E3 Ligase Subunit Fbxo15 and PINK1 Kinase Regulate Cardiolipin Synthase 1 Stability and Mitochondrial Function in Pneumonia


SUMMARY

Acute lung injury (ALI) is linked to mitochondrial injury, resulting in impaired cellular oxygen utilization; however, it is unknown how these events are linked on the molecular level. Cardiolipin, a mitochondrial-specific lipid, is generated by cardiolipin synthase (CLS1). Here, we show that S. aureus activates a ubiquitin E3 ligase component, Fbxo15, that is sufficient to mediate proteasomal degradation of CLS1 in epithelia, resulting in decreased cardiolipin availability and disrupted mitochondrial function. CLS1 is destabilized by the phosphatase and tensin homolog (PTEN)-induced putative kinase 1 (PINK1), which binds CLS1 to phosphorylate and regulates CLS1 disposal. Like Fbxo15, PINK1 interacts with and regulates levels of CLS1 through a mechanism dependent upon Thr219. S. aureus infection upregulates this Fbxo15-PINK1 pathway to impair mitochondrial integrity, and Pink1 knockout mice are less prone to S. aureus-induced ALI. Thus, ALI-associated disruption of cellular bioenergetics involves bioeffectors that utilize a phosphodegron to elicit ubiquitin-mediated disposal of a key mitochondrial enzyme.

INTRODUCTION

Acute lung injury (ALI) is a devastating disorder that occurs most often from severe bacterial pneumonia or sepsis and is commonly associated with multiorgan failure. One clinical hallmark of patients with ALI is a profound defect in cellular oxygen consumption and extraction, termed cytopathic dysoxia, the mechanisms of which are not understood at the basic level (Loiacono and Shapiro, 2010; Samsel and Schumacker, 1991; Schumacker and Samsel, 1989; Shoemaker et al., 1993; Singer, 2007). Indeed, significant efforts to enhance tissue oxygenation by increasing oxygen delivery in sepsis have proven ineffective, indicating that a mitochondrial defect contributes to the pathobiology of multiorgan failure. Thus, impaired oxygen utilization may be due to mitochondrial abnormalities and ensuing epithelial apoptosis, both of which have been extensively described in ALI subjects (Brealey et al., 2002; Protti and Singer, 2006; Singer, 2007; Svistunenko et al., 2006). Type II alveolar epithelial cells exhibit on a cellular basis the highest level of pulmonary oxygen utilization and contain ~50% of mitochondria in the lung (Crapo et al., 1978, 1980; Massaro et al., 1975). Thus, an intact mitochondrial apparatus for oxygen utilization at the epithelial cellular level is imperative for these cells to generate the chemical energy needed to maintain pulmonary homeostasis.

Cardiolipin is an integral component of inner mitochondrial membranes and essential for bioenergetics (Choi et al., 2007; McMillin and Dowhan, 2002). Hence, cardiolipin deficiency is lethal, as its impaired production is linked to impaired cell growth and viability (Ostrander et al., 2001). A final step for de novo mitochondrial synthesis of cardiolipin utilizes phosphatidylglycerol and cytidine diphosphate choline-diacylglycerol as substrates in a reaction catalyzed by the enzyme cardiolipin synthase 1 (CLS) (Schlame and Hostetler, 1997). The gene encoding CLS1 was recently cloned from yeast and mammalian sources, and CLS knockdown results in impaired cell growth and viability (Chang et al., 1998; Choi et al., 2007; Houkko and Li, 2006; Jiang et al., 1999; Lu et al., 2006). Interestingly, the CLS transcript (hCLS1) is ubiquitously expressed in tissues but low in whole lung suggestive of its posttranslational regulation (Chen et al., 2006). After initial de novo biosynthesis on the inner membrane of mitochondria, cardiolipin undergoes further remodeling in the mitochondria to incorporate unsaturated fatty acyl groups that confer its structural and biologic activity; this remodeling is
catalyzed either by CLS1 itself, tafazzin, or lysocardiolipin acyltransferase (Nie et al., 2010; Xu et al., 2010). CLS1, however, appears to exhibit reactivity activity for lysophosphatidylglycerol rather than lysocardiolipin. The data suggest that, on one hand, cardiolipin has a vitally important structural and physiologic role in mitochondria, as its deficiency leads to cytochrome C release and apoptosis (Choi et al., 2007; McMillin and Dowhan, 2002). However, because of its evolutionarily conserved resemblance to bacterial cardiolipin, the release of mammalian cardiolipin from dying cells into the extracellular space serves as a highly potent mitochondrial-derived damage-associated molecular pattern (DAMP-CL) that disrupts lung homeostasis (Ray et al., 2010).

