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Host and pathogen copper-transporting P-type ATPases function antagonistically during Salmonella infection.

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\textbf{Running Title:} Macrophage ATP7A requirement in Salmonella resistance
ABSTRACT
Copper is an essential, yet potentially toxic trace element that is required by all aerobic organisms. A key regulator of copper homeostasis in mammalian cells is the copper transporting P-type ATPase, ATP7A, which mediates copper transport from the cytoplasm into the secretory pathway, as well as copper export across the plasma membrane. Previous studies have shown that ATP7A-dependent copper transport is required for killing phagocytosed Escherichia coli in a cultured macrophage cell line. Herein, we expand on these studies by generating Atp7a<sup>LysM<sup>cre</sup> mice in which the Atp7a gene was specifically deleted in cells of the myeloid lineage including macrophages. Primary macrophages isolated from Atp7a<sup>LysM<sup>cre</sup> mice exhibit decreased copper transport into phagosomal compartments and a reduced ability to kill Salmonella typhimurium compared to macrophages isolated from wild type mice. The Atp7a<sup>LysM<sup>cre</sup> mice are also more susceptible to systemic infection by S. Typhimurium than wild type mice. Deletion of the S. Typhimurium copper exporters, CopA and GolT, was found to decrease infection in wild type mice, but not in the Atp7a<sup>LysM<sup>cre</sup> mice. These studies suggest that ATP7A-dependent copper transport into the phagosome mediates host defense against S. Typhimurium, which is counteracted by copper export from the bacteria via CopA and GolT. These findings reveal unique and opposing functions for copper transporters of the host and pathogen during infection.

INTRODUCTION
Copper is an essential nutrient with redox properties that endow copper-containing enzymes with the ability to function in a wide range of biological processes (1). However, copper can become toxic if concentrations exceed the capacity of homeostatic control mechanisms. Accordingly, organisms across all phyla possess sophisticated mechanisms for sensing, chelating, compartmentalizing and transporting copper such that concentrations of this metal are sustained...
at appropriate levels to meet essential demands, while maintaining a cytoplasmic milieu that is essentially devoid of free copper. There are several mechanisms by which copper is toxic, including the production of damaging reactive oxygen species, the displacement of metal ions such as iron from functional sites within enzymes, or the adventitious binding of copper at functionally important locations within enzymes (2-5). The primary mechanisms of copper tolerance in bacteria include copper export from the cytoplasm into the periplasm or extracellular milieu (6-9), copper sequestration by metallothioneins (10) and oxidation of Cu(I) by multi-copper oxidases to generate the less toxic Cu(II) ion (11-13). There is now considerable evidence that copper tolerance pathways may be a general determinant of virulence for different bacterial pathogens. Mutations that affect copper exporters have been shown to reduce virulence of Mycobacterium tuberculosis (14, 15), Pseudomonas aeruginosa (16) and Streptococcus pneumonia (17). In Salmonella enterica s.v. Typhimurium, inactivation of the copper exporting P-type ATPases, CopA and GolT, results in reduced survival within macrophages (18). These studies suggest that copper tolerance may be a general mechanism of virulence in bacteria and that interactions with the host immune system expose microbes to toxic levels of copper. However, the underlying mechanisms are not fully understood. Hypercupremia is a well-documented host response to infection that involves the increased hepatic secretion of ceruloplasmin, an acute phase protein containing ~90% of the serum copper (19-22). Recent studies indicate that urinary ceruloplasmin is a source of bactericidal copper that protects against urinary tract infections (23). Other studies suggest that copper may play a bactericidal role within macrophages of the innate immune system (22). Studies using the RAW264.7 macrophage cell line have shown that the proinflammatory agents, lipopolysaccharide (LPS) or interferon-γ (IFN-γ) increase expression of ATP7A and stimulate trafficking of this transporter from the Golgi to phagosomal compartments (24). The phagosomal compartment is a hostile site of containment and killing of microbes engulfed by the macrophage,
and has been shown to accumulate high concentrations of copper during infection with *Mycobacteria* (25). Silencing of ATP7A expression in RAW264.7 cells attenuated their ability to kill *Escherichia coli* K12, suggesting that copper transport by ATP7A into the phagosome of macrophages represents an important mechanism of copper-mediated killing of invading microbes (24). However, the extent to which ATP7A functions against other pathogenic bacteria has not been determined in primary macrophages or in whole animals. To this end, we generated *Atp7a<sup>LysM</sup>* mice in which the *Atp7a* gene was specifically deleted in myeloid cells of the innate immune system, and tested the susceptibility of these mice to wild type- and copper-sensitive strains of *Salmonella enterica sv. Typhimurium*.

