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Ferroportin 1 expression regulates intracellular growth of *Salmonella enterica serovar Typhimurium*, a link between innate immunity and hereditary hemochromatosis.

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Abbreviations

-	negative
%	percent
-/-	homocygous negative
^	to the power of
‘’	inches
+	positive
<	smaller than
>	greater than
±	plus/minus
≤	equal or smaller than
℃	degree Celcius
µ	micro
9-clone	FPN1-overexpressing clone of J774 cells
A	ampere
Ab	antibody
ABC	ATP-binding cassette transporter
APS	ammonium persulfate
ATP	adenosine 5'-triphosphate
BSA	bovine serum albumine
c	complimentary
ca.	circa
cfu	colony forming units
Cl	chloride
CMV	cytomegalovirus
CO_2	carbon dioxide
CpG	cytosin-phosphate-guanosin
Da	Dalton
dd	double-deionized

DMEM	Dulbecco's Modified Eagle's Medium
DMT	divalent metal transporter
DNA	deoxyribonucleic acid
EDTA	ethylenediamine tetraacetic acid
FBS	fetal bovine serum
Fe^{2+}	ferrous iron
Fe^{3+}	ferric iron
Fig.	figure
FPN1	ferroportin 1
g	G-force
g	gram/s
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
G-clone	GFP-overexpressing clone of J774 cells
GFP	green fluorescent protein
Gln	glutamine
h	hour/s
H_20	water
HCl	hydrogen chloride
Hep	hepcidin
His	histidine
HIV	human immunodeficiency virus
HJV	hemojuvelin
HRP	horse-radish peroxidase
IgG	immunoglobulin G
III	three
in.	inch/es
inc.	incorporation
iNOS	inducible nitric oxide synthase
IRE	iron responsive element
IREG	iron regulated gene
k	kilo/s

l	liter/s
lamp	lysosome associated-membrane glycoprotein
LB	Luria-Bertani
lbs	pounds
m	meter/s
m	milli
M	molar
m	mutant
M6PR	mannose-6-phosphate receptor
MGH	Massachusetts General Hospital
min	minute/s
Mn^{2+}	manganese
MOI	multiplicity of infection
mRNA	messenger ribonucleic acid
MTP	metal transporter protein
n	nano
NaCl	sodium chloride
NADPH	nicotinamide adenine dinucleotide phosphate
NO	nitric oxide
Nramp	natural resistance-associated macrophage protein
ODN	oligo deoxynucleotides
p	probability value
PBS	phosphate buffer saline
pH	power of hydrogen
PMSF	phenylmethylsulfonyl fluoride
RES	reticuloendothelial system
s	second/s
S.	*Salmonella*
SCV	*Salmonella* containing vacuole
SD	standard deviation
SDS	sodium dodecyl sulfate

SEM	standard error of the mean
SI 1344	*Salmonella enterica serovar Tyhimurium* type 1344
sq	square
ß	beta
TBST	tris buffer saline Tween 20
TfR-2	transferrin receptor-2
TLR	Toll-like receptor
TNF	tumor necrosis factor
Traf2	TNF receptor-associated factor 2
UV	ultraviolet
V	volt/s
wt	wild-type
x	amount X of substance dissolved per unit volume
x	multiplied by

1. Introduction

1.1. Hemochromatosis

Hereditary hemochromatosis is a genetically determined disorder in which mutations of certain genes involved in iron metabolism lead to increased intestinal iron absorption. The clinical manifestations of this disorder and of other forms of iron overload are related to iron deposition in tissues, such as liver, pancreas, and heart leading to tissue damage in these organs. Hemochromatosis is subcategorized in different types, mainly based on the chronological order, in which the genetic mutations have been described [1]. Currently, four subtypes of hemochromatosis are recognized. In Caucasian populations hereditary hemochromatosis is predominantly associated with mutations in the HFE gene, which was discovered in 1996 [2], and categorized as type 1 hemochromatosis. A more severe form is juvenile hemochromatosis, which is categorized as type 2, and comprises subtypes 2 A that is due to mutations in the HAMP gene coding for the antimicrobial peptide hepcidin [3, 4], and 2 B that is due to mutations in hemojuvelin (HJV) [5]. In type 3 hemochromatosis transferrin receptor-2 (TfR-2) is mutated, which is expressed in hepatocytes [6, 7]. In hemochromatosis type 4, also known as ferroportin disease, the iron exporter ferroportin 1 (FPN1) is mutated [8].

Type 4 hemochromatosis is an autosomal dominant form of hemochromatosis [9-17], which differs from the standard form type 1, in that the mononuclear phagocyte system cells retain iron. The affected individuals have heavy iron deposits in Kupffer cells and less pronounced iron overload in hepatocytes [12, 18]. FPN1 mutations have a heterogeneous clinical presentation. While some families have a severe disorder similar to type 1 hemochromatosis, other families have a milder form of the disease with high ferritin levels, mild anemia, mild organ disease [19, 20], and despite total body iron overload, a lower than expected transferrin saturation [10, 13, 15]. A possible explanation for the heterogeneous effects of these FPN1 mutations is related to the interaction of

FPN1 with hepcidin, a peptide that normally downregulates FPN1. Some mutations produce a protein with limited ability to export iron, resulting in excess accumulation of iron in macrophages, normal to reduced transferrin iron saturation and mild anemia. Other mutations produce FPN1 that is resistant to hepcidin. Hepcidin-resistant FPN1 retains full iron export capability and allows iron to be absorbed excessively through the gut. Individuals affected by this mutation have high levels of ferritin, increased transferrin saturation, and typical deposition of iron in the hepatic parenchyma [21-23]. Thus, while some of the FPN1 mutations suggest loss-of-function phenotypes [24], others suggest gain-of-function phenotypes [9, 11, 16].

A FPN1 mutation categorized as Q182H was first isolated in 2003 from a 63-year-old man with serum ferritin of 3018 µg/L (normal values are 50-350 µg/L) who had been previously diagnosed with bilateral cataracts at the age of 46 years. This patient had no mutation in the iron responsive element (IRE) of the L-ferritin gene, which is associated with the hyperferritinemia cataract syndrome [25-30]. He was found to have a heterozygous G>T change in exon 6 at position 850, leading to a Gln182His replacement [14]. This mutation has been shown to be located in a putative loop of the FPN1 protein between 2 transmembrane domains, where several other mutations have been found in humans [10-12, 18] and in zebra fish [31]. It also has been shown that hepcidin treatment does not result in downregulation of the Q182H mutation at the cell surface. The mutation causes a gain-of-function phenotype due to insensitivity to hepcidin [32].

1.2. Ferroportin 1

Ferroportin 1 is the only known cellular iron exporter identified to date and is also called FPN1, IREG-1, MTP-1, and Slc11a3 [31, 33, 34]. FPN1 was discovered simultaneously by three groups in 2000 [31, 33, 34]. It is a non-heme iron-export protein located on the basolateral membrane of duodenal enterocytes [31, 33, 34] and is abundantly expressed in reticuloendothelial macrophages of the liver, bone marrow, and splenic red pulp [35]. Mouse ferroportin cDNA encodes a 570-

amino-acid protein with a predicted mass of 62 kDa [34]. Sequence data show that FPN1 has ten putative transmembrane domains [31, 33, 34] and is extremely well conserved in humans, mice, and rats, showing more than 90% homology among these species [33]. Studies in cultured macrophages, which show marked increases in FPN1 after erythrophagocytosis [36], indicated a role for FPN1 in iron recycling. FPN1 plays not only an important role in type 4 hemochromatosis, but also in beta-thalassemia [37] and other iron overload syndromes.

FPN1 cell surface expression is post-transcriptionally regulated by hepcidin [21]. Binding of hepcidin to FPN1 results in internalization and degradation of FPN1, leading to a decrease in iron-export. Decreased plasma iron leads to a reduction in hepcidin synthesis, resulting in increased plasma membrane FPN1 and increased iron delivery into plasma [21]. After hepcidin binds to FPN1 on the plasma membrane, FPN1 is tyrosine phosphorylated. Mutants of FPN1 that do not get internalized or that are internalized slowly show either absent or impaired phosphorylation. Once internalized FPN1 is first dephosphorylated and then ubiquinated. Ubiquinated FPN1 is trafficked through the multivesicular body pathway to the late endosome where it is degradated [38].

1.3. Hepcidin

Hepcidin, a 25-amino-acid peptide secreted by the liver in response to iron loading [39] and inflammation [4, 40], has the structure of an amphipathic molecule composed of two distorted anti-parallel ß-sheets separated by a hairpin loop containing a vicinal disulfide bond [41]. Mammalian hepcidins were found to play an essential role in iron homeostasis. This was first suggested in studies using subtractive cloning approaches in mice subjected to dietary iron overload [39]. Hepcidin gene expression is up-regulated under iron overload conditions, and disruption of the hepcidin gene leads to accumulation of iron in the liver and pancreas, as well as iron depletion in resident macrophages [42]. Hepcidin was initially purified from human urine and plasma ultrafiltrates during screenings for

proteins and peptides with antimicrobial activity [43, 44]. Infection experiments in animal models show a strong upregulation of hepcidin expression 24 h post-infection [45]. In mice, hepcidin is upregulated by lipopolysaccharide [39], turpentine [42], Freund's complete adjuvant [46], and adenoviral infections [42]. A strong correlation between hepcidin expression and anemia of inflammation exist in patients with chronic, inflammatory and infectious diseases, including fungal, viral and bacterial infections [47].

1.4. Salmonella

The *Salmonellae* constitute a genus of over 2,300 serotypes that are highly adapted for growth in both human and animal hosts and cause a wide spectrum of diseases. They are non-spore-forming and facultative anaerobic bacteria that constitute a large genus of Gram-negative bacilli within the family *Enterobacteriaceae* [48]. *S. enterica serovar Typhi* causes a systemic febrile illness, typhoid. Approximately 16 million cases are reported each year worldwide, particularly affecting less industrialized countries. Around 10% of affected individuals develop severe or complicated disease, and without specific treatment, 5-30% of all patients with typhoid fever may die [49]. Clinical symptoms are prolonged fever, headache, abdominal discomfort and general lethargy. Certain individuals infected with *S. enterica serovar Typhi* become life-long carriers, periodically secreting high amounts of bacteria in their stools. In others, typhoid disease will relapse with the same *S. enterica serovar Typhi* strain several months after the initial infection (1-5% of antibiotic-treated individuals), which suggests the presence of a persistent reservoir of bacteria in these individuals [50]. Despite a very close genetic relationship between *S. enterica serovar Typhi* and *S. enterica serovar Typhimurium*, they display differing ranges of host specificity. *S. enterica serovar Typhi* is adapted and restricted to the human host, and therefore fails to establish infection in laboratory rodents. Most of what we know about the pathogenesis of typhoid fever is based on *in vitro* studies done in human or murine cell lines and on studies of mice infected with *S. enterica serovar Typhimurium* [49], which causes

a typhoid-like disease in these animals [51]. In humans, *S. enterica serovar Typhimurium* is an agent of food poisoning that produces acute gastroenteritis [52]. Clinical symptoms occur 6 to 48 h after exposure and are usually self-limited, although less than 1% of chronic carriage is reported. People at increased risk of disseminated infections are the very young, elderly and immunocompromised [48].

Since the beginning of the 1990's, several strains of *Salmonella* have emerged, which are resistant to a range of antimicrobials, including first-choice agents for the treatment of humans. These strains are threatening to become a serious public health problem. Their resistance results from the use of antimicrobials both in humans and animal husbandry [53]. Animal products contaminate food or water, which leads to *Salmonella* transmission, and bacteria are widely distributed through the centralization of food processing [48]. When contaminated food is ingested, *Salmonella* traverse the gastrointestinal tract to colonize the small intestines, where they provoke a complex interaction of intracellular signal transducers and epithelial receptors [54]. In nontyphoidal salmonellosis, the bacteria generally cause a localized infection resulting in an influx of neutrophils to the intestines [55-57]. In mice, where *S. enterica serovar Typhimurium* cause a typhoid-like disease [51], bacteria target the M cells overlying Peyer's patches. Invasion is mediated by the type III secretion system that induces cytoskeletal rearrangements and delivers to the host cell cytoplasm bacterial effector proteins that are required for the bacteria to gain entry into nonphagocytic cells [58-60]. Injection of these effectors shortly after bacterial adherence induces pronounced ruffling of the host cell membrane. Fusion of the tips of ruffling membranes generates the *Salmonella*-containing vacuoles (SCVs), which engulf bacteria by a process similar to macropinocytosis [61]. After crossing the epithelium, bacteria are subsequently taken into macrophages where they ultimately may reside for extended periods of time, particularly in the red pulp macrophages of the spleen [62] and Kupffer cells of the liver [63]. Once inside these cells, bacteria are protected from polymorphonuclear leukocytes, complement factors and

antibodies [48]. Upon invasion, *Salmonella* undergoes intracellular growth within the SCV, which transiently interacts with the early endosome to further mature as a specialized vacuole displaying limited interactions with the endocytic route [64-67]. Pathogenic *Salmonella* strains are capable of proliferating in both macrophages and in epithelial cells [68]. The replication begins after an initial lag period that lasts about 4-6 h in HeLa cells and 8-12 h in macrophages [66].

1.5. Iron

Altered iron availability is a key component in the host defense against bacteria. Excess iron inhibits the transcription of the inducible nitric oxide synthase (iNOS), impairing nitric oxide (NO) synthesis [69]. This may result in inhibition of microbicidal activity of macrophages [70]. Iron plays a critical role in metabolism for its facility to gain and lose electrons in both the host and bacteria. Many redox enzymes that function in the electron-transport chains of intermediary metabolism depend on iron. Iron is abundant in nature, but the extremely low solubility of Fe^{3+} at a neutral pH means that bacteria need special mechanisms for iron acquisition from their environment [71]. They solubilize Fe^{3+} by siderophores, which specifically chelate Fe^{3+} and deliver it to their cytoplasm [71, 72]. In addition to siderophores, many bacteria acquire iron directly from ferritin, lactoferrin, transferrin, and iron released by heme degradation [73, 74]. Under anoxic conditions Fe^{2+} is stable and more soluble than Fe^{3+} in Gram-negative bacteria. The Fur protein represses transcription of genes that encode enzymes for the synthesis of siderophores and iron transport proteins when loaded with Fe^{2+} [71, 75].