Ubiquitination of proteins brands them for degradation either by the proteasome or via the lysosome (Tanaka et al., 2008). The conjugation of ubiquitin to a target protein is orchestrated by a series of enzymatic reactions involving an E1 ubiquitin-activating enzyme, ubiquitin transfer from an E1-activating enzyme to an E2-conjugating enzyme, and last, generation of an isopeptide bond between the substrate’s ε-amino lysine and the carboxyl-terminus of ubiquitin catalyzed by a E3-ubiquitin ligase (Hochstrasser, 2000). Of the many E3 ligases, the SCF superfamily is among the best studied (Tyers and Willems, 1999). The SCF complex has a catalytic core complex consisting of Skp1, Cullin1, and the E2 ubiquitin-conjugating enzyme (Cardozo and Pagano, 2004; Zheng et al., 2002). The SCF complex also contains an adaptor receptor subunit, termed F-box protein, that targets hundreds of substrates through phosphospecific domain interactions (Cenciarelli et al., 1999). F-box proteins have two domains: an NH2-terminal F-box motif and a carboxy-terminal leucine-rich repeat motif or tryptophan-aspartic acid (WD) repeat motif. The SCF complex uses the F-box motif to bind Skp1, whereas the leucine-rich/WD repeat motif is used for substrate recognition (Ilyin et al., 1999). However, there is a subclass of F-box proteins that lacks a distinct carboxy-terminal motif, thus named F-box domain only proteins (Fboxos). Of this protein family, the promoter of FBXO15 was used as reporter to screen pluripotent cells (Okita et al., 2007); the authentication of FBXO15 as a ubiquitin E3 subunit and its definitive substrate remains unknown, although a recent study suggests it may control levels of p-glycoprotein (Katayama et al., 2013).

Phosphatase and tensin homolog (PTEN)-induced putative kinase 1 (PINK1) is a mitochondrial serine/threonine-protein kinase encoded by the PINK1 gene (Unoki and Nakamura, 2001). Loss of function mutations in the PINK1 gene are critically linked to mitochondrial dysfunction and early onset Parkinson’s disease (Valente et al., 2004). PINK1 contains an NH2-terminal mitochondrial-targeting sequence and is predicted to have an important role in mitochondrial function (Mills et al., 2008). PINK1 is able to access mitochondria to regulate degradation of mitochondrial respiratory chain subunits (Vincow et al., 2013). One model suggests that PINK1 accumulates on the outer mitochondrial membrane after cell stress to recruit parkin (a ubiquitin E3 ligase) and regulate mitochondrial removal by mitophagy (Bieler, 2009). Loss of function mutations in parkin are also etiologically linked to familial forms of Parkinson’s disease (Bonifati, 2005; Ziviani et al., 2010). To date, parkin, mitochondrial protease HtrA2, mitochondrial chaperone TRAP1, and Akt are the only known molecular targets described for Pink1 (Murata et al., 2011; Plun-Favreau et al., 2007; Pridgeon et al., 2007; Sha et al., 2010). In this study, we have identified the phospho-enzyme CLS1 as a PINK1 target. PINK1 docks at a CLS1 sequence containing Thr219 to signal recruitment of Fbxo15 in lung epithelial cells leading to ubiquitination and degradation of CLS1, reduced cardiolipin synthesis, and apoptosis. Moreover, S. aureus infection exploits this PINK1-Fbxo15 pathway to impair mitochondrial integrity and produce lung injury. These results provide a unique mechanistic model for studying dysregulated oxygen metabolism and impaired mitochondrial integrity observed in severe pneumonia-associated ALI.

RESULTS

CLS1 Is Essential for Maintaining Cardiolipin Production and Mitochondrial Function

Human lung epithelial (A549) cells were transfected with control plasmid, CLS1-small hairpin RNA (shRNA), or a plasmid encoding the CLS1 gene. To examine de novo cardiolipin synthesis, content of phospholipids was assessed by normal phase liquid chromatography- mass spectrometry (MS) revealing decreased cardiolipin and an increase in the substrate, phosphatidylglycerol after CLS1-shRNA (Figure 1A). Further, CLS1-shRNA resulted in reduced CLS1 protein levels, whereas CLS1 plasmid overexpression increased cardiolipin synthesis and CLS1 protein levels (Figure 1B). Cells transfected with control shRNA or CLS1-shRNA were also assayed for ATP levels, indicating significantly decreased ATP levels with CLS1 knockdown, raising the possibility that cell viability is impaired (Figure 1C). Indeed, CLS1 knockdown induced apoptosis indicated by caspase 3 cleavage (Figure 1D, arrows). To evaluate mitochondrial function, transfected cells were stained with fluorescent DIOC2(3). A low-fluorescent subpopulation was observed using fluorescence-activated cell sorting (FACS) with CLS1 knockdown, which indicates mitochondrial membrane potential loss (Figure 1E). Interestingly, CLS1 knockdown also dramatically changed the morphology of mitochondria within the cells from healthy rod-shaped to large dense granules (Figure 1F). Last, JC-1 dye was used to stain mitochondria. JC-1 is a cationic dye that exhibits potential-dependent accumulation in mitochondria, indicated by a fluorescence emission shift from green (~525 nm) to red (~590 nm). CLS1 knockdown led to mitochondrial depolarization as indicated by a decrease in the red/green fluorescence intensity ratio (Figure 1G).