**RESULTS**

IFN-γ and LPS stimulate ATP7A trafficking to the phagosome. Previous studies using the RAW264.7 macrophage-like cell line have shown that under basal conditions ATP7A is normally localized to the trans-Golgi network and that pro-inflammatory agents IFN-γ and LPS stimulate trafficking of ATP7A to post-Golgi vesicles that partially overlap with the phagosome (24). To verify whether such trafficking occurs in primary macrophages, immunofluorescence was used to evaluate the localization of ATP7A in peritoneal macrophages incubated with or without IFN-γ or LPS. As shown in Figure 1, ATP7A was localized to the perinuclear region of untreated macrophages and significantly overlapped with the Golgi marker protein, GM130. Treatment of macrophages with LPS or IFN-γ for 16 h resulted in a more dispersed ATP7A distribution that included both the perinuclear region as well as cytoplasmic puncta (Figure 1A). To test whether these dispersed ATP7A-containing puncta correspond to the phagosome, IFN-γ-activated macrophages were exposed to fluorescently-labeled heat-killed *E. coli* to permit labeling of the phagosomal compartments. Immunofluorescence detection of ATP7A in these cells revealed that ATP7A partially overlapped with *E. coli* within the phagosomal compartment (Figure 1B). These
results using primary macrophages confirmed previous findings with RAW264.7 cells that ATP7A undergoes trafficking to vesicles and phagosomal compartments in response to pro-inflammatory agents.

**Generation of myeloid-specific Atp7a knockout mice.** To test whether ATP7A is required for macrophage-mediated bacterial killing, we generated a mouse model in which the Atp7a gene was specifically deleted in cells of the myeloid lineage, hereafter called Atp7a<sup>Δlysmcre</sup> mice. These mice were generated by crossing homozygous floxed Atp7a<sup>flox</sup> females, generated previously in our laboratory (26), with LysM-Cre males in which CRE recombinase is expressed from the LysM gene promoter (27). The resulting male Atp7a<sup>Δlysmcre</sup> mice appeared phenotypically normal and exhibited no differences in growth compared to control mice. Markers of copper status in the Atp7a<sup>Δlysmcre</sup> mice were essentially normal at 2- and 12-months of age including ceruloplasmin activity, blood hemoglobin, and copper and iron levels in liver and spleen (Supplementary Figure S1). Western blot analysis and immunofluorescence microscopy revealed a marked diminution of ATP7A protein in peritoneal macrophages isolated from Atp7a<sup>Δlysmcre</sup> compared to wild type mice (Figures 2A and 2B). There was no reduction in ATP7A protein abundance in a range of other tissues tested, suggesting that Atp7a gene deletion was specific to the myeloid lineage (Figure 2C).

**Bactericidal activity is attenuated in Atp7a<sup>Δlysmcre</sup> macrophages.** Previous work has shown that silencing of ATP7A expression in RAW264.7 macrophage-like cells attenuated bactericidal activity against a non-pathogenic strain of *E. coli* (24). To evaluate these findings in a more physiologically relevant model, we investigated the bactericidal activity of primary peritoneal macrophages from Atp7a<sup>Δlysmcre</sup> mice against the pathogenic bacterium, *S. Typhimurium.* Macrophages obtained from either wild type or Atp7a<sup>Δlysmcre</sup> mice were activated by overnight treatment with IFN-γ and then infected with wild type *S. Typhimurium* for 30 minutes to allow...
bacterial uptake. The macrophages were then washed in PBS containing gentamycin to kill extracellular bacteria, lysed and plated to enumerate surviving intracellular bacteria (uptake group). To determine bacterial survival, a second set of samples were incubated for an additional 1- or 2 hours and then subjected to lysis and plating (kill group). While there was no significant difference in the initial uptake of bacteria between wild type and Atp7aLysMcre macrophages, by 2 h there was a significantly higher percentage of bacterial recovered from Atp7aLysMcre macrophages compared to wild type macrophages (Figure 3A). Incubations longer than 2 h resulted in no recoverable bacteria from either wild type or Atp7aLysMcre macrophages (data not shown). These findings support the hypothesis that expression of ATP7A is a determinant of early bacterial survival within macrophages.