The membrane of Gram-negative bacteria consists of an outer membrane, the periplasmic space and the inner or cytoplasmic membrane. The outer membrane of Gram-negative bacteria forms a permeability barrier that does not allow diffusion of the large sized Fe^{3+}-siderophores. Bacteria have highly specific receptor proteins that serve as transport proteins to translocate Fe^{3+} from the cell surface across the outer membrane into the periplasm. These receptor proteins

transport Fe^{3+} not only released from Fe^{3+}-siderophores but also from transferrin, lactoferrin and heme. This transport requires energy, although there is no energy source in the outer membrane or in the periplasm. Gram-negative bacteria use the proton motive force of the cytoplasmic membrane as an energy source for the transport across the outer membrane by a specialized system called TonB-ExbB-ExbD. Fe^{3+}-siderophores, heme and Fe^{3+} released from diferric transferrin or lactoferrin are transported by ABC-transporters from the periplasm across the cytoplasmic membrane of Gram-negative bacteria to their cytoplasm [71, 75, 76]. Once the iron is intracellular, it is built into heme or non-heme iron proteins, and a small proportion is incorporated into bacterioferritin as a storage pool. Unlike in humans, most of the iron is present in a poorly defined state [77].

In the human adult more than two-thirds of the body's iron content is incorporated into hemoglobin in developing erythroid precursors and mature red cells [78]. When the red cells are old or damaged, they are phagocytosed by a certain macrophage population that is specialized in recycling iron from hemoglobin for re-use [79]. The engulfed red cells are lysed, and hemoglobin is degraded with the help of heme oxygenase. The liberated heme crosses the phagolysosomal membrane via a specific transporter called Nramp2 [80]. A part of the recovered iron is stored in ferritin, and the remainder is exported to reload circulating transferrin. This is quantitatively important, since only 1-2 mg of iron enters the body through the intestine each day, yet 25 mg are needed for erythropoiesis and other processes. Therefore, nearly all the available iron pool is derived from macrophage recycling [81]. Thus, the macrophage, which is both the host's main iron recycler and the home for intracellular iron-dependent bacteria such as *Salmonella,* is the ideal battle field for this element.

1.5. Iron Metabolism and Infection

Bacterial growth in mouse macrophages is influenced by the natural resistance-associated macrophage protein 1 (Nramp1) or (Slc11a1), although there is no evidence of a role of this protein in human typhoid [82]. The Nramp1 gene, also

called Lsh/Ity/Bcg gene, was identified in mice in 1993 [83] for its role in regulating resistance and susceptibility to intracellular pathogens such as *Leishmania Donovani*, *Salmonella enterica serovar Typhimurium*, *Mycobacterium Bovis* and *Intracellulare* [84, 85]. Nramp1 expression is restricted to neutrophils and macrophages [86, 87], where it localizes to membranes of maturing phagosomes and directly depletes the intraphagosomal environment of Mn^{2+} and Fe^{2+} [88]. Exactly how Nramp1's function as an iron transporter relates to its multiple pleiotropic effects on macrophage activation and pathogen survival is still unclear [89].

Several studies have shown that patients and mice with iron overload are more susceptible to a number of intracellular and blood pathogens [90, 91], and even a modest increase in iron intake diminishes host resistance to infection [92]. Many intracellular pathogens such as *Mycobacterium Tuberculosis*, *Salmonella enterica serovar Typhi* or *Toxoplasma* require iron within reticuloendothelial cells in order to become virulent and replicate [93]. Patients with type 4 hemochromatosis have iron-overloaded reticuloendothelial cells caused by mutations in FPN1 [12, 18], the cell type that is infected by *Salmonella in vivo.*

1.6. Aims of the Study

I hypothesize that a decrease of iron in macrophages reduces the ability of iron-dependent organisms for colonization and improves the host's resistance to infection. FPN1 reduces intracellular iron levels as an iron exporter [31, 33, 34] and could therefore inhibit intracellular iron-dependent pathogens. The downregulation of FPN1 by hepcidin [21, 94] increases the intracellular iron pool and may favor the survival of iron-dependent organisms.

1.) The first aim of this study was to determine the effect of overexpression of wild-type FPN1 on intracellular growth of *Salmonella*.
2.) The second aim of this study was to determine the effect of downregulation of wild-type FPN1 by hepcidin on intracellular growth of *Salmonella*.

3.) The third aim of this study was to determine if 700 nM of hepcidin has direct effects on *Salmonella's* growth.

4.) The fourth aim of this study was to determine if the mutant Q182H variant of FPN1 has different effects than wild-type FPN1 on intracellular growth of *Salmonella*.

1.7. Experimental Strategy

In order to examine the role of FPN1 in intracellular growth of *Salmonella*, the human epithelial cancer cell line HeLa was transiently transfected with either wild-type FPN1 or the FPN1 Q182H mutant. Protein expression was confirmed by Western blotting. Cells were then infected with the wild-type *S.enterica serovar Typhimurium* strain Sl 1344, and after 2 h and 22 h post-infection, viable intracellular bacteria in cell lysates were quantitated using gentamicin protection assays. Similar studies were carried out using stable transfectants of the murine macrophage cell line J774 overexpressing FPN1. The results from both sets of experiments indicated that increased expression of FPN1 decreased the intracellular growth of *Salmonella*. Since FPN1 expression is regulated by hepcidin, we also examined the effect of hepcidin treatment on the intracellular replication of *Salmonella*. We confirmed that hepcidin decreased FPN1 expression in transfected HeLa and J774 cells and found that it increased the intracellular growth of *Salmonella*. In contrast, hepcidin had little or no effect on the expression of the FPN1 Q182H mutant and, correspondingly, did not influence the effect of the mutant protein on *Salmonella* growth.

1.8. Summary

Hemochromatosis type 4 is an iron overload disease, which is caused by mutations of ferroportin 1 (FPN1), the only known eukaryotic iron exporter to date. FPN1 is a transmembrane protein, and its expression on the cell surface is regulated post-transcriptionally by the interaction with hepcidin. Treatment of cells with hepcidin results in internalization and degradation of cell surface FPN1. The Q182H mutant of FPN1 appears to be unresponsive to hepcidin and does

not show the expected internalization upon hepcidin exposure [23], a peptide upregulated in response to iron overload, inflammation and infection. *Salmonella enterica serovar Typhimurium* is the causative agent of gastroenteritis in humans and a severe systemic disease resembling human typhoid fever in mice. In typhoid, pathogens survive intracellularly for extended periods of time in macrophages in a host cell compartment, the *Salmonella*-containing vacuole, which is segregated from the normal late endosomal trafficking pathway [95]. *Salmonella* require iron as a nutrient for survival. Iron is also believed to influence *Salmonella's* virulence [96]. FPN1 is expressed in cells of the mononuclear phagocyte system, the main cell type which is infected by *Salmonella in vivo*. In this study, I used transiently transfected HeLa cells and stably transfected J774 macrophages to evaluate the effect of FPN1 and hepcidin on intracellular growth of *Salmonella enterica serovar Typhimurium*. Overexpression of the wild-type FPN1 and the mutant variant Q182H inhibit intracellular *Salmonella* replication. Hepcidin treatment reverses the effect of wild-type FPN1 on *Salmonella*, while it does not influence the effects of the mutant Q182H. I found that increased expression of FPN1, either in transiently transfected HeLa cells or in stably transfected J774 macrophages, resulted in significant inhibition of bacterial growth. Conversely, hepcidin treatment of FPN1 expressing cells enhanced the intracellular growth of *Salmonella*. This effect was less pronounced with the FPN1 hepcidin-resistant mutant, Q182H. My findings indicate an important role for FPN1 in controlling the growth of *Salmonella* inside cells and suggest that altered FPN1 expression and function in hemochromatosis, and possibly other iron overload states, may explain the susceptibility to intracellular pathogens in these conditions.

2. Materials and Methods

2.1. Materials

2.1.1. Tools

Autoclaving machine	Steris
Autoclave tape	Fisher
Autoradiography cassette	Fisher
Autoradiographic film (X-Omat, 8 x 10 in)	Kodak
Autoradiographic film developer (X-Omat, 2000 A)	Kodak
Bacterial culture plates	Fisher
Bacterial culture tubes	Fisher
Bacterial cell spreader	Bel-Art
Bacterial incubator	Boekel
Cell culture hood	Baker Company
Cell culture incubator	ThermoForma
Cell scraper	Corning Incorporation
Chromatography paper	Fisher
Electrophoresis system	Fisher
Gel cassette	BioRad
Freezer (-20/ -80°C)	General Electrics
Heating plate	Thermolyne
Laboratory film	Parafilm
Microcentrifuge	Eppendorf
Microcentrifuge tubes	Fisher
Micropipettes	Gilson
Microplates	Fisher
Microplate reader machine	Emax
Microscope	Zeiss
Nitrocellulose membrane	BioRad
Pipettes, serological (1 ml/ 5 ml/ 10 ml/ 25 ml)	Fisher

Pipettes, glass 9''	Fisher
Plastic film	Saran
Rubber policemen	Fisher
Scale	Scout
Spectrophotometry machine	Backman Coulter
Tips (yellow, blue)	Fisher
Tubes (15 ml/ 50 ml), polypropylene conical	Falcon
Tissue culture plates	
(6-wells/ 24-wells/ 100 x 20 mm), polystyrene	Falcon
UV-marker	Stratagene
Vortex	Fisher
Waterbath	Fisher, Precision
Wipes	Kimberley-Clark

2.1.2. Chemicals

Acrylamide (40%)	Fisher
Agar	Becton-Dickinson
Aprotinin	Sigma
Ammonium persulfate (APS)	Fisher
Beta-metacaptoethanol (100%)	Fisher
Bovine serum albumin (BSA)	BioRad
Bromophenol blue (Free Acid)	Fisher
Chemiluminescent substrate (West Pico)	Fisher
DcProtein Assay Kit	
(containing reagent A, B and S)	BioRad
EDTA disodium salt	Fisher
Ethyl alcohol	AAPER
Ethidium bromide	Fisher
Fetal bovine serum (FBS)	Hyclone
Gentamicin	Sigma
Glycerol	Fisher

Hydrogen chloride (HCl)	Fisher
Isopropyl alcohol	Sigma
Lipofectamine 2000	Invitrogen
Leupeptin	Sigma
L-glutamine	Invitrogen
Marker, molecular weight	BioRad
Methanol	Fisher
Milk, non-fat dry instant	Stop & Shop
Mouse serum (normal)	Sigma
Penicillin	Invitrogen
Phosphate buffer saline (PBS)	Invitrogen
Phenylmethylsulfonyl fluoride (PMSF)	Sigma
Soap (SDS)	Steris
Sodium azide	Sigma
Sodium chloride (NaCl)	Fisher
Sodium dodecyl sulfate (SDS)	Fisher
Streptomycin	Invitrogen
TEMED	Fisher
Tris base	Fisher
Triton	Fisher
Tryptone	Becton-Dickinson
Trypsin-EDTA	Invitrogen
Tween 20	Fisher
Water	MGH
Yeast	Fisher

2.1.3. Cell Lines

HeLa	American Tissue Culture Collection
J774	American Tissue Culture Collection
J774-G clone	provided by Dr. Marianne Wessling-Resnick

J774-9 clone	provided by Dr. Marianne Wessling-Resnick

2.1.4. Plasmids

Ferroportin 1 wild type	provided by Dr. Marianne Wessling-Ressnick
Ferroportin 1 Q182H	provided by Dr. Doug Fishman, (Wessling-Resnick lab)
Nramp2	Cherayil lab
GFP	Cherayil lab
CMV	Cherayil lab
Traf2ΔR	Cherayil lab

2.1.5. Peptides and Antibodies

Hepcidin	Peptides International Inc.
Ferroportin 1-antibody	provided by Dr. David Haile
GAPDH-antibody	Research Diagnostic Inc.
HRP-goat anti-mouse IgG	Fisher
HRP-goat anti-rabbit IgG	Zymed

2.1.6. Bacteria

S. enterica serovar Typhimurium type Sl 1344	American Tissue Culture Collection

2.1.7. Media

Tissue Culture Medium

Dulbecco's Modified Eagle's Medium (DMEM)	Invitrogen

LB-Medium

5.0 g tryptone, 2.5 g yeast, and 5.0 g NaCl were weighed and dissolved in

ddH2O to a final volume of 500 ml. Medium was autoclaved for 20 min. The autoclave was run at 121°C with a pressure of 15 lbs/sq. Autoclave tape was included on the material to confirm sterilization.

2.1.8. Bacterial Plates

LB-Agar-Plates

5.0 g tryptone, 2.5 g yeast, and 5.0 g NaCl were mixed and topped up with ddH2O to 500 ml. Then, 7.5 g agar was added. The LB-agar-solution was autoclaved for 20 min. The autoclave was run at 121°C with a pressure of 15 lbs/sq. Autoclave tape was included on the material to confirm sterilization. When liquid was at approximately 50°C, solution was poured into tissue culture plates. The plates stayed at room temperature overnight before they were stored at 4°C. Before usage, plates were warmed to room temperature and dried in hood for 10 min.

Streptomycin Agar Plates

LB agar solution was prepared. After the liquid was cooled to approximately 50°C, streptomycin was added to a final concentration of 50 µg/ml. Then, the solution was poured into tissue culture plates. The plates stayed at room temperature overnight before they were stored at 4°C. Before usage, plates were warmed to room temperature and dried in hood for 10 min.

2.1.9. Solutions for Western Blotting

1M Tris-HCl pH 8.7 or pH 6.8

Tris base	121.1 g
HCl (10 M)	until pH was 8.7 or 6.8
ddH2O	to final volume of 1000 ml

SDS-Page Gel (10%)

ddH2O	3.6 ml ddH2O
TrisCl (1M) buffer pH 8.7	3.7 ml

SDS (10%)	100 µl
Acrylamide (40%)	2.5 ml
APS (10%)	50 µl
TEMED	5 µl

Stacking Gel (3%)

ddH2O	4.3 ml
TrisCl (1M) buffer pH 6.8	150 µl
SDS (10%)	50 µl
Acrylamide (40%)	375 µl
APS (10%)	75 µl
TEMED	2.5 µl

Triton-Lysis Buffer

Triton X-100 (1%) in 10 mM Tris pH 8.0
150 mM NaCl
Aprotinin 10 µg/ml
Leupeptin 10 µg/ml
PMSF (2 mM)

SDS-Lysis Buffer

Tris-HCl (60 mM), pH 6.8
SDS (2%)
Glycerol (5%)
ß-Mercaptoethanol (5%)
Aprotinin 10 µg/ml
Leupeptin 10 µg/ml
PMSF (2 mM)

4x Sample Buffer

SDS	8.0 g

Tris-HCl (1M) pH 6.8	25 ml
ddH2O	to volume of 60 ml
Glycerol	40 ml
Bromophenol blue	0.016 g

Molecular Weight Marker

Marker	10 µl
Sample buffer (1x)	13 µl

10x Stock for SDS-Page Running Buffer

Tris base	30.3 g
Glycine	144 g
ddH2O	to final volume of 1000 ml

1x SDS-Page Running Buffer

SDS (10x)-page running buffer	50 ml
SDS (10%)	5 ml
ddH20	to final volume of 500 ml

10x Stock for Western Transfer Buffer

Tris base	29.1 g
Glycine	12.0 g
ddH2O	to final volume of 500 ml

1x Western Transfer Buffer

Methanol	100 ml
SDS (10%)	2 ml
Western transfer buffer (10x)	50 ml
ddH2O	to final volume of 500 ml

Tris-Buffered Saline Tween 20 (TBST)

Tris-HCl (1M), pH 8.0	20 ml
NaCl (5M)	60 ml
ddH2O	to final volume of 2000 ml
Tween 20	1 ml

Blocking Solution (5% non-fat dry milk in TBST)

Non-fat dry milk (5%)

TBST

2.2. Methods

2.2.1. Cell Culture

The human epithelial cervical cancer cell line HeLa and the murine macrophage cell line J774 were maintained in Dulbecco's Modified Eagle's Medium supplemented with 10% FBS, 50 µg/ml streptomycin, 50 µg/ml penicillin, and 2 mM L-glutamine at 37°C in an atmosphere of 5% $C0_2$. The cells were regularly passaged every 2-3 days.

