL-9609
CaCO2	ATCC	HTB-37
HeLa	ATCC	CCL-2
<b>Experimental Models: Organisms/Strains</b>		
Mouse C57BL/6J	Janvier Laboratory	N/A
<b>Oligonucleotides</b>		
Primers for mutant construction, See <a href="#">Table S1</a>	This study	N/A
Primers for qRT-PCR, See <a href="#">Table S3</a>	This study	N/A
<b>Software and Algorithms</b>		
Flow Jo-v10	Flow Jo, LLC	<a href="https://www.flowjo.com">https://www.flowjo.com</a>
Prism	GraphPad Software	<a href="https://www.graphpad.com">https://www.graphpad.com</a>

## LEAD CONTACT AND MATERIALS AVAILABILITY

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Melanie HAMON ([melanie.hamon@pasteur.fr](mailto:melanie.hamon@pasteur.fr)). All unique/stable reagents generated in this study are available from the Lead Contact with a completed Materials Transfer Agreement.

## EXPERIMENTAL MODEL AND SUBJECT DETAILS

### Bacterial culture and cell infections

All *S. pneumoniae* strains used in this study are listed in Table S1. *S. pneumoniae* strains were grown in Todd Hewitt broth (Bacto, BD, USA) supplemented with 50 mM HEPES at 37°C with 5% CO<sub>2</sub> until the optical density at 600 nm = 0.6. *L. monocytogenes* strains were grown in brain-heart infusion medium (Difco, BD, USA) at 37°C with 5% CO<sub>2</sub> until the optical density at 600 nm = 1. Bacteria were washed twice with PBS and diluted in serum-low cell culture medium. For *S. pneumoniae* strains, a multiplicity of infection (MOI) of 50:1 for R6 and a MOI of 25:1 for TIGR4 were used unless otherwise indicated. After 3h of *S. pneumoniae* infection, cells were washed with PBS for 3 times, and then either collected for future process, or cultured in medium with penicillin (10 µg/ml) and gentamicin (100 µg/ml) for later time points. For *L. monocytogenes* strains, which are described previously (Eskandarian et al., 2013; Hamon et al., 2007), a multiplicity of infection (MOI) of 50:1 was used. After 1h of Listeria infection, cells were washed with PBS 3 times, and cultured in medium with 10 µg/ml gentamycin to carry out Listeria infection for late time points.

The adherence and intracellular assays of *S. pneumoniae* were performed using A549 cells. A total of  $5 \times 10^6$  R6 pneumococci were added to 24-well tissue culture plates containing  $1 \times 10^5$  cells each well. After 3h infection, cells were washed 3 times with PBS to remove the unattached bacteria. To determine the amount of adherent pneumococci, PBS washed cells were lysed using sterile ddH<sub>2</sub>O. Lysates and its serial dilutions were plated on Columbia blood agar plates (43059, BIOMERIEUX) overnight at 37°C with 5% CO<sub>2</sub>, and the colony forming units (CFUs) were counted as bacteria adherence. To determine the amount of intercellular pneumococci, PBS washed cells were cultured in medium with penicillin (10 µg/ml) and gentamicin (100 µg/ml) to kill extracellular bacteria. Sterile ddH<sub>2</sub>O was added at 4h time point after infection (namely 1h after PBS washing) or 6h time point after infection. Lysates and its serial dilutions were plated on Columbia blood agar plates overnight at 37°C with 5% CO<sub>2</sub>, and the colony forming units (CFUs) were counted as intercellular bacteria.