**ATP7A is required for copper delivery to bacteria within the phagosome.** To further explore the role of ATP7A in bacterial survival, we tested whether copper is delivered to bacteria upon phagocytosis by macrophages and whether this might be dependent on ATP7A. Wild type *S. Typhimurium* was grown overnight in M3 minimal medium to deplete copper content and then labeled with a copper probe, CF4, which fluoresces upon copper binding (28). Under these conditions, the CF4-labeled bacteria exhibit little detectable fluorescence, but showed a marked increase in fluorescence upon exposure to copper (Supplementary Figure S2). Phagocytosis of CF4-labeled *S. Typhimurium* by wild type macrophages resulted in a marked increase in fluorescence; however, this increase in fluorescence was significantly attenuated in macrophages from Atp7aLysMcre mice (Figures 3B and 3C). These findings support the hypothesis that ATP7A transports copper into the phagosome and that this process is required for copper-dependent bactericidal activity.

*S. Typhimurium* copper exporters, CopA and GolT, are required for bacterial survival in wild type, but not Atp7aLysMcre macrophages. We then investigated whether the bactericidal
activity conferred by ATP7A in macrophages might be altered in S. Typhimurium lacking the
copper exporters CopA and GolT. Both of these proteins are thought to confer copper
resistance by exporting cytoplasmic copper into the periplasmic space (18). We confirmed that
the ΔcopA/ΔgolT mutant was highly sensitive to copper added in the growth medium
(Supplementary Figure S3). Infection experiments indicated that compared to S. Typhimurium,
there was a significant reduction in the survival of the ΔcopA/ΔgolT mutant bacteria within
peritoneal macrophages from wild type mice (Figure 4). However, within macrophages derived
from At7aLysMcre mice there was no significant difference in survival of the wild type and
ΔcopA/ΔgolT strains of S. Typhimurium (Figure 4). These results suggest that the CopA/GolT
copper exporters are necessary for S. Typhimurium survival within wild type macrophages, but not
in At7aLysMcre macrophages.

Increased susceptibility of ATP7A^LysMcre mice to Salmonella is dependent on expression of
CopA/GolT. Next, we tested the susceptibility of At7a^WT and At7a^LysMcre mice to co-infection with
equal quantities of wild type and ΔcopA/ΔgolT strains of S. Typhimurium. At three days post-
infection, the ratios of wild type and ΔcopA/ΔgolT bacteria were enumerated in the liver and
spleen. The results show that there was a significant survival advantage for the wild type
compared to ΔcopA/ΔgolT S. Typhimurium in the liver and spleen of wild type mice (Figure 5).
However, in the At7a^LysMcre mice there were no survival differences between wild type and
ΔcopA/ΔgolT bacteria in the liver and spleen (Figure 5). Taken together, these results suggest
that CopA and GolT mediated copper export is required for S. Typhimurium survival in the host,
but only if ATP7A is functionally expressed in macrophages.

DISCUSSION

In this study, we provide evidence that copper-transporting ATPases of both the host and
pathogen function antagonistically during infection. Our results demonstrate that the loss of
ATP7A in primary murine peritoneal macrophages results in a significant attenuation in bactericidal activity against *S. Typhimurium*, consistent with the previous finding that ATP7A is required for *E. coli* killing in RAW264.7 macrophage-like cells (24). Like RAW264.7 cells, the pro-inflammatory agents LPS and IFN-γ were found to stimulate ATP7A trafficking to the phagosomal compartment in primary macrophages. This process was associated with ATP7A-dependent copper transport into the phagosomal compartment as indicated by the finding that CF4-labeled bacteria exhibited increased fluorescence upon phagocytosis by macrophages in an ATP7A-dependent manner. Taken together, these findings support a model in which ATP7A-dependent copper transport into the phagosomal compartment contributes to macrophage bactericidal activity.