HeLa Cells

During regular passage, HeLa cells were removed with trypsin at 100% confluency and diluted about 1:6 with fresh medium. To prepare cells for transfection, cells were diluted 1:5 in fresh medium without antibiotics and plated in 24-well or 6-well plates.

J774 Cells

Cells adhered tightly to the surface of the cell culture dish. During regular passage, cells were harvested with cell scrapers at 80-85% confluency and diluted 1:5 with fresh medium. To prepare cells for infection followed by gentamicin protection assays, cells were diluted 1:2.5 in fresh medium without antibiotics and grown in 24-well plates. For protein analysis by Western blotting, cells were grown in 6-well plates and used for experiments at 95-100% confluency.

2.2.2. Transient Transfection

HeLa cells were plated as described above. Transfections were performed using Lipofectamine 2000 according to manufacturer's protocol. DNA and transfection reagent were diluted in a ratio of 0.8 µg of DNA per 50 µl of serum-free and antibiotic-free medium, and 2 µl of Lipofectamine 2000 reagent was added to 50 µl of serum-free and antibiotic-free medium. After 5 min of incubation at room temperature, DNA and Lipofectamine 2000 were combined and incubated for 20 min at room temperature to allow the DNA-Lipofectamine 2000 complexes to

form. Then, 100 µl of DNA-Lipofectamine 2000 suspension was added per well to 24-well plates, and 500 µl per well to 6-well plates. To distribute the complexes, plates were rocked gently back and forth. Cells were incubated at 37°C in a 5% CO_2 atmosphere for 48 h until cells were ready to assay for transgene expression. Growth medium was replaced after 5-6 h to diminish exposure to the possibly toxic transfection reagent. After about 48 h, the pH of the cell-culture medium was acidic. To diminish cell stress, medium was replaced 1 h before infection or Western blot analysis. In experiments with hepcidin-containing medium, the medium was not replaced.

2.2.3. Hepcidin Treatment

Cells received a one-time treatment with hepcidin that occurred 18 h before lysates for Western blot analysis were obtained. Cells were infected with *Salmonella* 18 h after hepcidin treatment. One hour post-infection, medium was replaced by hepcidin-free medium containing 100 µg of gentamicin per ml. Cells were 2 h without hepcidin exposure. At 2 h post-infection, one plate was lysed. The other plate's medium was replaced by fresh medium containing lower concentration of gentamicin (10 µg/ml). Hepcidin was added to the appropriate wells of this plate. I used a 700 nM concentration of hepcidin for all experiments.

2.2.4. Bacterial Culture

The wild-type *S. enterica serovar Typhimurium* strain SI 1344, which is highly virulent to mice, was grown on a streptomycin-LB-agar plate at 37°C overnight, then stored at 4°C for several weeks. The plate was wrapped in laboratory film to prevent dry-out.

<u>HeLa Cells</u>

One to two colonies of SI 1344 were grown in 2 ml of LB-medium for several hours at 37°C while shaking vigorously until culture reached saturation. Then, culture was diluted 1:1000 and grown under static conditions in tightly-capped 12 ml tubes overnight at 37°C. Next morning, 0.5 ml of bacterial suspension was

transferred to a sterile tube. The sample was centrifuged at 12,000 x g for 3 min. Supernatant was discarded, bacteria washed twice with sterile PBS before pellet was resuspended in 0.5 ml of antibiotic-free medium. Then, sample was vortexed and diluted 10-fold in antibiotic-free medium.

J 774 Cells

One to two colonies of Sl 1344 were inoculated in 2 ml of LB-medium and grown overnight at 37°C while shaking vigorously. After culture had reached saturation the next morning, 0.5 ml of bacterial suspension was transferred to a sterile tube. Sample was centrifuged at 12,000 x g for 3 min at room temperature. Supernatant was discarded, pellet washed twice with sterile PBS and re-suspended in 0.5 ml of antibiotic-free medium. The sample was vortexed and diluted 10-fold by adding 50 µl of diluted bacterial suspension to 350 µl of antibiotic-free medium and 100 µl normal mouse serum. Preparation was incubated for 10 min at room temperature to allow complement factors to coat bacteria. Then, 25 µl aliquots of appropriate suspension (ca. 10^7 bacteria) were added to wells containing HeLa or J774 cells. Suspension was vortexed each time before an aliquot was collected. Multiplicity of infection (MOI) was about 20:1. Cells were incubated for 1 h at 37°C.

2.2.5. Gentamicin Protection Assay

After infection, plates were incubated at 37°C for 1 h. Then, culture medium was aspirated, and cells were washed twice with sterile PBS before being overlaid with fresh medium containing 100 µg of gentamicin per ml to kill extracellular bacteria [97]. At 2 h post-infection, one plate was lysed. Medium was replaced on the other plate by fresh medium containing a lower concentration of gentamicin (10 µg/ml) and incubated at 37°C in 5% CO_2 atmosphere. The second plate was lysed 22 h post-infection. To determine the number of viable intracellular bacteria, cell lysates were obtained by disrupting cell membranes solely by osmotic pressure. Cells were washed twice with 0.5 ml of ice-cold sterile PBS and then lysed with 200 µl of sterile 1% Triton-X 100 in water for 10 min at 4°C

by rocking gently. The 1%-triton-cell-bacterial-suspensions were transferred to sterile tubes on ice. Serial dilutions of the lysates were made, aliquots spread on LB-agar-plates and incubated overnight at 37°C. Colony forming units (cfu) were counted the following day.

2.2.6. Microplate Protein Assay

To determine cell growth, all plates were regularly observed under the microscope. Additionally, protein assays were performed for all samples of the gentamicin protection assays according to manufacturer's protocol. When cells were lysed with 1% sterile Triton, aliquots of 20 µl per sample were collected in sterile tubes on ice and stored at -20°C until analysis. Samples were centrifuged at 12,000 x g for 10 min at 4°C. Aliquots of 10 µl of the supernatant fractions were collected in sterile tubes and 25 µl of a mix of reagent A and S were added. The mix was prepared with 25 µl of reagent S and 1 ml of reagent A. Protein standard dilutions containing from 0.2 mg/ml to 1.5 mg/ml protein were prepared with BSA and the same triton-buffer as the sample contained. Then, 200 µl of reagent B was added to each tube before tubes were vortexed. After 15 min of incubation at room temperature, aliquots of 100 µl per sample were transferred to microplate. Absorbance was measured at 750 nm wave length in a microplate reader.

2.2.7. Western Blot Analysis

To analyze protein expression, semi-dry Western blotting was performed.

<u>Preparation of Samples</u>

HeLa and J774 cells were grown as described above. Cell monolayers were washed once in ice-cold PBS and lysed in 0.2 ml of ice-cold lysis buffer, either Triton-buffer or SDS-buffer. To enhance lysis, rubber policemen were used to scrape cells off the wells. Cell lysates obtained by the SDS-buffer were sonicated for 10 s. Samples were mixed with 4x Sample buffer to 25% final concentration and ß-mercaptoethanol to 5% final concentration. Samples lysed with the SDS-

buffer were loaded without prior heating on to the 10% SDS-polyacrylamide gel, whereas samples lysed with the Triton-buffer as well as the molecular weight markers were heated for 5 min prior to loading. Molecular weight standards were run in parallel on each gel.

SDS-Page

Glass plates and combs were cleaned with water, soap, and alcohol before the cassette was set up. The running gel was poured into the cassette, and about 100 µl ddH20 was dropped on top to minimize contact with air. After polymerization was completed 1 h later, the water was removed with chromatography paper. The stacking gel was poured on top of the running gel, and combs were inserted to form the loading wells. After 45 min polymerization was completed, samples were loaded and tank filled with electrophoresis buffer. The gel was run at 200 V constant voltage for about 1 h. After disassembling the cassette, the stacking gel was removed from the running gel with a clean razor.

Transfer to Nitrocellulose Membrane

Gel and nitrocellulose membrane, both equal in size, were pre-soaked in 1x transfer buffer for 15 min. Eight pieces of chromatography paper, a little larger in size than the gel, were pre-wetted with 1x transfer buffer. The transfer sandwich was constructed by placing four pieces of buffer-soaked chromatography paper on the anode, on top of that the membrane, then, the gel and an additional four pieces of buffer-soaked chromatography paper. Components were stack neatly, with edges parallel, without trapped air bubbles. The cathode was then assembled. Transfer was carried out at 200 mA constant current for 1 h.

Blocking and Antibodies

After transfer, the nitrocellulose membrane was blocked in TBST/milk for 1 h at room temperature by gentle rocking. Then, the membranes were incubated with the primary antibodies in blocking solution overnight at 4°C by gentle rocking. The next morning, blots were washed 4 times 5 min with TBST at room

temperature by gentle rocking and incubated with the secondary antibody, a peroxidase-linked IgG in blocking solution, for 30 min at room temperature and gentle rocking. Afterwards, blots were washed 4 times 5 min with TBST.

Visualization of Immunoreactivity

Any adherent liquid on the blots was absorbed by chromatography paper. The blots were then placed on a clean plastic sheet, protein-side up. Chemiluminescent substrate was prepared as instructed by the manufacturer (1:1 mixture of component A and B), and membranes were covered with the mixture. Membranes were incubated for 5 min at room temperature by gentle rocking. Blots were picked up with clean forceps, the excess reagent briefly dried with chromatography paper and plastic paper used as a sheet protector. The light-activated marker was placed next to blots in the autoradiography cassette. In the dark, autoradiographic film was placed on the blot for varying periods of exposure time. The film was immediately developed.

2.2.8. Spectrophotometry

One to two colonies of SI 1344 grown on streptomycin-agar were suspended in 2 ml of LB-medium. Suspension was incubated overnight at 37°C and vigorously shaken. The next morning, culture had reached saturation and was diluted 1:100 in LB-medium. Equal amounts were transferred to two 50 ml tubes. To evaluate any direct effect of hepcidin on growth of *Salmonella*, 700 nM of the peptide was added to one culture. Both cultures were kept with loosely closed caps to allow sufficient oxygenation at 37°C while vigorously shaking. Absorbances of both cultures were monitored every 60 min with a spectrophotometer using a wavelength of 600 nm. LB-medium was used as a blank in each case.

2.2.9. Statistical Analysis

The data are shown in means ± standard error of the mean (SEM). Statistical analysis was performed by using unpaired Student's t-test.

3. Results

Overexpression of wild-type FPN1 and mutant Q182H FPN1 in HeLa cells inhibits intracellular growth of *Salmonella*.

HeLa cells were transiently transfected with either wild-type FPN1 or the FPN1 Q182H mutant. Cell lysates were prepared about 48 h after transfection, and Western blotting was carried out to analyze expression of FPN1 and the housekeeping protein GAPDH. As shown in Fig.1, wild-type and mutant FPN1 were expressed at approximately equal levels.

Also 48 h after transfection, HeLa cells were infected with the wild-type *S. enterica serovar Typhimurium* strain SI 1344 at a MOI of approximately 10:1. Viable intracellular bacteria in cell extracts were measured using gentamicin protection assays at 2 and 22 h post-infection (Fig. 2). At 2 h post-infection, extracts from cells transfected with either wild type or mutant FPN1, and mock cells, grew a similar amount of colony-forming units (cfu). At this time, the amount of cfu was representative of the amount of bacterial invasion, because bacteria begin to replicate only after an initial lag period of about 4-6 h after entry into the cell [66]. Therefore, both wild-type and mutant FPN1 overexpression in HeLa cells do not seem to influence bacterial invasion. However, at 22 h post-infection, cell extracts of mock and GFP transfected cells grew large amounts of cfu, while cell extracts of wild-type and mutant FPN1 transfected cells grew fewer cfu. The amount of cfu measured in cell extracts obtained 22 h post-infection represent the extent of bacterial replication. Thus, both wild-type and mutant FPN1 overexpression in HeLa cells seem to inhibit bacterial replication. Statistical significance was calculated based on the difference of cfu between GFP and FPN1 expressing cells. GFP was used as a control, because GFP is not known to influence bacterial invasion or replication. Interestlingly, the amount of cfu obtained from lysates of GFP expressing cells was less than the amount of cfu obtained from mock cells that were not transfected with any plasmid. This

decrease in cfu may be a side effect of the transient transfection procedure. The data in Fig. 2 show that overexpression of wild-type and mutant FPN1 inhibits intracellular replication of *Salmonella* in HeLa cells to an approximately equal extent. The mechanism of inhibition is unrelated to the transfection procedure.

Figure 1

HeLa cells

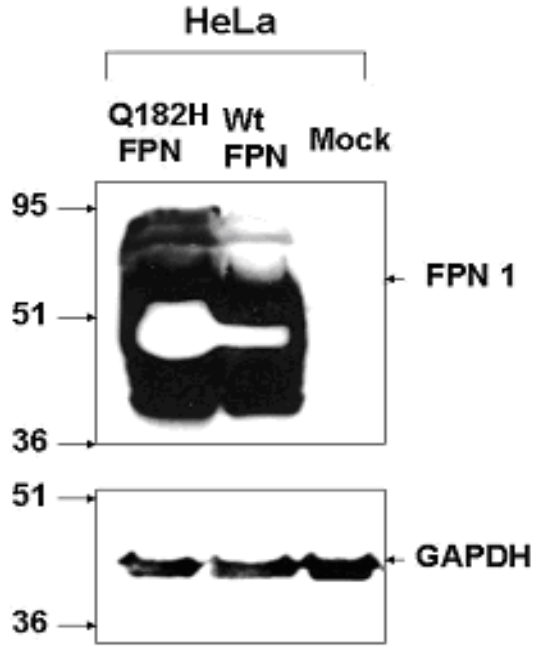


Fig. 1: Wild-type and Q182H mutant FPN1 in HeLa cells are expressed at approximately equal levels as shown by Western blotting. HeLa cells were transiently transfected with wild-type and mutant FPN1. GAPDH was used as control. Transiently transfected HeLa cells were grown in tissue culture medium supplemented with 10% FBS. Cell extracts were obtained 48 h after transfection. Cells were lysed with triton-buffer. Protein expression detected with anti-FPN1 antibody diluted 1:3000 and anti-GAPDH antibody diluted 1:2000 in blocking solution.