FBXO15 Targets CLS1 Protein for Ubiquitination and Degradation, thereby Disrupting Mitochondrial Function

We embarked on an unbiased screen testing proteins that might be involved in CLS1 degradation. Over 27 randomly selected F-box proteins (FBXL, FBXW, and FBXO family members) were ectopically expressed in lung epithelial cells (Figures S1A–S1C) and cells collected and lysates analyzed for expression of F-box proteins and endogenous CLS1. Only expression of FBXO15 decreased CLS1 levels. To confirm the specificity of FBXO15 targeting, we performed coimmunoprecipitation (co-IP) experiments. A549 cells were lysed and subjected to CLS1 IP.
Of six E3 ligases subunits tested, only Fbxo15 was detected in association with CLS1 (Figure S1D). Further, ectopic Fbxo15 expression significantly decreased half-life (t1/2) of CLS1 (Figure 2A). Addition of the proteasomal inhibitor, MG132, to cells led to a significant increase in CLS1 protein half-life, whereas this was not seen with the lysosomal inhibitor, leupeptin (Figure 2B). Thus, endogenous Fbxo15 targets CLS1 for possible ubiquitination. To identify the ubiquitination acceptor site within CLS1, we used a variety of biochemical assays and microscopy techniques to monitor mitochondrial function and structure. These included assays for cardiolipin and phosphatidylglycerol levels, ATP levels, caspase 3 activity, mitochondrial membrane potential, and mitochondrial morphology.

Figure 1. CLS1 Is Essential for Preserving Mitochondrial Function and Structure

(A) A549 cells were transfected with control plasmid shRNA, CLS1-shRNA, or a plasmid encoding CLS1 for 48 hr. Shown is LC-MS assessment of cardiolipin and phosphatidylglycerol in A549 cells. Typical negative-mass spectra of cardiolipin (left, CL) and phosphatidylglycerol (right, PG) obtained from A549 cells above. Below is shown content of CL and PG in control A549 cells and cells after knockdown of CLS1. Content of phospholipids was assessed by normal-phase LC-MS. The results are presented as mean ± SD; n = 3. Statistical analyses were performed by Student’s t test. The statistical significance of differences was set at p < 0.05.

(B) A549 cells were transfected with control (CON) plasmid, CLS1-shRNA, or a plasmid encoding CLS1 for 48 hr. Cells were also labeled with [3H]-palmitic acid for an additional 24 hr after plasmid transfection. Cells were collected and lipids extracted and separated by thin-layer chromatography (TLC). The lipids were quantified using a plate reader, and the radioactivity of the spots was counted and graphed (results are presented as mean ± SE; n = 3 experiments; *p < 0.05 versus CON). Below: cell lysates were assayed for levels of immunoreactive hCLS1 and actin proteins.

(C) ATP levels were assayed in cells using the Cell-Glo ATP assay following the manufacturer’s protocol (results are presented as mean ± SE; n = 5 experiments; *p < 0.01 versus CON). RLU, relative light units.

(D) A549 cells were transfected with control shRNA or CLS1-shRNA for 48 hr, and cells were collected and assayed for caspase 3 immunoblotting, indicating cleavage products (arrows).

(E) A549 cells were transfected with control shRNA or CLS1-shRNA for 48 hr, and cells were stained with fluorescent DiOC2(3) dye followed by FACS analysis.

(F) Cells were stained with MitoTracker Green to visualize mitochondria; cells were then analyzed by confocal microscopy.

(G) A549 cells were stained with JC-1 dye; the fluorescence emission at channel green (∼525 nm) and red (∼590 nm) was recorded. Scale bar represents 10 μM.
CLS1, a candidate-mapping approach was used where CLS1 lysine mutants were constructed and synthesized in vitro before testing using ubiquitination assays (Figure S2A). Of several CLS1 point mutants tested, only CLS1-K174R exhibited partial resistance to SCFFbxo15-directed polyubiquitination (Figure S2B). Finally, the K174R mutant exhibited an extended t1/2 compared to wild-type CLS1 (Figure S2C).

To evaluate the biological role of Fbxo15, A549 cells were transfected with an empty plasmid, Fbxo15-shRNA, or a plasmid encoding the Fbxo15 gene prior to assays for cardiolipin synthesis. Fbxo15 plasmid expression significantly decreased cardiolipin synthesis and CLS1 protein levels, whereas Fbxo15 knockdown significantly increased cardiolipin production and CLS1 protein levels (Figures 2C and 2D). Hence, Fbxo15 regulates cardiolipin biosynthesis via CLS1 in cells. Next, cells were transfected with Fbxo15 plasmid to assess mitochondrial morphology. Interestingly, ectopic expression of Fbxo15 plasmid dramatically changed the morphology of mitochondria within cells from healthy rod-shaped structures to large dense granules (Figure 2E). JC-1 staining revealed that Fbxo15 plasmid overexpression leads to mitochondrial depolarization as indicated by a decrease in the red/green fluorescence intensity ratio (Figure 2F). These results were also confirmed by FACS analysis and DiOC2(3) staining indicative of a low-fluorescent subpopulation with Fbxo15 plasmid overexpression, suggestive of mitochondrial membrane potential loss (Figure 2G). Fbxo15 plasmid