### Cell Culture

The human alveolar epithelial cell line A549 (ATCC CCL-185) cells were cultured in F-12K culture medium supplemented with 10% fetal calf serum (FCS) and 1% glutamine. The human bronchial epithelial cell line BEAS-2B (ATCC CRL-9609) cells were cultured in DMEM culture medium supplemented with 10% FCS and 1% glutamine. The human cervical carcinoma epithelial cell line HeLa (ATCC CCL-2) cells and human colon carcinoma cell line CaCO<sub>2</sub> (ATCC HTB-37) cells were cultured in MEM culture medium supplemented with 1% glutamine, 1 mM sodium pyruvate (GIBCO), 0.1 mM nonessential amino acid solution (GIBCO), and 10% (HeLa) or 20% (CaCO<sub>2</sub>) FCS. Cells were seeded in 6-well or 24-well plates 2 days before infection. When cells were grown to semi-confluence (24h time point), they were serum-starved (0.25% FCS) for 24h before use in experiments.

### Mouse Husbandry

All protocols for animal experiments were reviewed and approved by the CETEA (Comité d'Ethique pour l'Expérimentation Animale - Ethics Committee for Animal Experimentation) of the Institut Pasteur under approval number Dap170005 and were performed in accordance with national laws and institutional guidelines for animal care and use. Wild-type C57BL/6 female 8-9 week old mice purchased from Janvier Labs (France) and allowed a minimum of 7 day to acclimatize before any infections were carried out.

## METHODS DETAILS

### Use of chemical and biological and reagents

For experiments involving chemical inhibitors, cells were pretreated for 3h before infection with Tautomycin (0.8 µM, Tocris Bioscience, 119757-73-2) or Okadaic acid (0.1 µM, Sigma, N0636). Hydrogen peroxide (Sigma, 7722-84-1) was added to the cells for 1h at indicated concentrations. Purified PLY and LLO were obtained as described previously (Hamon et al., 2007). Cells were treated with 6 nM LLO or 6 nM PLY for 20 or 30 min.

### Construction of *S. pneumoniae* mutants

All primers used in construction of *S. pneumoniae* mutants are listed in Table S2. Mutants of *S. pneumoniae* were generated by transforming donor DNA into pneumococci using competence stimulating peptide (CSP) as described previously (Bricker and Camilli, 1999). To create a deletion allele with selection marker, antibiotic-resistance cassettes were fused with the upstream and

downstream flanking regions of target genes by overlapping PCR. Specifically, *ply* gene was replaced by with *erm* cassette in  $\Delta$ *ply* mutant; *lytA* gene was replaced by with *erm* cassette in  $\Delta$ *lytA* mutant; in  $\Delta$ *spxB* mutant, *spxB* gene was replaced by *kan+sacB* (KS) cassette, which is a cassette amplified from Sweet Janus cassette (Li et al., 2014). The KS cassette permits allelic replacement or marker-free knock in through sequential positive and negative selection. Therefore, we constructed *in situ* complement strains using KS cassette. Briefly, using kanamycin-resistance positive selection, targeted sequence was replaced by KS cassette in chromosome. Using sucrose sensitivity negative selection, KS cassette was further replaced by either wild-type genes or genes carrying point mutation. Point mutation was also created by overlapping PCR.

### **In vivo infections**

*In vivo* infections were performed by intranasal injection of  $5 \times 10^5$  bacteria per mouse. Lungs were collected after 20h from infection and digested using the Lung Dissociation Kit according to the manufacturer's instructions (Miltenyi Biotec) plus added Dispase II at 0.1U/ml (Roche). Briefly, lungs were instilled with 1ml dispase solution through the trachea before adding them to C Tubes and being digested on Octo Dissociator (Miltenyi Biotec). Single cell suspensions were filtered on 70um strainer before being stained to identify lung epithelial cells (CD45-CD31-CD326+). Cells were further permeabilized with Transcription Factor Staining Buffer Set (eBioscience) and stained for H3S10ph followed by secondary. Data was acquired on a MACSQuant cytometer (Miltenyi Biotec).

### **Cell synchronization and cell cycle analysis**

Cells were synchronized by a thymidine block as described (Bostock et al., 1971). For cell cycle distribution, cells were detached in PBS and fixed in 70% ethanol for 1h at  $-20^{\circ}\text{C}$ . Cells were washed and re-suspended in PBS containing 10  $\mu\text{g/ml}$  of propidium iodide and 100  $\mu\text{g/ml}$  of RNase (DNase free).