Figure 2

HeLa cells

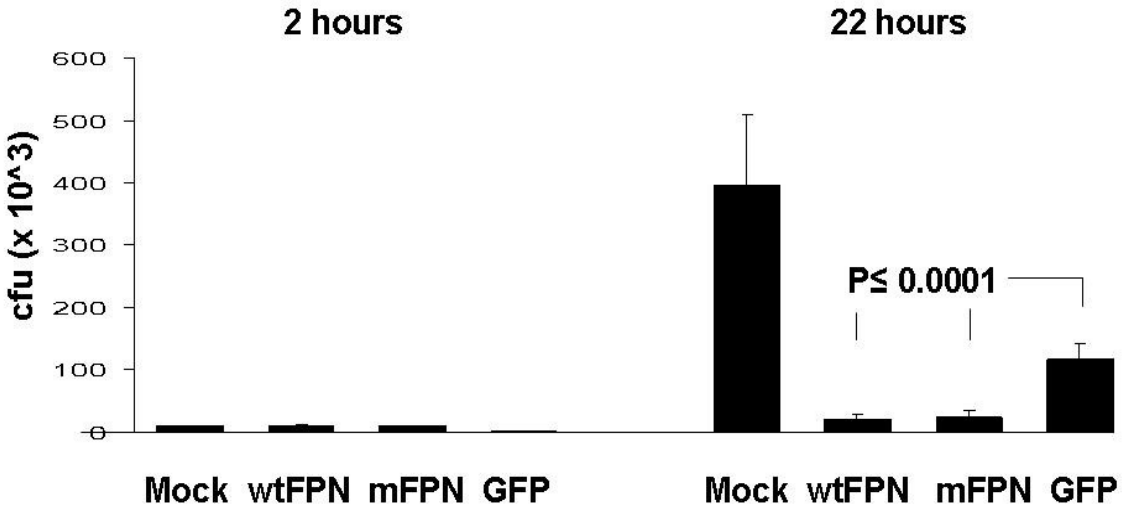


Fig. 2: Effect of FPN1 overexpression in HeLa cells on intracellular survival of *Salmonella* measured by gentamicin protection assays. HeLa cells were grown in tissue culture medium supplemented with serum and transiently transfected with expression plasmids encoding wild-type FPN1, the Q18H mutant, and GFP. Mock cells were not transfected and shown as control. Transiently transfected HeLa cells were grown in tissue culture medium supplemented with 10% FBS, infected with *Salmonella* and number of viable intracellular bacteria determined by gentamicin protection assays at 2 and 22 h post-infection. Lysates of gentamicin protection assays were obtained 48 h after transfection. Data of one experiment done with triplicate samples are expressed as means ±SD. The experiment is representative of four similar experiments performed on different days, each in triplicate.

Extent of intracellular bacterial growth inhibition is serum-dependent.

Since FPN1 is an iron exporter, I evaluated changes in iron levels on bacterial growth by changing the iron content of the culture medium. Thus, in one series of experiments I used tissue culture medium free of FBS, while in all other experiments I used tissue culture medium supplemented with 10% FBS. Commercial FBS contains ferritin in a range between 0.8 to 6.0 µg/ml with an iron saturation of about 20% and a transferrin load of 55% to 92% in relation to the ferritin content [98].

HeLa cells were grown in tissue culture medium without supplement of FBS beginning 16 h after transfection. The bacteria used for infection were also suspended in tissue culture medium without FBS supplement. Gentamicin protection assays were performed to determine viable intracellular bacteria at 2 and 22 h post-infection. At 2 h post-infection, cell extracts of FPN1-transfected and mock cells grew a similar amount of cfu, supporting the observation that bacterial invasion does not seem to be altered by FPN1 overexpression. At 22 h post-infection, cell extracts obtained from HeLa cells expressing wild-type or mutant FPN1 showed less cfu than mock and GFP expressing cells. Statistical significance was calculated based on the difference of cfu in both lysates of wild-type and mutant FPN1 expressing HeLa cells compared to the amount of cfu grown from GFP-expressing HeLa cells at 22 h post-infection.

Comparing the extent of bacterial growth inhibition by FPN1 (Fig.2 and 3) at 22 h post-infection, it is noticeable that in the absence of FBS (Fig.3) the amount of cfu was approximately 2-fold decreased as compared to the 10-fold decrease seen in the presence of FBS (Fig.2). Unlike invasion, where no significant difference between the two series of experiments is noticeable, extent of bacterial growth inhibition seems to be dependent on serum, which suggests that extent of bacterial growth inhibition may be related to the amount of available iron.

Figure 3

HeLa cells

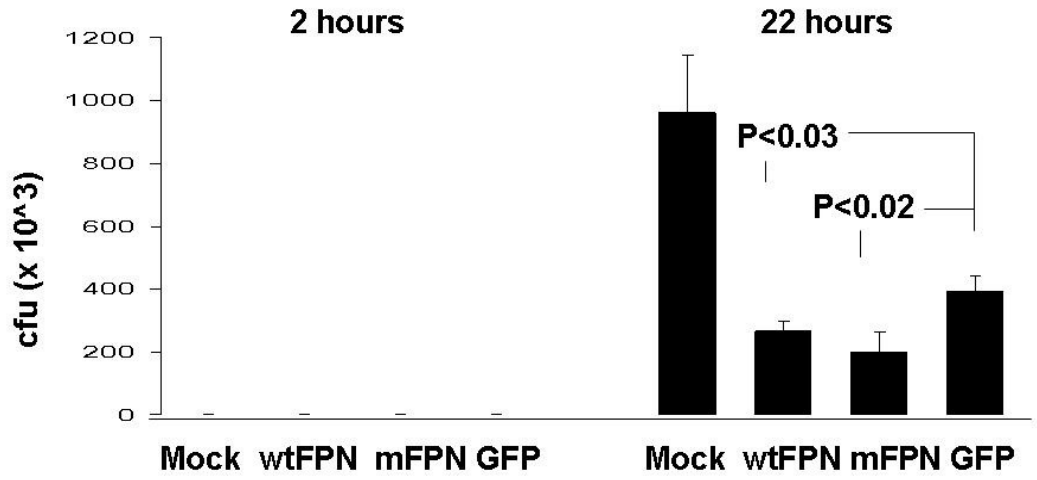


Fig. 3: Effect of both wild-type and mutant FPN1 overexpression in HeLa cells on intracellular growth of *Salmonella* measured by gentamicin protection assays. HeLa cells were grown in tissue culture medium free of FBS. Extent of inhibition of bacterial replication by FPN1 overexpression seems to be related to serum and likely to the amount of available iron. HeLa cells were transiently transfected with expression plasmids encoding wild-type FPN1, the Q18H mutant, and GFP. Mock cells were not transfected and shown as control. Transiently transfected HeLa cells were grown in tissue culture medium free of FBS, infected with *Salmonella* and the number of viable intracellular bacteria determined by gentamicin protection assays at 2 and 22 h post-infection. Lysates for gentamicin protection assays were obtained 48 h after transfection. Data of one experiment are expressed as means ±SD. The experiment is representative for three similar experiments performed on different days, each in triplicate.

Variables influence intracellular bacterial growth in transiently transfected HeLa cells.

Bacterial invasion was approximately equal in FPN1 overexpressing cells and control cells (Fig. 2 and 3), unlike bacterial replication which was significantly decreased in both wild-type and mutant FPN1 overexpressing cells, and interestingly, also in GFP overexpressing cells as compared to mock cells that were not transfected with any plasmid. Several factors may be responsible for this effect such as for instance interference of the lipid transfection reagent with the endosomal pathway. To evaluate for an inhibitory effect of the transfection reagent Lipofectamine 2000 on bacterial growth, I compared *Salmonella* survival in cells treated with Lipofectamine 2000 with cells that were not treated with Lipofectamine 2000. Both of these cells were not transfected with any plasmid (Fig. 4). As analyzed by gentamicin protection assays, the transfection reagent Lipofectamine 2000 does not seem to alter bacterial growth.

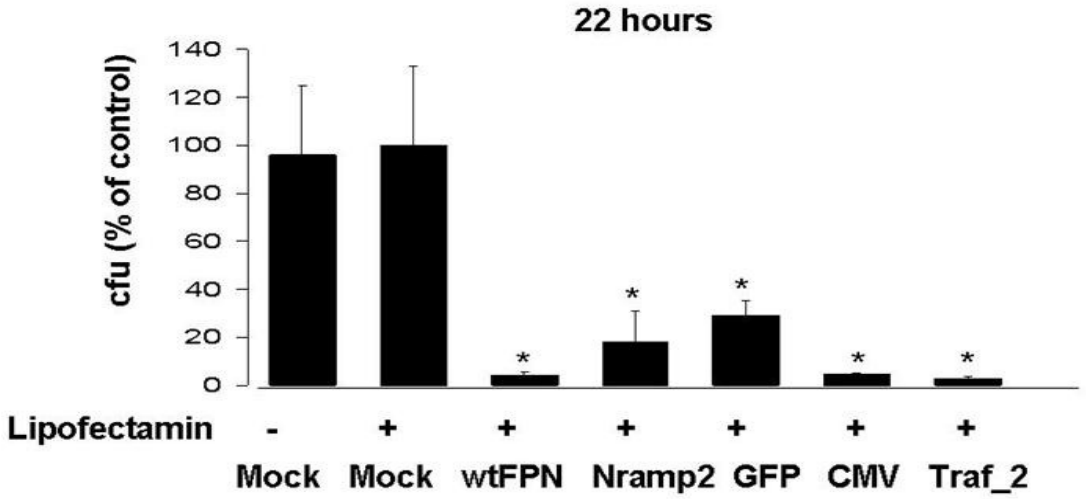
To further evaluate for variables that influence bacterial growth, all cells were regularly observed under the microscope. As noted during the course of the experiments, increased cell death occurred after transient transfection. To evaluate the extent of cell death in my experimental setting, I measured the protein content of each cell extract sample that was later analyzed by gentamicin protection assays. The protein content was measured by using protein assays and is considered an indicator for the amount of viable cells present in the sample. Culture dishes were washed twice with ice-cold PBS before analysis, which usually eliminates dead cells from culture dishes. Only viable cells were likely to be left and contribute to the amount of protein in the sample. I analyzed 20 µl of each cell extract sample by protein assays, before gentamicin protection assays were carried out. I measured a decrease in protein content in lysates of transiently transfected cells, particularly in lysates of FPN1 overexpressing cells, as compared to lysates of mock transfected cells (data not shown). This decrease of protein content correlated partly with the decrease of cfu (Fig. 2, 3, 4, 7, 8). These data indicate that overexpression of FPN1 and also of other

proteins causes cell death to some extent that might be partly responsible for the decrease in viable *Salmonella* as measured by gentamicin protection assays. However, the amount of cell death does not satisfactorily explain the 2- to 10-fold decrease (Figs. 2 and 3) of cfu seen in FPN1 overexpressing HeLa cells as compared to the GFP transfected HeLa cells. I conclude that the decrease in bacterial replication in FPN1 overexpressing cells is caused by a mechanism that occurs in addition to cell death.

A smaller amount of cfu was seen in lysates of all HeLa cells that were transiently transfected with DNA 22 h post-infection as compared to the amount of cfu obtained from lysates of mock transfected cells. To test the possibility that DNA transfection itself could affect bacterial growth, we transfected different DNA plasmids including the cytomegalovirus (CMV) promoter, Traf2ΔR, which is a dominant negative construct of the signal-transducing adaptor molecule Traf2 [99], GFP, and the iron-transporter Nramp2. These plasmids were intended to serve as controls for the transfection procedure, since none of these molecules is known to inhibit bacterial growth. The amount of cfu measured in the cell extracts of these transgene expressing cells was decreased in comparison to the amount of cfu obtained from mock cell extracts. *Salmonella* survival thus seems decreased in all of these transiently transfected HeLa cells (Fig. 4). A possible explanation may be that plasmids can interact with the TLR-9 receptor through regions of CpG DNAs [100] and inhibit bacterial growth through the activation of TLR-9. In fact, the TLR-9 receptor is known to play a role in infection, although its exact function is not clear to date [101].

Figure 4

HeLa cells



F

Fig. 4: Effect of transiently transfected plasmids in HeLa cells on intracellular growth of *Salmonella* was measured by gentamicin protection assays at 22 h post-infection. A significant smaller amount of cfu was obtained from lysates of all HeLa cells that were transiently transfected with DNA as compared to the amount of cfu obtained from lysates of mock cells. Cells were transiently transfected with expression plasmids encoding the CMV promoter, Traf2ΔR, GFP, Nramp2 and wild-type FPN1. Mock cells were not transfected, column one shows cells free of Lipofectamine 2000, column two to seven show cells treated with Lipofectamine 2000. Transiently transfected cells were grown in tissue culture medium supplemented with 10% FBS, infected with *Salmonella* after 48 h and the number of viable intracellular bacteria determined by gentamicin protection assays at 22 h post-infection. Results are from different cell lysates, obtained on different days. To account for day-to-day variability in cell growth, results are shown as relative differences of cells as compared to the mock cells that were treated with Lipofectamine 2000. Experiments were performed at least in triplicate. Data are expressed as means ± SEM (*$p \leq 0.001$ compared to the controls).

Overexpression of FPN1 in J774 cells inhibits intracellular growth of *Salmonella.*

The data in Figs. 2, 3, and 4 suggest that FPN1 plays a role in the regulation of intracellular growth of *Salmonella*. To test if these observations also apply to macrophages, the cell type which *Salmonella* infects in *vivo,* I carried out experiments with the murine macrophage cell line J774. I used stable transfectants of the parental J774 cell line that were transduced with retroviral vectors containing GFP (G-clone) or GFP plus FPN1 (9-clone). Knutson *et al.* selected GFP-expressing cells by flow cytometry and identified individual clones overexpressing FPN1. Their analysis of these clones indicated that the level of FPN1 in the 9-clone is approximately 3-fold higher than in the G-clone. Furthermore, their functional experiments showed that the 9-clone releases about 70% more iron than the G-clone following erythrophagocytosis [94].