Figure 2. Fbxo15 Targets CLS1 Protein for Ubiquitination and Degradation to Disrupt Mitochondrial Function
(A) CLS1 protein half-life determination with Fbxo15 overexpression or without (CON; n = 3 experiments; *p < 0.01 versus CON).
(B) CLS1 protein half-life determination using cyclohexamide (CHX) without or with MG132 or leupeptin (LEU) (n = 3 experiments; *p < 0.01 versus CON).
(C and D) A549 cells were transfected with control shRNA, Fbxo15-shRNA, or a plasmid encoding Fbxo15 for 48 hr. Cells were labeled with [3H]-palmitic acid for additional 24 hr. Cells were collected and lipids extracted and separated by TLC. The TLC plate was quantified using a plate reader, the radioactivity of spots counted and graphed in (D), and samples processed for immunoblotting below (n = 3 experiments; *p < 0.05 versus CON or empty plasmid).
(E and F) A549 cells were transfected with Fbxo15 before staining with MitoTracker Green (E) or JC-1 (F).
(G) A549 cells were transfected with increasing amounts of Fbxo15 plasmid before staining with DIOC2(3); cells were then analyzed by FACS.
(H) ATP levels were assayed in Fbxo15-transfected A549 cells using a Cell-Glo ATP assay (n = 5 experiments; *p < 0.01 versus CON).
(I) Cells were transfected with increasing amounts of Fbxo15 plasmid. Cells were collected and cell lysates analyzed for V5, CLS1, and β-actin immunoblotting (n = 2). Scale bar represents 10 μM.

Results in (A), (B), (D), and (H) are presented as mean ± SE.
overexpression also significantly decreased ATP levels (Figure 2H) coupled with a dose-dependent decrease in immunoreactive CLS1 content (Figure 2I).

**PINK Regulates CLS1 Stability**

To determine the Fbxo15 docking site within the CLS1, a deletion-mapping approach was used where truncated CLS1 mutants were constructed and synthesized in vitro. These synthesized his-tagged mutant CLS1 proteins were then subjected to copurification with Fbxo15 using a cobalt column. All CLS1 constructs were successfully synthesized in vitro, and CLS1 C200 and C150 mutants lacked ability to bind Fbxo15 (Figure S3A). Thus, Fbxo15 docks within the enzyme spanning the region of 200–250 amino acids (aas) within CLS1. Closer examination of this region revealed several potential phosphorylation sites determined by a Net-Phos program (Blom et al., 1999). Site-directed mutagenesis of candidate CLS1 phosphorylation sites was performed, followed by in vitro synthesis and copurification with Fbxo15 using a cobalt column. Of several mutants tested, only a CLS1-T219A lacked ability to bind Fbxo15 (Figure S3B). Thus, T219 within CLS1 serves as a potential molecular recognition site for Fbxo15 kinase, consistent with the behavior of SCF members to target phosphodegrons within substrates. Alternatively, this site could serve as a docking or phosphorylation site for a kinase that regulates binding of Fbxo15 to CLS1 indirectly.

To first identify if CLS1 is phosphorylated, A549 cells were lysed and subjected to CLS1 IP and samples probed using phospho-threonine antibodies; the results revealed a band that migrates at the predicted size of CLS1 (Figure 3A). To identify a kinase that phosphorylates CLS1, we performed co-IP experiments. A549 cells were lysed and subjected to CLS1 IP and samples also probed with antibodies against several candidate kinases. Of seven kinases tested, only PINK1 was detected in the CLS1 immunoprecipitates (Figure 3B). In vitro kinase assays using recombinant proteins under various control conditions indicated that PINK1 directly phosphorylates CLS1 (Figure 3C). Additional mapping and pull-down studies indicated that PINK1 kinase also docks within the 200–250 aas region of CLS1 requiring T219 (Figures 3D and 3E). Similar to Fbxo15, knockdown of PINK1 kinase using shRNA increased CLS1 levels, whereas overexpression of PINK1 plasmid decreased CLS1 levels (Figure S4A). Last, protein half-life studies demonstrated that the T219A CLS1 mutant exhibited a significantly extended t1/2 compared to wild-type enzyme and other mutants tested (Figures S4B and S4C). Physiologically, PINK1 plasmid expression in cells reduced cardiolipin synthesis, ATP levels, and mitochondrial morphologic integrity (Figures 3F–3I), whereas PINK1 knockdown stimulated cardiolipin synthesis (Figure 3F). Additional studies suggest that CLS1 is degraded within the cytosol rather than in the mitochondria because ectopic PINK1 or Fbxo15 plasmid expression was sufficient to reduce immunoreactive CLS1 in soluble fractions of cells and Fbxo15 levels were not detected in mitochondria even after ectopic Fbxo15 plasmid expression (Figures S5A and S5B). Last, PINK1 depletion using shRNA resulted in reduced levels of polyubiquitinated CLS1 suggesting that the kinase was
required for Fbxo15 recruitment to the cardiolipin biosynthetic enzyme (Figure S5C).