The CopA and GolT proteins are P-type ATPases that function in copper export across the plasma membrane of *S. Typhimurium* and confer tolerance to high copper concentrations (18). A role for CopA and GolT in bacterial survival within macrophages was previously suggested by studies using murine bone marrow derived macrophages (18), which we now confirm using primary peritoneal macrophages. The finding that the ΔcopA/ΔgolT strain was less able than wild type *S. Typhimurium* to colonize the liver and spleen of wild type mice indicates that such a requirement also exists *in vivo*. This supports the hypothesis that interactions with the host immune system expose *S. Typhimurium* to growth restrictive concentrations of copper, and that copper export via CopA and/or GolT is needed for bacterial survival. The finding that deletion of ATP7A in macrophages results in similar levels of survival of both wild type and ΔcopA/ΔgolT *S. Typhimurium in vitro and in vivo* suggests that ATP7A-mediated copper loading of the phagosome is a mechanism for restricting *S. Typhimurium* growth.

It is also important to note that certain Gram negative bacteria, including *S. Typhimurium*, possess one or more copper-dependent SodC superoxide dismutases in the periplasm. Copper acquisition by these SOD proteins appears to occur in the periplasm and has been shown to require the...
activity of CopA and GolT as well as the periplasmic protein, CueP (29). Thus, the contribution of CopA and GolT to survival within the macrophage phagosome may not be solely due to increased copper tolerance, but also enhanced resistance to superoxide anions through activation of SodC proteins. One caveat to this hypothesis is that in the presence of copper, dismutation of superoxide to \( \text{H}_2\text{O}_2 \) by SodC may be detrimental to bacteria due to copper-catalyzed production of highly toxic hydroxyl radicals via Fenton-like chemistry (30). Clearly, further experiments are warranted to decipher the contribution of copper homeostatic proteins to mechanisms of \( S. \) Typhimurium survival and pathogenicity.

In summary, the finding that the innate immune system exploits the biocidal properties of copper adds a new layer of complexity to our general concept of nutritional immunity. Although traditionally defined by mechanisms of metal withholding from pathogenic microbes (31), it is now clear that nutritional immunity also encompasses mechanisms that harness the toxic properties of metals. Since copper tolerance genes are common to all bacteria, it will be important to determine the extent to which copper functions against different types of pathogens and at different sites of infection.

MATERIALS AND METHODS

Mice. Floxed \( \text{Atp7a} \) mice (\( \text{Atp7a}^{fl/fl} \)), in which exon 16 of the \( \text{Atp7a} \) gene is flanked by LoxP sites, were generated previously (26). To generate the conditional myeloid knockout (\( \text{Atp7a}^{l+y} \)), female \( \text{Atp7a}^{fl/y} \) mice were crossed with C57B6.129P2-Lyz2\(^{tm1(cre)If0}/J \) mice (Jackson Laboratory). As the \( \text{Atp7a} \) gene is located on the X chromosome, only male hemizygous \( \text{Atp7a}^{fl/y} \) mice were used in this study and these were identified as Cre positive or negative using genotyping methods described previously (26). All mice were approximately 8-10 weeks of age at the time of experiments unless otherwise stated.
Bacteria. Wild type and ΔcopA/ΔgolT strains of *Salmonella enterica* sv. *Typhimurium* on the SL1344 background were described previously (18). The ΔcopA/ΔgolT strain harbors an antibiotic resistance marker (*cat*, conferring chloramphenicol resistance) to allow discrimination of wild type and mutant bacteria in competition infection assays. Bacteria were grown to mid log-phase in Luria Broth (LB) and harvested by centrifugation, resuspended in PBS containing 15% (v/v) glycerol, and frozen at -80°C. The colony forming units (cfu) of these bacterial stocks were calculated for each strain prior to infection experiments.

Isolation of peritoneal macrophages. Mice were intraperitoneally injected with 0.8 mL of 4% (v/v) thioglycolate in PBS to elicit infiltration of macrophages into the peritoneal cavity. Five days post injection, the mice were euthanized. Ice-cold PBS (10 mL) was injected into the peritoneal cavity of each mouse. After 2 minutes of gentle agitation, the cell suspension was pelleted by centrifugation at 800 x g and the cells were plated on untreated Petri dishes in RPMI medium (Life Technologies) containing 20% (v/v) FBS (ThermoFisher). Petri dishes were incubated for 4 h at 37°C in a 5% CO2 incubator to allow macrophage attachment. Cells were then briefly rinsed in PBS to remove debris and fresh RPMI medium was added. Macrophages isolated using this method were typically greater than 95% of the cell population as verified by positive CD11b immunofluorescence staining.