I used these J774 clones for infection experiments with *Salmonella*. The experiments were carried out in a similar way to the HeLa cell experiments. Viable intracellular bacteria were determined by gentamicin protection assays. I observed that the amount of bacterial invasion in the J774-9 clone did not differ significantly from the amount of invasion seen in the J774-G clone, unlike bacterial replication. There was a 70% decrease of bacterial replication in the FPN1-overexpressing 9-clone as compared to the GFP-overexpressing G-clone (Fig. 5). The 70% decrease of *Salmonella* replication in the 9-clone in my experiments compared to the 70% increase of iron export in the experiments published by Knutson *et al.* may suggest that the amount of iron transported by FPN1 might correlate with the inhibition of *Salmonella* replication.

Figure 5

J774 cells

G-clone: expressing stably GFP

9-clone: expressing stably GFP and FPN1

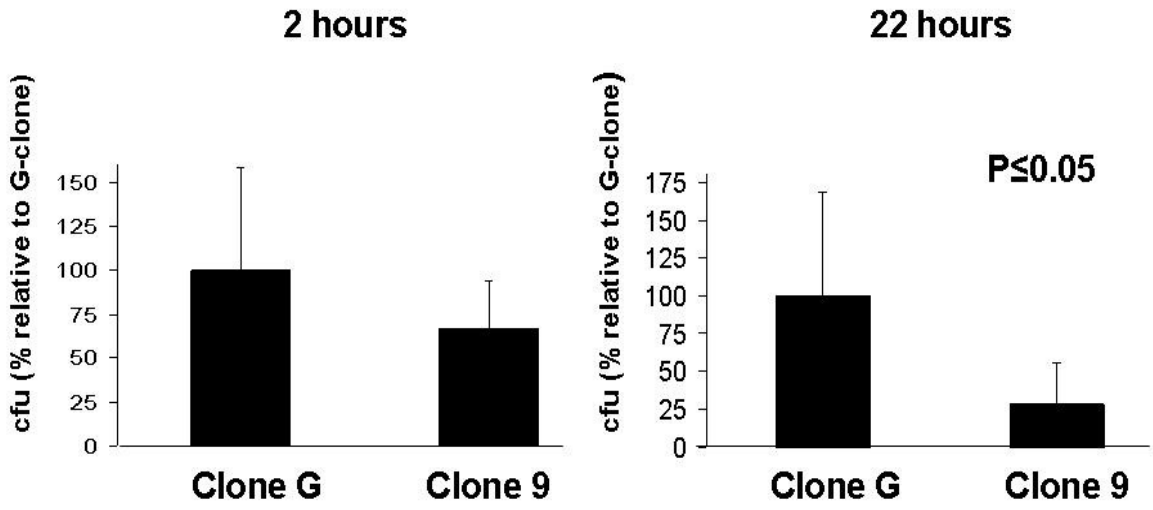


Fig. 5: Effect of FPN1 in J774 macrophages on intracellular growth of *Salmonella* was measured by gentamicin protection assays. The GFP containing G-clone and the GFP plus FPN1 containing 9-clone were infected with *Salmonella*, and the number of viable intracellular bacteria determined by gentamicin protection assays at 2 and 22 h post-infection. Cells were grown in tissue culture medium supplemented with 10% FBS. Results are from two different cell lysates, obtained on different days. To account for day-to-day variability in cell growth, results are shown as relative differences of the FPN1 expressing 9-clone as compared to the GFP expressing G-clone. Data are expressed as means ±SEM for two independent experiments, performed in triplicate.

Hepcidin downregulates surface expression of wild-type FPN1 in HeLa cells.

To elucidate further the role of FPN1 on *Salmonella's* growth in HeLa cells, we induced FPN1 downregulation by treating cells with hepcidin. We used a concentration of 700 nM of hepcidin in all experiments. HeLa cells were first transiently transfected with wild-type FPN1. After 32 h, one aliquot of the cells was treated with 700 nM of hepcidin for the following 17 h, until lysates for Western blot analysis were obtained. Treatment with hepcidin reduced FPN1 expression in HeLa cells as analyzed by Western blotting (Fig. 6).

In the course of the experiments, I noticed that hepcidin functioned not reliably after the peptide was stored at -80℃ for a few weeks. The inconsistency of function may be due to the highly unusual feature of the hepcidin molecule, which contains a disulfide linkage between adjacent cysteines near the turn of the hairpin. Compared to most disulfide bonds, those which are formed between adjacent cysteines are stressed and can have a greater chemical reactivity [41]. To determine if hepcidin was efficiently downregulating wild-type FPN1 in each of our experiments, Western blot analysis of FPN1-overexpressing HeLa cells was carried out before each series of experiments where hepciding was used (Fig. 6).

Figure 6

HeLa cells

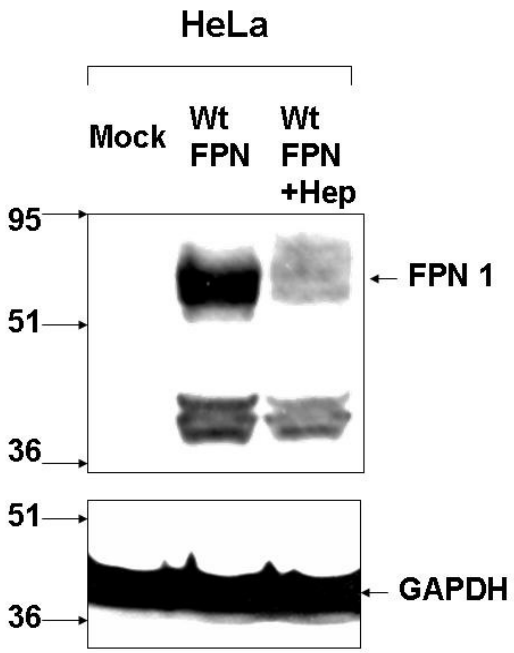


Fig. 6: Downregulation of wild-type FPN1 in HeLa cells by hepcidin is shown by Western blotting. HeLa cells were transiently transfected to express wild-type FPN1. One set of cells was treated with hepcidin. As seen in lane 3, expression of wild-type FPN1 after treatment with hepcidin was decreased. The housekeeping protein GAPDH was approximately equally expressed in all lanes. Transiently transfected HeLa cells that overexpress wild-type FPN1 were grown in tissue culture medium supplemented with 10% FBS. After 32 h of transfection, cells were treated with 700 nM of hepcidin. Lysates for Western blotting were obtained after 17 h of hepcidin exposure. Lysates were obtained by SDS-buffer, samples were sonicated, and protein expression detected with anti-FPN1 antibody 1:3000 diluted or anti-GAPDH antibody 1:2000 diluted in blocking solution.

Wild-type FPN1 in HeLa cells downregulation by hepcidin results in enhanced intracellular growth of *Salmonella*.

After downregulation of wild-type FPN1 was confirmed by Western blot analysis, the effect of FPN1 downregulation by hepcidin on intracellular *Salmonella* growth was evaluated by gentamicin protection assays at 2 and 22 h post-infection. I overexpressed wild-type FPN1 in HeLa cells by transient transfection. As controls for the transfection procedure I used CMV or Traf∆2R. Half of the control cells as well as the FPN1 overexpressing cells were treated with hepcidin. All cells were infected with *Salmonella*, and viable intracellular bacteria were determined by gentamicin protection assays (Fig. 7). At 2 h post-infection we see a trend that hepcidin enhances bacterial invasion in CMV, Traf∆2R and FPN1 overexpressing cells as compared to the corresponding group of cells that were not treated with hepcidin. This observation is only statistically significant in the CMV and Traf∆2R cells.

At 22 h post-infection, the effect of hepcidin differs between the cells transfected with the control plasmids and the ones transfected with wild-type FPN1. Statistically significant increased bacterial replication is seen only in wild-type FPN1 overexpressing cells that were treated with hepcidin as compared to wild-type FPN1 overexpressing cells without treatment. Bacterial replication was not significantly altered by hepcidin in cells which were transiently transfected with CMV or Traf∆2R as compared to cells transfected with the same plasmids that were not exposed to hepcidin. In fact, in these cells there was a slight decrease of bacterial replication after hepcidin treatment, although it was not statistically significant. Thus, since hepcidin specifically downregulates wild-type FPN1 surface expression [21], I conclude that the expression level of wild-type FPN1 influences intracellular bacterial growth within the first 22 h of infection.

Figure 7

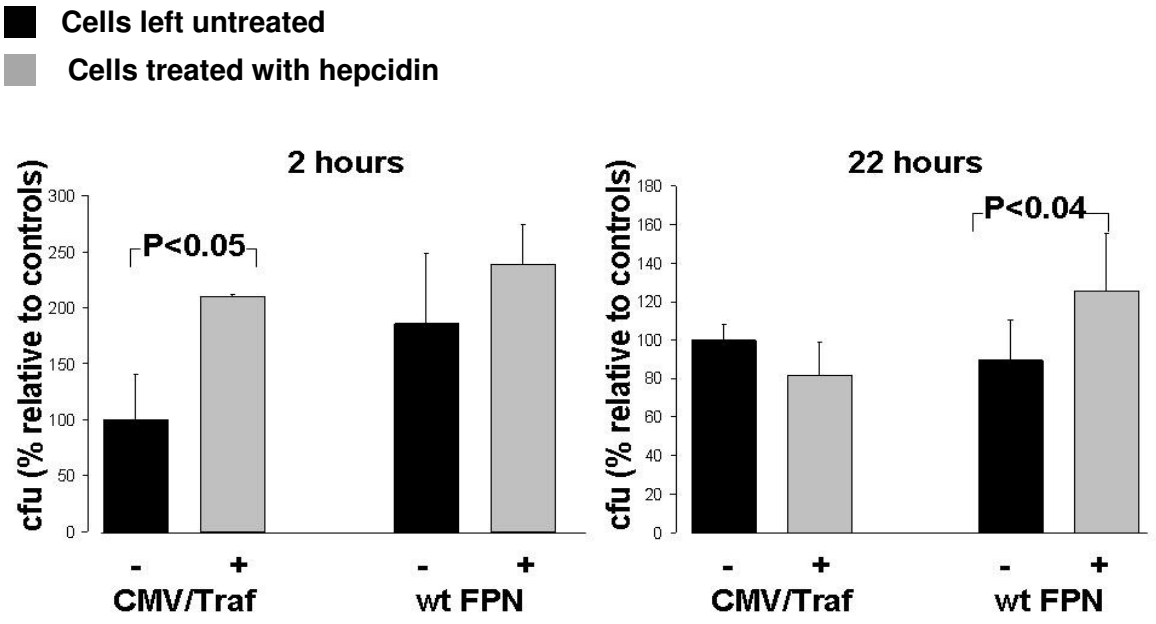


Fig. 7: Wild-type FPN1 in HeLa cells is downregulated by hepcidin and results in enhanced intracellular growth of *Salmonella* as measured by gentamicin protection assays. Transiently transfected HeLa cells that express wild-type FPN1, CMV, and Traf were grown in tissue culture medium supplemented with 10% FBS. After 32 h of transfection, cells were treated with 700 nM of hepcidin. Lysates for gentamicin protection assays (2-h-time point) were obtained after 17 h or after 37 h (22-h-time point) of hepcidin treatment. For functional analysis cells were infected, the number of viable intracellular bacteria was determined by gentamicin protection assays at 2 and 22 h post-infection. Results are from two different cell lysates, obtained on different days. To account for day-to-day variability in cell growth, results are shown as relative differences to the CMV or Traf overexpressing control cells (100%) at 2 and 22 h post-infection respectively. Data are expressed as means ±SEM for two independent experiments, one carried out with CMV overexpressing control cells and one with Traf overexpressing control cells, performed in triplicate.

Mutant FPN1 in HeLa cells is not downregulated by hepcidin, and therefore hepcidin does not alter intracellular growth of *Salmonella*.

Hepcidin does not downregulate surface expression of the Q182H mutant [32]. To evaluate the role of this mutant FPN1 during *Salmonella* infection, I infected HeLa cells that overexpressed the Q182H mutant. The mutant was introduced by transient transfection. HeLa cells overexpressing the CMV promoter served as control for the transfection procedure. Half of all of these cells were treated with hepcidin while the other half remained as control for the effects of hepcidin treatment. Cells were infected with *Salmonella*, and gentamicin protection assays were carried out at 2 h and 22 h post-infection. At 2 h post-infection bacterial invasion was measured, and we observed a significant enhancement of bacterial invasion in all cells that were treated with hepcidin (Fig. 8). Thus, hepcidin appears to facilitate *Salmonella* invasion into cells that express either CMV or mutant FPN1.

At 22 h post-infection, intracellular bacterial replication was measured by using gentamicin protection assays. Interestingly, hepcidin seems to decrease *Salmonella* replication in cells that overexpress the CMV promoter, although it is not statistically significant. More important and as expected, hepcidin does not have any effect on *Salmonella* replication in cells overexpressing mutant FPN1 (Fig. 8). Mutant FPN1 lacks sensitivity to hepcidin. My experiments indicate a functional consequence of this observation. Cells that overexpress mutant FPN1 do not show changes in *Salmonella* survival after hepcidin treatment (Fig. 8), while cells overexpressing wild-type FPN1 show improved bacterial growth in response to hepcidin treatment (Fig. 7).