S. aureus Activates an Fbxo15/PINK1 Pathway to Impair Mitochondrial Function via CLS1

When S. aureus was given to mice (1 × 10⁷ cfu/mouse i.t.) for 3 hr or 6 hr, mice were euthanized and lungs harvested and assayed for CLS1, Fbxo15, PINK1, actin, and phospho-threonine (phospho-T) protein levels. (B) Lung tissues from (A) were collected, lysed, and subjected to CLS1 IP, followed by Fbxo15, PINK1, actin, and phospho-threonine immunoblotting. HC, heavy chain; LC, light chain.

To evaluate effects of S. aureus on murine epithelia, lung type II cells were isolated and cultured with the pathogen. We observed morphologic changes and depolarization in mitochondria by MitoTracker and JC-1 staining (Figure S6A). Similarly, S. aureus also increased levels of immunoreactive Fbxo15 and PINK1 and decreased levels of CLS1 in murine type II cells (Figure S6B).

S. aureus also reduced cardiolipin production and increased cardiolipin levels.

**Figure 4. S. aureus Impairs Cardiolipin Biosynthesis and Mitochondrial Function**

(A) S. aureus was given to mice (1 × 10⁷ cfus/mouse i.t.) for 3 hr and 6 hr. Mice were euthanized and lungs harvested and assayed for CLS1, Fbxo15, PINK1, actin, and phospho-threonine (phospho-T) protein levels.

(B) Lung tissues from (A) were collected, lysed, and subjected to CLS1 IP, followed by Fbxo15, PINK1, actin, and phospho-threonine immunoblotting. HC, heavy chain; LC, light chain.

(C and D) A549 cells were infected with S. aureus at moi = 10 and 100 for 16 hr before labeling with [3H]-palmitic acid and assayed for cardiolipin production (C) and secretion (D).

(E) Cells were transfected with CON shRNA, PINK1-shRNA, and/or Fbxo15-shRNA before exposure to S. aureus (S. au). Cells were then stained with JC-1 and observed under confocal microscopy.

(F) A549 cells were transfected with plasmids encoding either Fbxo15 or PINK1 or exposed to S. aureus at moi = 50 for 16 hr; cells were washed and analyzed using Seahorse technology to measure oxygen consumption rates.

(G) A549 cells were transfected with either empty plasmid or CLS1 plasmid for 24 hr before exposure to S. aureus at moi = 20 or 100. Cells were then collected and assayed for ATP levels and CLS1 immunoblotting. n = 3 experiments; *p < 0.05 versus 0 moi.

(H) Human lungs (n = 6 control and six pneumonia lung tissue samples) were assayed for CLS1, Fbxo15, and PINK1 protein levels by immunoblotting; relative protein levels were graphed using densitometry (n = 3 in C, D, and G; n = 4 in F; n = 6 in H; *p < 0.05 versus CON in C, D, F, and H; data presented as mean ± SE). Scale bar represents 10 μM.
release of this mitochondrial-specific DAMP into culture medium (Figures 4C and 4D). JC-1 staining revealed that double knockdown of Fbxo15 and PINK1 protein attenuated mitochondrial depolarization caused by S. aureus (Figure 4E). Importantly, oxygen consumption rates in cells were significantly reduced by PINK1 or Fbxo15 plasmid overexpression or after S. aureus infection (Figure 4F). These results correlated with decreased cardiolipin mass in cells after PINK1 or Fbxo15 plasmid overexpression (Figure S6C). Further, expression of CLS1 plasmid in cells was sufficient to rescue S. aureus-induced decreases in ATP production (Figure 4G). Immunoblot analysis from subjects with pneumonia revealed increased PINK1 and Fbxo15 content and decreased CLS1 protein levels (Figure 4H).

**Lentiviral Fbxo15 Gene Transfer Induces Acute Lung Injury**

To assess the biological significance of Fbxo15, we expressed the F box protein in vivo. We hypothesized that Fbxo15 gene transfer will decrease CLS1 protein levels, thus reducing mitochondrial function, accentuating pulmonary injury. In mice, lentiviral Fbxo15 gene transfer decreased CLS1 protein levels (Figure 5A). These effects were associated with increased lung resistance and elastance (lung stiffness), decreased compliance (volume/pressure; Figures 5B–5E), and increased lavage protein concentration and lavage cell counts (Figures 5F and 5G); the F box protein also significantly produced pulmonary edema evidenced by extravasation of Evans blue dye into the lung fluid (Figure 5H). Thus, overexpression of Fbxo15 mimics many of the physiologic effects seen with S. aureus infection in vivo.