Immunological Techniques. Immunofluorescence microscopy and Western blot analysis were performed as described previously (32).

Phagosomal labeling. Fresh cultures of primary peritoneal macrophages were activated by a 16 h treatment in serum-free medium containing 25 ng/mL IFN-γ at 37°C in a 5% CO2 incubator. The media were then inoculated with Alexa 488-labelled heat-killed *E. coli* (ThermoFisher) at a multiplicity of infection (MOI) of 10:1. After a 30 min incubation to allow for bacterial uptake, the
macrophages were washed with PBS to remove extracellular bacteria, fixed with paraformaldehyde and processed for confocal immunofluorescence microscopy.

Detection of copper delivery to phagosomal \textit{S. Typhimurium}. Copper-deficient wild type \textit{S. Typhimurium} was prepared by culturing overnight in M9 minimal media containing 100 µM iron. Bacteria were pelleted and resuspended in ice-cold PBS containing 4 µM of CF4, a copper sensor that fluoresces upon copper binding (28) (33, 34) (a kind gift of Chris Chang, University of California, Berkeley). Bacteria were pelleted and washed three times with ice-cold PBS to remove unbound CF4. The CF4-labeled bacteria were then added at a MOI of 10:1 to primary macrophages that had been pre-treated for 16 h with 25 ng/mL IFN-γ at 37°C in a 5% CO2 incubator. After 15 min at 37°C to allow for phagocytosis, the macrophages were centrifuged onto coverslips to allow detection of bacterial fluorescence by microscopy.

Cell culture and macrophage infection assays. Cultured peritoneal macrophages isolated from \textit{Atp7a}^WT and \textit{Atp7a}^{LysMcre} mice were activated by overnight treatment in serum-free medium containing 25 ng/mL IFN-γ. Cells were detached from Petri dishes by scraping into ice-cold serum-free medium, washed, and resuspended in triplicate in serum-free medium. For infection assays, wild type and mutant bacteria were mixed in equal numbers in PBS, added to the peritoneal macrophages at a MOI of 10:1 and incubated at 37°C for 30 min to allow bacterial phagocytosis. The media were then removed, and the samples rinsed twice with PBS, followed by an incubation for 10 minutes at 37°C in medium containing 100 µg/mL gentamicin to kill extracellular bacteria. The number of surviving intracellular bacteria was assessed by determination of the viable counts on non-selective and chloramphenicol selective LB agar to allow discrimination of recovered wild-type and mutant bacteria (uptake group). A second set of identically treated macrophages (kill group) were incubated for indicated times at 37°C in serum-free medium to allow bacterial killing to occur, lysed, and then plated onto LB agar. Bacterial survival was determined by dividing the
number of colonies in the kill group by those in the uptake group. All infection assays were carried out in triplicate for each condition and repeated at least four times. Statistical significance was determined using an unpaired Student's t test.

**Salmonella infections.** A 1:1 volume mix of wild type and ΔcopA/ΔgolT *S. Typhimurium* in 200 µl of PBS was delivered via intraperitoneal injection into *Atp7a*<sup>WT</sup> and *Atp7a*<sup>LysMcre</sup> mice (10<sup>4</sup> cfu/strain). At 4 days post-infection, the mice were euthanized and the livers and spleens were homogenized in ice-cold PBS using a stomacher and enumerated by plating serial dilutions. The competitive index was calculated by determining the ratio of wild type to ΔcopA/ΔgolT in each tissue divided by the ratio of wild type to ΔcopA/ΔgolT delivered in the original inoculum.

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**FIGURE LEGENDS**

**FIG1. ATP7A traffics to the phagosomal compartment in activated macrophages.** (A) Immunofluorescence analysis of the ATP7A protein in primary mouse peritoneal macrophages that were stimulated with LPS (100 ng/ml) or IFN-γ (25 ng/ml) for 16 h. Cells were labeled with antibodies against ATP7A protein (green) and the Golgi marker, GM130 (red). Nuclei were labeled with DAPI (blue). (B) Co-localization of ATP7A with phagocytosed *E. coli*. Peritoneal macrophages were stimulated with IFN-γ (25 ng/ml) for 16 h and exposed to fluorescently-labelled
E. coli (red) to allow phagocytosis. Cells were washed to remove extracellular bacteria and processed to detect ATP7A by immunofluorescence as above. Regions of co-localization between ATP7A and E. coli are shown in yellow in the merged panel (arrows).