Figure 8

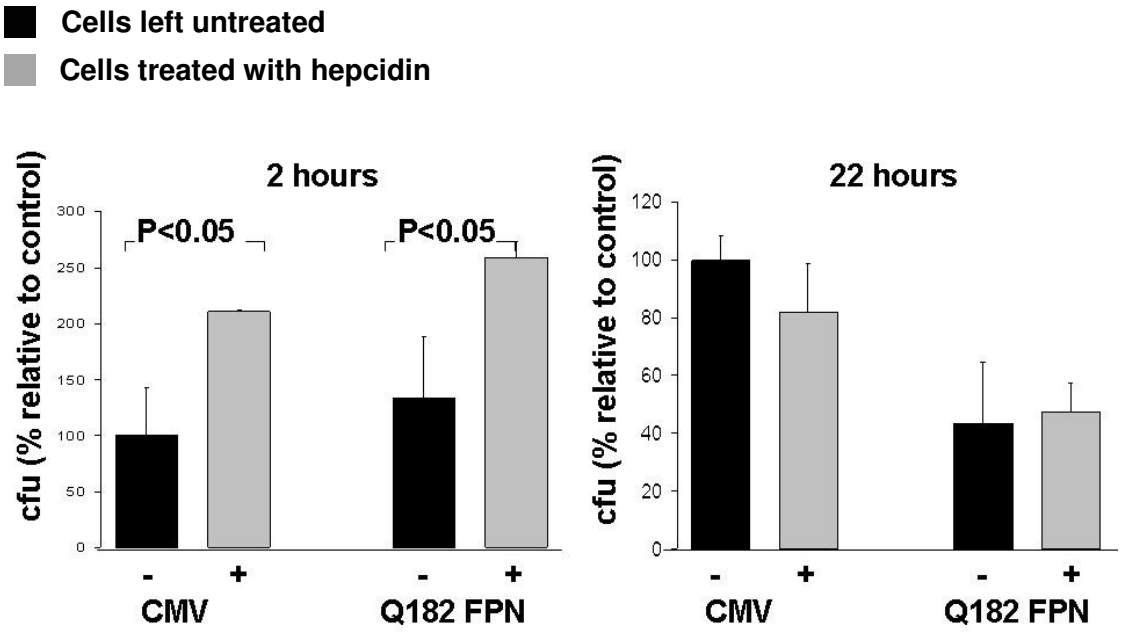


Fig. 8: Mutant FPN1 in HeLa cells is not downregulated by hepcidin. Treatment of hepcidin does not alter intracellular replication of *Salmonella* measured by using gentamicin protection assays at 2 and 22 h post-infection. Transiently transfected HeLa cells that overexpress mutant FPN1 were grown in tissue culture medium supplemented with 10% FBS. After 32 h of transfection, cells were treated with 700 nM of hepcidin. Lysates for gentamicin protection assays (2-h-time point) were obtained after 17 h or after 37 h (22-h-time point) of hepcidin treatment. For functional analysis, cells were infected with *Salmonella*, the number of viable intracellular bacteria determined by gentamicin protection assays. Results are from two different cell lysates, obtained on different days. To account for day-to-day variability in cell growth, results are shown as relative differences to the CMV overexpressing control cells (100%) that were not treated with hepcidin at 2 and 22 h post-infection respectively. Data are expressed as means ±SEM for one experiment, performed in triplicate.

Wild-type FPN1 in J774 macrophages is downregulated by hepcidin.

I tested the effects of hepcidin on wild-type and mutant FPN1 overexpressing HeLa cells and discovered different functional consequences in *Salmonella* infection. *In vivo, Salmonella* infect macrophages, where they reside for extended periods of time. To determine if my observations from HeLa cells are also applicable to macrophages, I infected the murine macrophage cell line J774. I used parental J774 cells and the previously described J774 9-clone that stably overexpresses wild-type FPN1. Cells of the 9-clone were treated with hepcidin for 4 h and 24 h before cell lysates were prepared. Western blot analysis was carried out to analyze FPN1 expression as well as expression of the housekeeping protein GAPDH (Fig. 9). Westerm blot analysis shows low expression of FPN1 in the parental J774 cell line as seen in lane 1 and higher FPN1 expression in the 9-clone as seen in lane 2 to 4. Lysates of the J774 9-clone cells were treated with hepcidin for either 24 h or 4 h. These lysates show decreased FPN1 expression as seen in lane 3 and 4 as compared to lysates of the J774-9 clone that were not treated with hepcidin as seen in lane 2 (Fig. 9). The housekeeping protein GAPDH was expressed at approximately equal levels in all lanes.

Figure 9

J774 cells

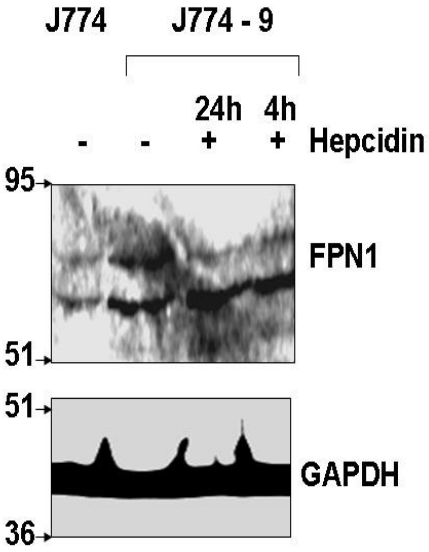


Fig. 9: Downregulation of wild-type FPN1 in J774 cells by hepcidin is shown by Western blot analysis. J774-9 cells stably overexpress FPN1. Western blot analysis shows decreased expression of wild-type FPN1 of the 9-clone, as seen in lane 3 and 4, after treatment with 700 nM of hepcidin. GAPDH was used as control for both J774 and J774-9 cells. J774 cells grown in tissue culture medium supplemented with 10% FBS were treated for either 4 h or 24 h with hepcidin before lysates were obtained by SDS-buffer, samples sonicated, and protein expressions detected with anti-FPN1 antibody 1:3000 diluted or anti-GAPDH antibody 1:2000 diluted in blocking solution.

Wild-type FPN1 in J774 macrophages downregulated by hepcidin results in enhanced intracellular growth of *Salmonella*.

To evaluate functional consequences of FPN1 downregulation by hepcidin in J774 macrophages, I treated J774 macrophages with hepcidin, infected them with *Salmonella* and measured viable intracellular bacteria by gentamicin protection assays at 2 and 22 h post-infection (Fig.10). I used parental J774 cells and the previously described J774 9-clone that stably overexpresses wild-type FPN1. Cells were treated for approximately 17 h with hepcidin, and then infected with *Salmonella* at a MOI of approximately 10:1. After 2 h and 22 h, viable intracellular bacteria in cell extracts were measured using gentamicin protection assays. At 2 h post-infection, a slight trend is noticeable that hepcidin treatment enhances bacterial invasion in the parental J774 cells and that hepcidin treatment inhibits bacterial invasion in the 9-clone (Fig. 10), although these observations are not statistically significant.

At 22 h post-infection, the FPN1 overexpressing J774-9 clone shows approximately 25% less cfu than the parental cells, confirming our previous results that FPN1 overexpression in J774 macrophages inhibits *Salmonella* replication (Fig. 5). More important, Fig. 10 shows that *Salmonella* replication in J774 macrophages is affected by hepcidin. Hepcidin enhances *Salmonella* replication in the 9-clone. A significant ($p<0.03$) increase of bacterial replication of approximately 2-fold is seen the 9-clone cells that were treated with hepcidin as compared to cells of the 9-clone that were not treated with hepcidin. In the parental J774 cells, an increase in bacterial growth is seen with hepcidin treatment as compared to parental J774 cells that were not treated with hepcidin, although there is no statistical significance. Thus, we conclude that hepcidin enhances intracellular bacterial growth in macrophages, the cell line which is chronically infected *in vivo* with *Salmonella*.

Figure 10

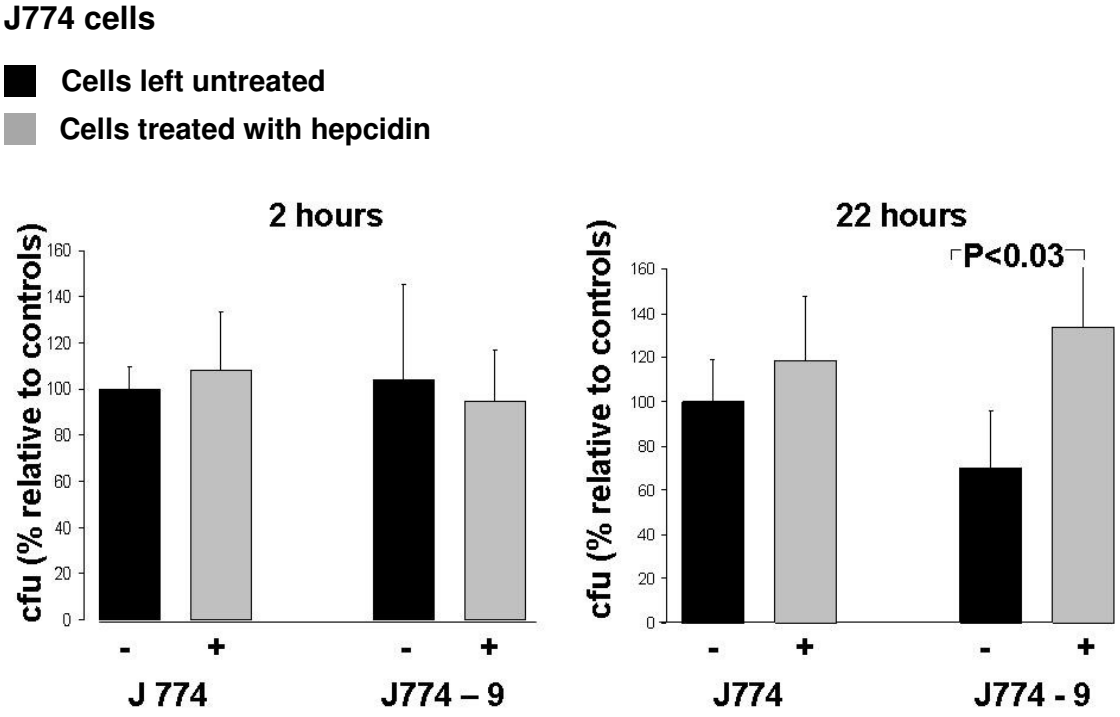


Fig. 10: Wild-type FPN1 in J774 cells downregulation by hepcidin results in enhanced intracellular replication of *Salmonella* as measured by gentamicin protection assays at 2 and 22 h post-infection. J774 cells grown in tissue culture medium supplemented with 10% FBS were treated with 700 nM of hepcidin for 17 h before they were infected with *Salmonella.* The number of viable intracellular bacteria was determined by gentamicin protection assays. Results are from two different cell lysates, obtained on different days. To account for day-to-day variability in cell growth, results are shown as relative differences to the untreated J774 control cells (100%). Cfu determined at 2 h post-infection are shown in relation to cfu of the untreated J774 control cells obtained at 2 h post-infection. Cfu determined at 22 h post-infection are shown in relation to cfu of the untreated J774 control cells obtained at 22 h post-infection. Data are expressed as means ±SEM for two independent experiments, performed in triplicate.

700 nM of hepcidin does not directly influence growth of *Salmonella.*

Hepcidin downregulates FPN1 surface expression and, as indicated in the literature, also has microbicidal properties [102]. To evaluate the direct effect of 700 nM of hepcidin on the replication of *Salmonella*, I compared the growth curve of two *Salmonella* cultures. To one of the cultures I added 700 nM of hepcidin. Both cultures contained 10 µl saturated *Salmonella* culture per ml of LB-medium. Bacterial growth rates in these two cultures were determined by spectrophotometry at 600 nm wave length (Fig. 11). Both growth curves were identical. Consequently, a concentration of 700 nM hepcidin does not directly influence *Salmonella's* growth *in vitro*.

Figure 11

Bacterial Cultures

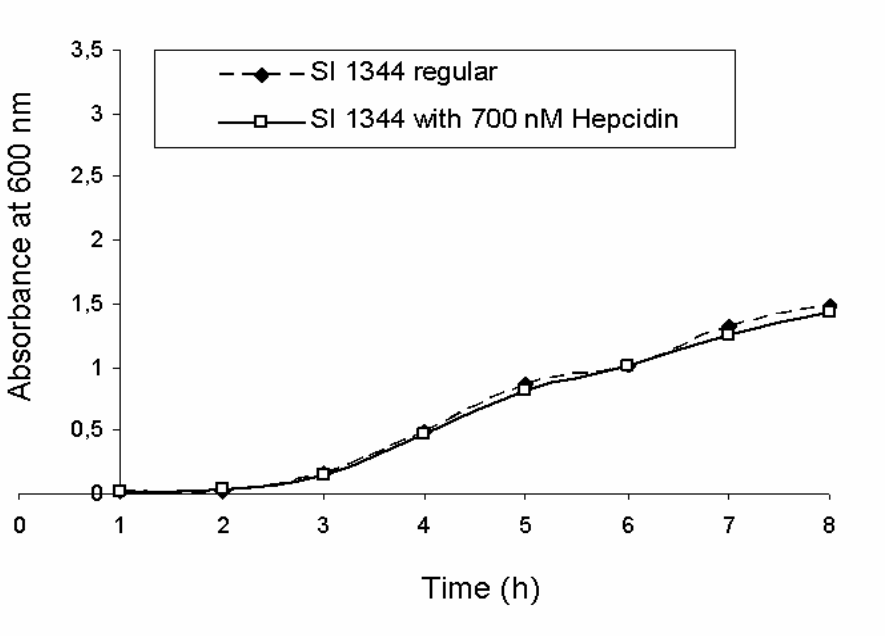


Fig. 11: 700 nM of hepcidin does not directly influence growth of *Salmonella* as measured by spectrophotometry at 600 nm wave length. Bacterial growth was compared in two LB-cultures. Both cultures contained 10 µl saturated *Salmonella* culture per ml of LB-medium. One of the cultures contained in addition 700 nM of hepcidin.

4. Discussion

Here I report that changes in FPN1 expression modulate intracellular bacterial survival in HeLa cells and J774 macrophages. I found that increased expression of FPN1, either in transiently transfected HeLa cells or in stably transfected J774 macrophages, resulted in significant inhibition of bacterial growth. Conversely, when I treated FPN1 expressing cells with hepcidin, a hepatic peptide that induces FPN1 degradation, intracellular growth of *Salmonella* was enhanced. This effect was less pronounced with a hepcidin-resistant mutant of FPN1. This study demonstrates a direct functional role of FPN1 and an indirect functional role of hepcidin on *Salmonella* infection. Moreover, this is the first study, to my knowledge, demonstrating functional differences of wild-type and mutant FPN1 during intracellular bacterial infection.

The macrophage is both the host's main iron recycler and the home for intracellular iron-dependent bacteria such as *Salmonella*. To fight infections, macrophages require iron themselves as a cofactor for the execution of important antimicrobial effector mechanisms, including the NADPH dependent oxidative burst and the production of nitrogen radicals catalyzed by the inducible nitric oxide synthase [91, 103]. Several mechanisms in iron regulation have been identified to date. In the macrophage, iron metabolism is partly regulated by Nramp1, which transports iron and other metals into and/or out of the phagosome [104]. Nramp1 is a pH-dependent efflux pump at the phagosomal membrane that directly depletes the intraphagosomal environment of Mn^{2+} and Fe^{2+}. This depletion seems to create a stressful and growth-limiting environment that is actively sensed by the bacteria and results in increased bacteriostasis and/or bactericidal activity [105]. Mutations of the Nramp1 gene cause susceptibility to infection by intracellular microbes [82, 106]. Nramp1 expression thus inhibits bacterial growth. Deprivation of nutritional iron in mice is associated with prolonged survival and decreased mortality from acute *Salmonella* infection

[107]. NRAMP homologues have been also identified in bacteria. They are involved in acquiring divalent metals from the extracellular environment. Interestingly, bacterial and mammalian NRAMP proteins compete for the same essential substrates within the microenvironment of the phagosome [87]. Nramp2, the divalent metal iron transporter 2 (DCT1/DMT1), was identified as the major intestinal iron absorptive mechanism at the apical membrane of the duodenum [108, 109]. It also localizes in recycling endosomes where it transports iron into the cytosol [110, 111]. Like Nramp1, Nramp2 has been shown to be recruited to the phagolysosome in J774 cells [112], suggesting that this protein plays a role in intracellular iron handling in the macrophage. However, the potential roles Nramp1 and Nramp2 play in iron recycling remain unknown. The only known cellular iron exporter identified to date is FPN1 [31, 33, 34], which is a nonheme iron-export protein located on the basolateral membrane of duodenal enterocytes [31, 33, 34] and is abundantly expressed in reticuolendothelial macrophages of the liver, bone marrow, and splenic red pulp [35]. FPN1 is important for iron recycling [36], and FPN1's expression is post-translationally regulated by hepcidin [21].