**Pink1 Knockout Mice Have Reduced Severity of S. aureus-Induced Acute Lung Injury**

To assess the biological significance of PINK1, mice deficient in the kinase were infected with S. aureus (Figure 6). The baseline levels of values of lung mechanics in control mice differed in Pink1/C0/C0 mice versus mice examined in Figure 5 because of experimental design (strain differences; all mice were infected with S. aureus). Nevertheless, Pink1−/− mice compared to wild-type littermates had better compliance and reduced lung resistance and elastance (Figures 6A–6D) and decreased lavage protein concentration (Figure 6E). Interestingly, lavage cell counts, bacterial load, and lung histology was not altered between the groups (Figures 6F–6H). Importantly, levels of cardiolipin in lung fluid were significantly reduced in Pink1−/− mice versus wild-type mice (Figure 6I), consistent with reduced mitochondrial injury. These results suggest that PINK1 regulates vulnerability to microbial lung injury with limited effects on alveolar inflammation. Moreover, the upregulation of PINK1 by S. aureus plays an important role in the pathophysiology of this infection. However, these studies were not designed to assess the long-term effects of PINK1 depletion on other parameters such as survival after S. aureus infection. This will be important to fully understand the biological role of this kinase in experimental pneumonia.

**DISCUSSION**

A profound mitochondrial defect exists during ALI and multorgan failure, the molecular basis of which is unknown. Here, we show that S. aureus infection of epithelia implicated in severe ALI degrades the indispensable mitochondrial enzyme, CLS1, required for cardiolipin biosynthesis. S. aureus is a major cause of nosocomial and community-acquired pneumonia but also severe sepsis and endocarditis (Watkins et al., 2012). S. aureus-induced pneumonia is common and potentially life-threatening (Osiyemi and Dickinson, 2000; Ragle et al., 2010; Rello et al., 2005; Sidorova and Domnikova, 1999). In recent years, more...
and more S. aureus isolates exhibit methicillin resistance, further presenting a significant challenge in its eradication. Thus, based on its importance in the clinical arena and ability to trigger a mitochondrial apoptotic pathway, this pathogen was selected to assess effects on cardiolipin metabolism (Haslinger et al., 2003). We provide evidence of the molecular regulation of CLS1 protein, demonstrating that a poorly characterized F box protein, Fbxo15, is recruited to CLS1, resulting in its polyubiquitination and proteasomal elimination. Indeed, Thr219 within CLS1 is a critical molecular recognition or docking site for PINK1; this site also signals Fbxo3 recruitment leading to CLS1 polyubiquitination at K174. Fbxo15 alone is sufficient to significantly impair mitochondrial oxygen utilization and lung stability, whereas PINK1 is required to mediate adverse effects of S. aureus on pulmonary injury. As a whole, our data suggest that S. aureus severely disrupts cardiolipin biosynthesis perhaps through ubiquitination at the CLS1 step, which then leads to extracellular release of preformed cardiolipin (Figures 4D and 7). We have previously shown that extracellular cardiolipin potently disrupts lung homeostasis and recapitulates many features of pneumonia (Ray et al., 2010). Hence, our data suggest that, as one mechanism, severe bacterial infection triggers phosphorylation-dependent ubiquitination of a key substrate (CLS1) as a means to release a mitochondrial-derived DAMP to elicit pulmonary dyshomeostasis.

Aside from few initial studies (Chen et al., 2006; Su and Dowhan, 2006), the structure-function and posttranslational control of CLS1 is largely unknown. There appears to be tight coordination between translation and transcription of CLS in yeast (Su and Dowhan, 2006). Further, thyroxine has been shown to increase hepatic enzyme activity (Hostetler, 1991) and lipopolysaccharide decreases CLS mRNA levels in the liver (Lu et al., 2011). CLS1 normally resides within the inner membrane of mitochondria (Chen et al., 2006), where it could potentially colocalize with a pool of PINK1 (Meissner et al., 2011). However, due to lack of a canonical mitochondrial targeting signal, it is unlikely that Fbxo15 traffics inside the mitochondria. More likely, our data suggest that Fbxo15 mediates ubiquitinylation and degradation of CLS1 within the endoplasmic reticulum (ER) after its biosynthesis, perhaps as a feedback regulatory mechanism. Indeed, fractionation studies suggest that mitochondria are devoid of significant concentrations of Fbxo15 under native conditions or after ectopic expression of Fbxo15 (Figure S5). Yet F box protein or PINK1 plasmid expression reduces CLS1 immunoreactive levels in the cytosol and in the mitochondria (Figure S5). These results suggest that, once Fbxo15-mediated CLS1 depletion occurs in
The ability of PINK1 to mediate turnover of mitochondrial proteins is not unusual given its role in modulating stability of membrane-bound respiratory chain subunits (Vincow et al., 2013). Further, compared to wild-type littermates, Pink1<sup>−/−</sup> mice were less prone to S. aureus-induced injury, as reflected by better lung mechanics and lower protein concentrations, with no effect on inflammatory cell recruitment in lung fluid. These results suggest that S. aureus primarily impairs lung biophysical properties (e.g., reduces surfactant activity) in a PINK1-dependent manner. Interestingly, S. aureus also exploits PINK1 to phosphorylate other substrates such as AKT and Parkin, as PINK1 knockdown effectively prevents S. aureus-induced phosphorylation of AKT and Parkin (Figure S7).