**FIG 2. Deletion of Atp7a in macrophages of Atp7aLysMcre mice.** (A) Immunoblot analysis of ATP7A protein levels in primary peritoneal macrophages derived from Atp7aWT and Atp7aLysMcre mice. Each lane represents a different mouse. (B) Immunofluorescence analysis of the ATP7A protein levels in peritoneal macrophages from Atp7aWT and Atp7aLysMcre mice. Cells were labeled with antibodies against ATP7A protein (green) and the Golgi marker, GM130 (red). Nuclei were labeled with DAPI (blue). (C) Immunoblot analysis of ATP7A protein levels in the indicated tissues derived from Atp7aWT and Atp7aLysMcre mice.

**FIG 3. Loss of ATP7A in macrophages impairs copper loading of the phagosomal compartment and enhances survival of phagocytosed S. Typhimurium.** (A) Survival of wild type S. Typhimurium in murine peritoneal macrophages derived from Atp7aWT or Atp7aLysMcre mice. Macrophages were pretreated overnight with 25 ng/ml IFN-γ and then incubated with wild type S. Typhimurium for 30 minutes to allow phagocytosis. The percentage of surviving bacteria in macrophages was determined after 1 h and 2 h (mean ± S.D.; n = 3; Student t-test). (B) Loss of ATP7A reduces copper delivery by macrophages to phagocytosed S. Typhimurium. S. Typhimurium was grown overnight in M9 minimal medium to deplete copper content and then pre-loaded with the 4 µM CF4. The bacteria were then exposed to peritoneal macrophages derived from Atp7aWT or Atp7aLysMcre mice to allow for phagocytosis. Macrophages were then washed extensively in PBS and imaged. Each panel is representative of macrophages isolated from a different mouse. (C) Fluorescence measurements of phagocytosed bacteria within Atp7aWT or Atp7aLysMcre macrophages (mean ± S.D.; n = 15 cells in at least 5 different fields; Student t-test).
Experiments were repeated three times with essentially the same results.

**FIG 4.** Survival of *S. Typhimurium* is dependent on the CopA/GolT copper exporters in *Atp7a*<sup>WT</sup>, but not *Atp7a<sup>LysMcre</sup>*, macrophages. Peritoneal macrophages were pretreated overnight with 25 ng/ml IFN-γ and then infected at a MOI of 10:1 with the wild type (SL1344) or ΔcopA/ΔgolT strain of *S. Typhimurium*. The percentage survival was calculated as the proportion of bacteria recovered at the indicated time points relative to the initial uptake of bacteria (mean ± S.D.; n = 5; Student t-test). Note that the ΔcopA/ΔgolT mutant exhibits reduced survival in WT but not *Atp7a<sup>LysMcre</sup>* macrophages.

**FIG 5.** Copper export via CopA/GolT is required for *S. Typhimurium* survival in *Atp7a*<sup>WT</sup> mice, but not *Atp7a<sup>LysMcre</sup>* mice. Equal amounts of wild type (SL1344) and ΔcopA/ΔgolT *S. Typhimurium* were intraperitoneally injected into *Atp7a*<sup>WT</sup> or *Atp7a<sup>LysMcre</sup>* mice. At 4 days post-infection, the competitive index was calculated in the liver and spleen, defined as the ratio of wild type to ΔcopA/ΔgolT bacteria recovered from each organ divided by the ratio of wild type to ΔcopA/ΔgolT bacteria injected (mean ± S.D.; n = 5; Student t-test). Note the competitive advantage of wild type *S. Typhimurium* over the ΔcopA/ΔgolT strain in wild type mice.

**References**


Figure 2
Figure 4
Figure 5

Liver

Spleen

**p<0.01**

Competitive index

Atp7a<sup>WT</sup> Atp7a<sup>LysMcre</sup>

Atp7a<sup>WT</sup> Atp7a<sup>LysMcre</sup>