This in vitro study was carried out using transiently transfected HeLa cells and stably transfected J774 macrophages. FPN1 was consistently and adequately expressed in HeLa cells after transient transfection. Overexpression of FPN1 resulted in significant inhibition of bacterial growth as measured by gentamicin protection assays. This effect could be caused either by FPN1 itself or by other effects related to the transfection procedure. The high level of FPN1 expression attained after transient transfection could be deleterious for the cell. In fact, high levels of FPN1 have been reported to be toxic to cells in culture [113]. I observed under the microscope morphological changes in cells after transfection of FPN1 which suggest cell death. This possibility was confirmed by measuring total protein concentration in the lysates of transfected cells. I found that these lysates had less total protein concentration, suggesting diminished cells per dish due to cell death. It is known that dead cells release intracellular bacteria that usually

infect neighboring cells. In my experimental setting, bacteria released from dead cells were most likely killed by the gentamicin present in the culture medium, which could have affected the results of the gentamicin protection assays. Therefore, cell death is one major confounding variable in my overexpression experiments with HeLa cells.

Several plasmids were used to serve as controls for effects of the transfection procedure. Interestingly, the insertion of plasmids resulted in decreased bacterial growth in all of my cells. I used plasmids coding for proteins with no known function in iron-transport or infection such as GFP, CMV or Traf2ΔR, and also the iron-transporter Nramp2 which is suspected to act on the SCV, but its precise function is unclear so far. None of these proteins is known to inhibit bacterial growth, but all cells that were transfected with the mentioned plasmids showed dramatic decrease in *Salmonella* survival measured at 22 h post-infection. Diminished bacterial growth thus appears to be triggered by a common mechanism caused by the introduction of plasmids into HeLa cells. I suspect interference of the plasmids with the pattern recognition receptor (PRR), also called toll-like receptor 9 (TLR9) that is expressed in HeLa cells [114] and that plays a role in modulating bacterial infections [100, 101]. Unmethylated CpG DNAs activate the vertebrate innate immune system through TLR9 [100]. The plasmids used in my study may contain unmethylated CpG DNA regions, because studies on plasmids coding for different eukaryotic and viral promoters have shown that sequence specific DNA-methylation [115], particularly methylation of 5´-CG-3´ regions [116], inactivate plasmid promoters, possibly by interaction with transcription factor binding [117, 118]. Therefore, reporter gene expression is >90% decreased upon transient transfection into cultured mammalian cells if all CpG sites of expression plasmids are methylated [116, 119, 120]. The plasmids used in my study expressed their proteins at high levels; therefore I conclude that the plasmids contained unmethylated CpG DNA regions. Unmethylated CpG DNA activates signaling cascades through the TLR9 receptor leading to the secretion of a number of cytokines and chemokines [100].

A study in mice showed that TLR9 may play a role in controlling infection with *Salmonella enterica serovar Typhimurium*. A 15-fold increase in TLR9 mRNA expression was observed over the first 7 days of infection [101]. However, it is not known to date how CpG-ODNs, TLR9 and *Salmonella* interact exactly. But there seem to be sufficient data to assume that these variables influence each other. The results of my experiments could be explained by these interactions, although further studies must be undertaken to confirm my hypothesis that some of the growth inhibitory effects on *Salmonella* seen in the transfected cells may be related to activation of TLR9-dependent mechanisms.

Bacterial growth measured in transiently transfected HeLa cells may be also affected by other variables of the transfection procedure. Therefore, I mostly used HeLa cells expressing GFP as controls for the transfection procedure, a protein that has no known function in bacterial replication. Statistical significance was determined based on the difference of cfu in GFP-expressing and FPN1-expressing cells. My data show that overexpression of FPN1 results in significant decrease of bacterial growth compared to GFP overexpressing cells. Therefore, I can conclude that FPN1 overexpression is responsible for decreased *Salmonella* survival in these HeLa cell experiments.

The results obtained in HeLa cells were confirmed by experiments carried out in J774 macrophages. I evaluated overexpression of FPN1 in J774 macrophages by using retrovirally transduced cell lines. Besides avoiding the possibility of TLR stimulation, these cell lines offer other advantages over the transiently transfected HeLa cells. First, genes delivered into the cell by retroviral transduction are integrated into the host genome, an approach that allows for the generation of stable cell lines overexpressing FPN1. Second, retrovirally transduced genes are often expressed at more modest levels and may thus provide a better representation of functional changes reflecting activity of native protein levels. As previously reported, the stable J774 9-clone displayed basal FPN1 protein levels that were about 3 times higher than those measured in the

GFP overexpressing G-clone [94]. The results of FPN1 overexpression and GFP overexpression in J774 macrophages are in agreement with the results of the HeLa cell experiments. These findings correspond to the first aim of my study which was to determine the effect of overexpression of wild-type FPN1 on the intracellular growth of *Salmonella*.

To evaluate further the role of FPN1 on bacterial growth, the iron exporter was downregulated by treating the cell cultures with hepcidin. FPN1 downregulation resulted in enhanced bacterial growth compared to bacterial growth in FPN1 expressing cells not exposed to hepcidin, which promotes FPN1's lysosomal degradation and consequently decreases protein expression on the cell surface. The enhancement of *Salmonella*'s growth is likely due to hepcidin's regulation of FPN1. These findings correspond to the second aim of my study which was to determine the effect of FPN1 downregulation by hepcidin on the intracellular growth of *Salmonella*.

An hepcidin-concentration as low as 0.1 µM (0.3 µg/ml) induces FPN1 internalization within 1 h, whereas concentrations 10 times lower result in FPN1 internalization over a 3-h time course [21]. These values are consistent with estimates of plasma hepcidin concentration based on urinary hepcidin excretion in either iron-loaded or infected individuals [40, 121]. My data show that 700 nM of hepcidin has no direct effect on bacterial growth in culture medium. This corresponds to the third aim of my study which was to determine if 700 nM of hepcidin has direct effects on *Salmonella's* growth.

Several mechanisms can explain how FPN1 and hepcidin modulate *Salmonella* survival. FPN1 may inhibit bacterial growth by either acting on the mammalian membrane as an iron-exporter, or by acting on the endosomal membrane of the SCV as an iron-importer. Considering FPN1's functions on the mammalian membrane as an iron-exporter, the consequent decrease in cytoplasmic iron level may lead to very low iron levels in the microenvironment of the SCV

resulting in insufficient iron being transported into the SCV. Lack of iron inside the SCV could deteriorate bacterial housekeeping functions such as the respiratory chain and thus inhibit bacterial growth. Low cytoplasmic iron levels might also disturb the endosomal pathway. Direct interaction between SCVs and the late endosomal compartments normally does not occur, allowing *Salmonella* to escape from the host defense after invasion and to remain alive inside the SCV [122]. A study showed that chelator-mediated depletion of intracellular iron in macrophages stimulates recruitment of the mannose-6-phosphate-receptor (M6PR) to the SCV [105]. M6PR is essential for recruitment of lysosomal enzymes to the endosome and enables the lysosomes to fuse with SCVs. This fusion induces elimination of the bacteria from the host cell. Low levels of cytoplasmic iron in the host cell may thus hinder *Salmonella* from maintaining the integrity of the SCV and deprive the bacteria of its essential niche or deprive the bacteria of iron that is required for bacterial replication inside the SCV.

Another reasonable hypothesis is that FPN1 acts on the endosomal membrane of the SCV as an iron-importer. The SCV contains plasma membrane proteins that have been shown to aggregate at the site of bacterial invasion [123]. Upon invasion, *Salmonella* is engulfed by the host cell membrane to form the SCV [61]. Since the outside of the cell turns into the inside of the SCV, FPN1 might function as an iron-importer in the SCV. The SCV seems to contain sufficient iron for *Salmonella* to meet its iron requirement without stress [124], indicating that an iron-supply mechanism might be available. A microarray study investigated *Salmonella's* gene expression during the first 12 h of intracellular residence in J774-A1 macrophages, and no upregulation of iron-dependent genes was seen [124]. It is possible to deduce from this study if *Salmonella* was lacking iron in their experimental condition, since expression level of iron-dependent genes is regulated according to iron availability. In the presence of high iron concentrations, the Fur protein binds to Fe^{2+} and represses the expression of genes involved in iron uptake in *Salmonella* [125]. The study shows on the one hand that the Fur-dependent entABCE and iroDEN genes are highly down-

regulated during intracellular growth [125, 126]; and on the other hand that an alternative iron-responsive mechanism, which involves the PmrAB two-component system induced by high levels of Fe^{3+} [127], is also down-regulated. These data suggest that the SCV contains Fe^{2+} [124], since the Fur-dependent genes are down-regulated and Fur is activated by high levels of Fe^{2+} and that the SCV contains low levels of Fe^{3+}, since the Fe^{3+} dependent systems are down-regulated. *Salmonella's* iron requirements therefore seemed to be satisfied within the first 12 h inside the J774-A1 macrophage. It seems possible that FPN1 may act as an iron-importer in the SCV and provide the required iron.

In our experiments, if FPN1 acts on the SCV as an iron-importer, available iron might have exceeded *Salmonella's* requirements due to FPN1 overexpression and resulted in toxic levels of iron as well as free radical formation that killed the bacteria. In agreement with this model, downregulation of FPN1 reversed the situation and enhanced *Salmonella* survival in cells overexpressing FPN1. It would be unclear, though, why hepcidin enhanced *Salmonella* survival in parental J774 macrophages that display FPN1 at physiologic level. This might be explained either if generally FPN1 mediated iron-import into the SCV disturbs bacterial functions, or if FPN1 does not directly transport iron into the SCV. Recent data show that FPN1 deprives intracellular *Salmonella* of iron and thus support the hypothesis that iron deprivation is the likely mechanism by which FPN1 inhibits *Salmonella's* growth [128, 129].

Several studies have shown that patients and mice with iron overload are more susceptible to a number of intracellular and blood pathogens [90, 91], and even relatively modest increase in iron intake may diminish host resistance to infection [92]. Many intracellular pathogens such as *Mycobacterium Tuberculosis*, *Salmonella enterica serovar Typhi* or *Toxoplasma* require iron within reticuloendothelial cells in order to become virulent and replicate [93]. I speculated that a decrease of iron in macrophages would reduce the ability of iron-dependent organisms for colonization and improve the host's resistance to

infection. My experimental findings support this hypothesis. I also speculated that hepcidin, a peptide that increases the intracellular iron pool [21, 94], should favor the survival of iron-dependent organisms. Individuals with the Q182H mutation, which lacks sensitivity to hepcidin [32], may have increased resistance to infections with iron-dependent organisms. I showed that bacterial growth in cells expressing the Q182H mutant remains unchanged after hepcidin exposure, a positive effect for the host cell. *Salmonella enterica serovar Typhi*, *Mycobacterium tuberculosis* and other intracellular pathogens, might have selected for the mutation in the human host in the past. This possibility may explain the preservation of this mutation in the human population. This corresponds to the fourth aim of my study that was to determine if the mutant Q182H variant of FPN1 has different effects than wild-type FPN1 on the intracellular growth of *Salmonella*.

My study shows that downregulation of FPN1 by hepcidin enhances bacterial growth. Interestingly, the changes in hepcidin levels and iron metabolism that occur during inflammatory states have generally been considered to be inhibitory for infection. During states of infection secretion of hepcidin is upregulated. The upregulation of hepcidin may result in decreased iron content in the plasma and prevent infections in the extracellular compartment such as in the blood stream. However, my observations raise the possibility that hepcidin promotes growth of intracellular bacteria. If an iron dependent organism such as *Salmonella* is once intracellularly, then hepcidin seems to have an effect that is in opposition to the host's interest. Bacteria such as *Salmonella* may be able to survive intracellularly in macrophages for extended periods of time because of hepcidin's favorable effect. However, my experimental setting was limited and may not be representative for the complex interactions occurring between hepcidin, ferroportin, *Salmonella* and other factors in a mouse or human being.

Patients with type 4 hemochromatosis have iron-overloaded reticuloendothelial cells [12, 18]. It seems contradictory, since lack of FPN1 downregulation should

result in augmented iron-export from the cell and would lead to low cytoplasmic iron levels. Hepcidin's mechanisms in iron-transport seem to be more complex than pure downregulation of FPN1 on the cell surface, and most likely other variables are of great importance as well. Clearly, patients and mice with iron overload are far more susceptible to intracellular and blood pathogens [90, 91]. Elucidating the causative mechanisms of iron-overload in macrophages may lead to new insights into iron-regulatory mechanisms during infections with intracellular pathogen.

Knowledge about the precise mechanisms of interaction might provide a target for new therapeutic agents. New therapeutic options are particularly important since multi-drug microbial resistance of *Salmonella* and also of other intracellular pathogens such as *Mycobacterium* constitutes a severe health problem these days. If hepcidin follows the pattern of other peptide hormones or cytokines, its actions will be mediated by cell surface receptors [44]. Elucidation of the receptor and its transduction pathways can lead to the development of hepcidin agonists and antagonists. On the one hand, a cell-type specific modulation of FPN1-expression, eventually possible through cell-type-specific hepcidin antagonists, may be useful in therapies for chronic carriers of intracellular iron-dependent pathogens and may create an effect similar to the phenotype displayed by the FPN1 mutation Q182H, which is unresponsive to hepcidin and shows increased resistance to bacteria such as *Salmonella*. On the other hand, an hepcidin agonist could be used to fight early infections before an iron-dependent bacteria reaches its intracellular residence. Improved understanding of the host defense role of iron sequestration would facilitate such applications.