Importantly, cardiolipin levels were reduced in lung fluid of infected Pink<sup>1<sup>−/−</sup></sup> mice versus control mice, indicating that PINK1 depletion attenuates the ability of S. aureus to trigger release of cardiolipin-DAMP. Hence, PINK1 depletion may be protective to microbial virulence under some conditions and its varying roles might simply relate to differences in the cellular systems studied. This is similar to the effects of PINK1 on mitochondrial fission, in which apparently opposite effects are observed in cultured mammalian neurons compared to Drosophila muscle and spermatids (Dagda et al., 2009; Deng et al., 2008; Lutz et al., 2009).

In this regard, a key feature of lung epithelia is that they are highly enriched with mitochondria, suggesting a need in these cells for mechanisms to provide feedback inhibition to ensure tight homeostatic control of levels of proteins within the mitochondrial apparatus. Hence, PINK1 and Fbxo15 might represent a constitutive mechanism to limit an overabundance of CLS1, and yet this pathway could to be exploited in settings of pulmonary inflammation during severe infection with highly virulent bacterial pathogens.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Transfection**

A549 cells were cultured in F12/K medium (Gibco) supplemented with 10% fetal bovine serum. For half-life studies, cells were treated with cycloheximide (40 μg/ml) at different time points in blank medium. Cells lysates were prepared by brief sonication in 150 mM NaCl, 50 mM Tris, 1.0 mM EDTA, 2 mM dithiothreitol, 0.025% sodium azide, and 1 mM phenylmethylsulfonyl fluoride (buffer A) at 4°C. All plasmids were delivered into cells using Fugene HD. All plasmid constructs were generated using PCR-based strategies using appropriate primers; point mutants were generated using a site-directed mutagenesis kit (Chen and Mallampalli, 2009).

**S. aureus Infection**

A549 or murine type II cells were cultured in 6-well dishes at 0.5 million cells/plate for 24 hr before infection. S. aureus (A) was purchased from American Type Culture Collection (ATCC). Inoculums were freshly prepared prior to experiments using frozen stocks of S. aureus (ATCC strain 29213, frozen at midlog phase; optical density 625 = 0.8). S. aureus was maintained in tryptic soy broth minimal agar. Cultures were plated and grown overnight from frozen stock. Overnight plate cultures were then inoculated in tryptic soy broth and grown by rotary shaking at 37°C to log phase. Cells were then infected with S. aureus at multiplicity of infection (moi) = 10, 50, or 100 for 1, 2, or 16 hr.

**Coimmunoprecipitation**

Five hundred micrograms of total protein from cell lysates was preclarified with 20 μl of protein A/G beads for 1 hr at 4°C. Five micrograms of primary antibody was added for 18 hr incubation at 4°C. Forty microliters of protein A/G beads

---

**Figure 7. Model of Mitochondrial Damage during ALI**

S. aureus infection induces expression of a kinase, PINK1, that phosphorylates an indispensable protein CLS1, which in turn triggers CLS1 ubiquitination and degradation by the F box protein (SCFFbxo15), leading to decreased cardiolipin production. Extracellular release of a preformed pool of cardiolipin, and mitochondrial dysfunction. Fbxo15-PINK1 activation may contribute to cytopathic dysoxia observed in pneumonia. This pathway partakes in the pathobiology of pneumonia and acute lung injury (ALI).
were added for an additional 6 hr of incubation. Beads were slowly centrifuged and washed five times using 90 mM HEPES, 150 mM NaCl, 0.5 mM EGTA, 50 mM NaF, 10 mM Na3VO4, 1 mM phenylmethylsulfonyl fluoride, 20 μM leupeptin, and 1% (v/v) Triton X-100 (radio-immunoprecipitation assay) buffer, as described (Mallampalli et al., 2000). The beads were heated at 100°C for 5 min with 80 μl of protein sample buffer prior to SDS-PAGE and immunoblotting. CLS1 (Novus; Santa Cruz Biotechnology), Fbxo15 (Novus; GeneTex), and PINK1 primary antibodies (Abcam) were used at 1:500 to 1:1,000 dilution in immunoblotting.

Mass Spectrometry and Liquid Chromatography
Cellular lipids were extracted using the method of Folch (Folch et al., 1957). Cardiolipin resolved using high-performance thin-layer chromatography and analyzed by liquid chromatography (LC)/MS using a Prominance high-performance liquid chromatography system (Shimadzu) with a reverse phase C8 column (Luna; 5 micron; 4.6 mm × 15 cm; Phenomenex). An isotropic solvent system (2-propanol: water: triethylamine: acetic acid; 450:50:2:5:2.5: v/v/v/v) was used at a flow rate of 0.4 ml/min. Spectra were analyzed on a Q-TOF Premier mass spectrometer (Waters). Parameters of MS and details were described previously (Tyurina et al., 2011).