### **FACS Analyses**

Cells infected with GFP-expressing *L. monocytogenes*, cells stained with propidium iodide, and cells from lung of infected mice and stained with antibodies, were analyzed on a FACSCalibur. Data was analyzed using the FlowJo software.

### **Transfection of siRNA**

Lipofectamine 2000 (Invitrogen, 11-668-019) was used to introduce interference RNA into A549 cells. Pan PP1 siRNA (Santa Cruz, sc-43545) was transiently transfected (25 nM final concentration in well) to knockdown all isoforms of PP1. Scramble siRNA (ON-TARGETplus SMARTpool) was transiently transfected as control. Cells were assayed 48h after siRNA transfection.

### **Immunofluorescence**

Cells were grown on glass cover slides. After infection, cells were washed 3 times with PBS and fixed in 4% paraformaldehyde for 10 min at room temperature. After 3 washes, cells were permeabilized and blocked with 3% BSA with 0.5% Tween20 for 45 min. Immunostaining was performed with primary antibodies in 3% BSA+ 0.5% Tween20 for 90 min, and then Alexa Fluor 488 or 647 conjugated anti-immunoglobulin G (IgG) secondary antibodies in 3% BSA+ 0.5% Tween20 for 45 min.

### **Western blot analyses**

For western blot analysis, cells were lysed using 2  $\times$  Laemmli buffer (4% SDS, 20% glycerol, 200mM DTT, 0.01% bromophenol blue and 0.1 M Tris HCl, pH 6.8). Samples were sonicated for 5 s, boiled for 10 min, and then subjected to SDS-PAGE. Proteins were transferred to membrane under 2.5A/25V condition for 7min using a semidry transfer system (Trans-Blot Turbo, BIO-RAD). Transferred membranes were first blocked by TBS-Tween20 (0.1%) with 5% milk, and then incubated with primary antibodies overnight at  $4^{\circ}\text{C}$ . Membranes were washed with TBS-Tween20 (0.1%) and incubated with secondary peroxidase-conjugated anti-immunoglobulin G (IgG) antibodies for 1h at room temperature. After washing with TBS-Tween20 (0.1%), the immunoreactive bands were visualized using ECL substrate (Clarity Western ECL substrate, BIO-RAD) and imaged with a Western Blot detection system (ChemiDoc Imaging Systems, BIO-RAD). Quantification of western blots was performed using Image Lab (BIO-RAD).

### **RNA extraction and Quantitative PCR analyses**

The mRNA was extracted from cells using an RNeasy kit (QIAGEN, 74104). DNase treatment was performed using a DNase set (QIAGEN, 79254). cDNA was then synthesized using 1  $\mu\text{g}$  input RNA by BIO-RAD iScript gDNA Clear cDNA Synthesis Kit (1725035). Real-time PCR was performed using the SYBR Green kit (iTaQ Universal SYBR Green Supermix, BIO-RAD, 1725124) on a BIO-RAD CFX384. Data was obtained using BIO-RAD CFX manager. Relative gene expression analysis was performed using the  $2^{-\Delta\text{CT}}$  method as described (Livak and Schmittgen, 2001). All primers used in quantitative PCR analyses are listed in Table S3.



## QUANTIFICATION AND STATISTICAL ANALYSIS

The data are presented as the mean  $\pm$  SD. Student's *t* test was used for statistical analysis when compare two groups: \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ . One-way ANOVA method (Dunnett's Post Hoc test when compared to control group, and Turkey Post Hoc test when compared between groups) was used for statistical analysis when compare multiple groups: \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ . Statistical calculations were performed using SPSS.

## DATA AND CODE AVAILABILITY

This study did not generate any unique datasets or code.