Microscopy and Immunostaining
All the microscopy work was performed using a Nikon A1 confocal microscope using a 60× oil objective. The microscope was equipped with Ti Perfect Focus system and Tokai Hit live cell chamber providing a humidified atmosphere at 37°C with 5% CO2. Transfected cells (2 × 104) were plated at 70% confluency on 35 mm MatTek glass bottom culture dishes before being labeled with either MitoTracker Red (1:5,000) or JC-1 (1:100) for 20 min. Image analysis was by Nikon NIS-element and imageJ software.

Mitochondrial Lipids and Bioenergetics
A549 cells were transfected with control shRNA, Fbxo15-shRNA or PINK1 shRNA, or plasmids encoding either Fbxo15 or PINK1 for 48 hr. Cells were labeled with [3H]-palmitic acid for additional 24 hr for determination of cardiolipin synthesis. Cells and medium were collected and lipids extracted and separated by thin-layer chromatography (TLC) (Ray et al., 2010). Individual phospholipids on the TLC plates were quantified using a plate reader. ATP was assayed using a CellTiter-Glo Assay kit (Promega). Oxygen consumption in cells was assayed using an XF Analyzer (Seahorse Biosciences).

Animal Studies
Male C57BL/6 mice (purchased from Jackson Laboratories) were acclimated to all federal and institutional animal care guidelines and under a University of Pittsburgh Institutional Animal Care and Use Committee-approved protocol. Pink1 knockout mice are maintained as a heterozygous line on a C57/129/C57/129 background and crossed to generate PINK1+/+ and PINK1−/− littermates (Dagda et al., 2011). Mice were deeply anesthetized with ketamine (80–100 mg/kg intraperitoneally [i.p.]) and xylazine (10 mg/kg, i.p.), and then intubation with a 3/400 24-gauge plastic catheter. Replication-deficient lentivirus (Lenti) alone or Lenti-Fbxo15 (108 plaque-forming units in 50 μl of protein sample buffer prior to SDS-PAGE and immunoblotting, CLS1 (Novus; Santa Cruz Biotechnology), Fbxo15 (Novus; GeneTex), and PINK1 primary antibodies (Abcam) were used at 1:500 to 1:1,000 dilution in immunoblotting.

SUPPLEMENTAL INFORMATION
Supplemental Information includes seven figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2014.02.048.

AUTHOR CONTRIBUTIONS
R.K.M. was responsible for oversight of the studies; B.B.C. designed the study, performed experiments, analyzed the data, and wrote the manuscript; T.A.C., J.R.G., C.Z., B.E., T.D., C.K., A.C.M., S.R., T.L., and S.S. performed experiments; C.T.C., J.C., and C.L. assisted with animal experiments and provided Pink1 knockout mice; Y.Y.T. performed LC/MS of cardiolipin; C.T.C., A.R., P.R., and V.E.K. assisted with direction of studies and editorial revisions; and R.K.M. revised the manuscript and directed the study.

ACKNOWLEDGMENTS
This material is based upon work supported, in part, by the US Department of Veterans Affairs, Veterans Health Administration, Office of Research and Development, Biomedical Laboratory Research and Development. This work was supported by a Merit Review Award from the US Department of Veterans Affairs and National Institutes of Health R01 grants HL096376, HL097376, HL098174, HL081784, and P01HL114453 (to R.K.M.); HL116472 (to B.B.C.); NS065789 (to C.T.C.); and 12SDG12040330 (to C.Z.). The contents presented do not represent the views of the Department of Veterans Affairs or the United States Government.

Received: June 10, 2013
Revised: September 20, 2013
Accepted: February 28, 2014
Published: April 3, 2014

REFERENCES
cytochrome c from the inner mitochondrial membrane and accelerates stimulati-
Crapo, J.D., Barry, B.E., Foscutt, H.A., and Shelburne, J. (1980). Structural and biochemical changes in rat lungs occurring during exposures to lethal and adap-
Dagda, R.K., Cherra, S.J., 3rd, Kulich, S.M., Tandon, A., Park, D., and 
son’s disease genes parkin and parkin promote mitochondrial fission and/or 
Houtkooper, R.H., Akbari, H., van Lenthe, H., Kulik, W., Wanders, R.J., Frent-
Katayama, K., Noguchi, K., and Sugimoto, Y. (2013). FBXO15 regulates P-glycoprotein/ABCB1 expression through the ubiquitin—proteasome 
Lu, B., Xu, F.Y., Jiang, Y.J., Choy, P.C., Hatch, G.M., Grunfeld, C., and Fein-
diolipin synthase-1 mRNA expression does not correlate with endogenous cardiolipin synthase enzyme activity in vitro and in vivo in mammalian lipopoly-
saccharide models of inflammation. Inflammation 34, 247–254.