Iron retrieval is an essential component of bacterial pathogenesis and is a source of battle between the host and pathogen. Hepcidin may be the principal iron-regulatory hormone, and a bridge between iron metabolism and innate immunity. Studies of the molecular mechanisms of hepcidin activity, FPN1 function and *Salmonella's* iron requirements for virulence could transform our understanding

of the regulation of iron transport and innate immunity and may lead to new therapies for hemochromatosis, anemia of inflammation and infections with intracellular pathogens such as *Salmonella.*

5. Final Summary

5.1. Summary in English

Hemochromatosis type 4 is an iron overload disease which is caused by mutations of ferroportin 1 (FPN1), the only known eukaryotic iron-exporter to date. FPN1 is a transmembrane protein that is post-transcriptionally regulated by hepcidin. Treatment of cells with hepcidin results in internalization and degradation of cell surface FPN1. The Q182H mutant of FPN1 appears to be unresponsive to hepcidin and does not show the expected internalization on exposure to hepcidin. Hepcidin is upregulated in response to iron overload, inflammation and infection. *Salmonella enterica serovar Typhimurium*, the causative agent of gastroenteritis in humans, produces a severe systemic disease resembling human typhoid fever in mice. In typhoid, pathogens survive for extended periods of time in a host cell compartment, the *Salmonella*-containing vacuole, which is segregated from the normal late endosomal trafficking pathway. *Salmonella* require iron as a nutrient for survival and for maintaining full virulence. Correspondingly, the mammalian metal transporter Nramp1 functions in innate defense against the organism by altering intracellular iron concentrations. I carried out experiments to test the idea that FPN1, another macrophage iron transporter, might also be involved in regulating the intracellular growth of *Salmonella*. Moreover, I tested the idea that the Q182H mutant may differently affect *Salmonella's* growth than wild-type FPN1. In this study, I used transiently transfected HeLa cells and stably transfected J774 macrophages to evaluate the effect of FPN1 and hepcidin on intracellular growth of *Salmonella enterica serover Typhimurium*. Viable intracellular bacteria were quantified by gentamicin protection assays at 2 and 22 h post-infection. I found that increased expression of FPN1, either in transiently transfected HeLa cells or in stably transfected J774 macrophages, resulted in significant inhibition of bacterial growth. Conversely, when I treated FPN1 expressing cells with hepcidin, intracellular growth of *Salmonella* was enhanced. This effect was less

pronounced with the hepcidin-resistant mutant Q182H of FPN1. My findings indicate an important role for FPN1 in controlling the growth of *Salmonella* inside cells and suggest that altered FPN1 expression and function in hemochromatosis, and possibly other iron overload states, may explain the susceptibility to intracellular pathogens in these conditions. While upregulation of hepcidin in response to infection is generally considered to be protective, my observations indicate that the opposite is true for intracellular pathogens. Hepcidin-resistant FPN1 mutations like Q182H may thus confer an advantage to the infected host by preserving resistance to intracellular pathogens even in the presence of elevated hepcidin. My data provide the first molecular evidence for a possible mechanism that can explain why the mutant variant Q182H of FPN1 persisted during evolution.

5.2. Zusammenfassung auf Deutsch

Hämochromatose Typ 4 ist eine durch Eisenüberladung gekennzeichnete Eisenstoffwechselstörung, die durch Mutationen in Ferroportin 1 (FPN1) verursacht wird, welches das einzige zur Zeit bekannte Eisenexportprotein ist. FPN1 ist ein Transmembranprotein, dessen Expression an der Zelloberfläche posttranskriptionell von Hepzidin reguliert wird. Werden Zellen mit Hepzidin behandelt, wird FPN1 vermindert an der Zelloberfläche exprimiert, internalisiert und lysosomal degradiert. Die Q182H Mutante des FPN1 scheint nicht auf Hepzidin zu reagieren, denn die erwartete verminderte Expression von FPN1 an der Zelloberfläche nach Kontakt mit Hepzidin erfolgt nicht. Hepzidin ist ein Peptid, das in Folge von Eisenüberladung, Entzündung oder Infektion von der Leber sezerniert wird. *Salmonella enterica serovar Typhimurium* verursacht Gastroenteritis im Menschen und eine schwere systemische Erkrankung in der Maus, die dem menschlichen Typhus ähnlich ist. Bei Typhus überlebt das Bakterium intrazellulär für einen längeren Zeitraum in einer Vakuole, die vom späten endosomalen Stoffwechsel getrennt ist. *Salmonellen* brauchen Eisen sowohl zum Überleben als auch um ihre Virulenz vollständig aufrechtzuerhalten. Der Eisentransporter Nramp1 ist Teil der angeborenen Abwehr gegen

Organismen, indem er die intrazelluläre Eisenkonzentration verändert. In der vorliegenden Dissertationsschrift habe ich untersucht, ob FPN1, ein anderer Eisentransporter des Monozyten-Makrophagen-Systems, ebenfalls eine Rolle bei der Regulation des intrazellulären Wachstums von *Salmonellen* spielt. Ausserdem habe ich untersucht, ob die Q182H Mutante anders als natives FPN1 *Salmonellen* reguliert. In dieser Arbeit benutze ich transient transfizierte HeLa Zellen und stabil transfizierte J774 Makrophagen, um den Effekt von FPN1 und Hepzidin auf das intrazelluläre Wachstum von *Salmonella enterica serovar Typhimurium* zu evaluieren. Lebende intrazelluläre Bakterien wurden 2 und 22 Stunden nach der Infektion quantifiziert. Meine Ergebnisse von Gentamizin-Protektions-Assays zeigen, dass vermehrte Expression von Ferroportin das intrazelluläre Wachstum von Salmonellen vermindert. Dieser Effekt wird durch Hepzidin revidiert. Die Q182H Mutante des FPN1 ist resistent gegenüber Hepzidin und mindert Hepzidin's fördernden Effekt auf das bakterielle Wachstum. Meine Ergebnisse weisen eine wichtige Rolle von FPN1 in der Abwehr gegen intrazelluläre *Salmonellen* auf und lassen vermuten, dass Änderungen in der FPN1 Expression and Funktion, wie es bei der Hämochromatose und anderen durch Eisenüberladung gekennzeichneten Krankheiten der Fall ist, die Anfälligkeit für intrazelluläre Bakterien erklären. Während Hochregulation von Hepzidin bei Infektion allgemein als Schutz angesehen wird, lassen meine Beobachtungen vermuten, dass das Gegenteil bei intrazellulären Infektionen zutrifft. Hepzidin-resistente FPN1 Mutationen wie z.B. Q182H können zum Vorteil für den infizierten Wirt sein, indem sie Resistenz sogar bei vermehrtem Hepzidin gegen intrazelluläre Bakterien vermitteln. Das ist der erste Nachweis auf molekularer Ebene, der erklären kann, warum die Q182H Mutation von FPN1 während der Evolution persistiert hat.

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7. References

1. Pietrangelo, A., *Hereditary hemochromatosis.* Biochim Biophys Acta, 2006. **1763**(7): p. 700-10.
2. Feder, J.N., A. Gnirke, W. Thomas, et al., *A novel MHC class I-like gene is mutated in patients with hereditary haemochromatosis.* Nat Genet, 1996. **13**(4): p. 399-408.
3. Roetto, A., G. Papanikolaou, M. Politou, et al., *Mutant antimicrobial peptide hepcidin is associated with severe juvenile hemochromatosis.* Nat Genet, 2003. **33**(1): p. 21-2.
4. Nicolas, G., M. Bennoun, I. Devaux, et al., *Lack of hepcidin gene expression and severe tissue iron overload in upstream stimulatory factor 2 (USF2) knockout mice.* Proc Natl Acad Sci U S A, 2001. **98**(15): p. 8780-5.
5. Papanikolaou, G., M.E. Samuels, E.H. Ludwig, et al., *Mutations in HFE2 cause iron overload in chromosome 1q-linked juvenile hemochromatosis.* Nat Genet, 2004. **36**(1): p. 77-82.
6. Camaschella, C., A. Roetto, A. Cali, et al., *The gene TFR2 is mutated in a new type of haemochromatosis mapping to 7q22.* Nat Genet, 2000. **25**(1): p. 14-5.
7. Deaglio, S., A. Capobianco, A. Cali, et al., *Structural, functional, and tissue distribution analysis of human transferrin receptor-2 by murine monoclonal antibodies and a polyclonal antiserum.* Blood, 2002. **100**(10): p. 3782-9.
8. Pietrangelo, A., *The ferroportin disease.* Blood Cells Mol Dis, 2004. **32**(1): p. 131-8.
9. Sham, R.L., P.D. Phatak, C. West, et al., *Autosomal dominant hereditary hemochromatosis associated with a novel ferroportin mutation and unique clinical features.* Blood Cells Mol Dis, 2005. **34**(2): p. 157-61.

10. Montosi, G., A. Donovan, A. Totaro, et al., *Autosomal-dominant hemochromatosis is associated with a mutation in the ferroportin (SLC11A3) gene.* J Clin Invest, 2001. **108**(4): p. 619-23.
11. Njajou, O.T., N. Vaessen, M. Joosse, et al., *A mutation in SLC11A3 is associated with autosomal dominant hemochromatosis.* Nat Genet, 2001. **28**(3): p. 213-4.
12. Devalia, V., K. Carter, A.P. Walker, et al., *Autosomal dominant reticuloendothelial iron overload associated with a 3-base pair deletion in the ferroportin 1 gene (SLC11A3).* Blood, 2002. **100**(2): p. 695-7.
13. Cazzola, M., L. Cremonesi, M. Papaioannou, et al., *Genetic hyperferritinaemia and reticuloendothelial iron overload associated with a three base pair deletion in the coding region of the ferroportin gene (SLC11A3).* Br J Haematol, 2002. **119**(2): p. 539-46.
14. Hetet, G., I. Devaux, N. Soufir, et al., *Molecular analyses of patients with hyperferritinemia and normal serum iron values reveal both L ferritin IRE and 3 new ferroportin (slc11A3) mutations.* Blood, 2003. **102**(5): p. 1904-10.
15. Roetto, A., A.T. Merryweather-Clarke, F. Daraio, et al., *A valine deletion of ferroportin 1: a common mutation in hemochromastosis type 4.* Blood, 2002. **100**(2): p. 733-4.
16. Rivard, S.R., C. Lanzara, D. Grimard, et al., *Autosomal dominant reticuloendothelial iron overload (HFE type 4) due to a new missense mutation in the FERROPORTIN 1 gene (SLC11A3) in a large French-Canadian family.* Haematologica, 2003. **88**(7): p. 824-6.
17. Arden, K.E., D.F. Wallace, J.L. Dixon, et al., *A novel mutation in ferroportin1 is associated with haemochromatosis in a Solomon Islands patient.* Gut, 2003. **52**(8): p. 1215-7.
18. Wallace, D.F., P. Pedersen, J.L. Dixon, et al., *Novel mutation in ferroportin1 is associated with autosomal dominant hemochromatosis.* Blood, 2002. **100**(2): p. 692-4.

19. Pietrangelo, A., G. Montosi, A. Totaro, et al., *Hereditary hemochromatosis in adults without pathogenic mutations in the hemochromatosis gene.* N Engl J Med, 1999. **341**(10): p. 725-32.
20. Cremonesi, L., G.L. Forni, N. Soriani, et al., *Genetic and clinical heterogeneity of ferroportin disease.* Br J Haematol, 2005. **131**(5): p. 663-70.
21. Nemeth, E., M.S. Tuttle, J. Powelson, et al., *Hepcidin regulates cellular iron efflux by binding to ferroportin and inducing its internalization.* Science, 2004. **306**(5704): p. 2090-3.
22. Drakesmith, H., L.M. Schimanski, E. Ormerod, et al., *Resistance to hepcidin is conferred by hemochromatosis-associated mutations of ferroportin.* Blood, 2005.
23. Schimanski, L.M., H. Drakesmith, A.T. Merryweather-Clarke, et al., *In vitro functional analysis of human ferroportin (FPN) and hemochromatosis-associated FPN mutations.* Blood, 2005. **105**(10): p. 4096-102.
24. Harris, Z.L., A.P. Durley, T.K. Man, et al., *Targeted gene disruption reveals an essential role for ceruloplasmin in cellular iron efflux.* Proc Natl Acad Sci U S A, 1999. **96**(19): p. 10812-7.
25. Beaumont, C., P. Leneuve, I. Devaux, et al., *Mutation in the iron responsive element of the L ferritin mRNA in a family with dominant hyperferritinaemia and cataract.* Nat Genet, 1995. **11**(4): p. 444-6.
26. Cazzola, M., G. Bergamaschi, L. Tonon, et al., *Hereditary hyperferritinemia-cataract syndrome: relationship between phenotypes and specific mutations in the iron-responsive element of ferritin light-chain mRNA.* Blood, 1997. **90**(2): p. 814-21.
27. Martin, M.E., S. Fargion, P. Brissot, et al., *A point mutation in the bulge of the iron-responsive element of the L ferritin gene in two families with the hereditary hyperferritinemia-cataract syndrome.* Blood, 1998. **91**(1): p. 319-23.
28. Camaschella, C., G. Zecchina, G. Lockitch, et al., *A new mutation (G51C) in the iron-responsive element (IRE) of L-ferritin associated with*

hyperferritinaemia-cataract syndrome decreases the binding affinity of the mutated IRE for iron-regulatory proteins. Br J Haematol, 2000. **108**(3): p. 480-2.

29. Bosio, S., A. Campanella, E. Gramaglia, et al., *C29G in the iron-responsive element of L-ferritin: a new mutation associated with hyperferritinemia-cataract.* Blood Cells Mol Dis, 2004. **33**(1): p. 31-4.
30. Phillips, J.D., C.A. Warby, and J.P. Kushner, *Identification of a novel mutation in the L-ferritin IRE leading to hereditary hyperferritinemia-cataract syndrome.* Am J Med Genet A, 2005. **134**(1): p. 77-9.
31. Donovan, A., A. Brownlie, Y. Zhou, et al., *Positional cloning of zebrafish ferroportin1 identifies a conserved vertebrate iron exporter.* Nature, 2000. **403**(6771): p. 776-81.
32. Liu, X.B., F. Yang, and D.J. Haile, *Functional consequences of ferroportin 1 mutations.* Blood Cells Mol Dis, 2005.
33. Abboud, S. and D.J. Haile, *A novel mammalian iron-regulated protein involved in intracellular iron metabolism.* J Biol Chem, 2000. **275**(26): p. 19906-12.
34. McKie, A.T., P. Marciani, A. Rolfs, et al., *A novel duodenal iron-regulated transporter, IREG1, implicated in the basolateral transfer of iron to the circulation.* Mol Cell, 2000. **5**(2): p. 299-309.
35. Yang, F., X.B. Liu, M. Quinones, et al., *Regulation of reticuloendothelial iron transporter MTP1 (Slc11a3) by inflammation.* J Biol Chem, 2002. **277**(42): p. 39786-91.
36. Knutson, M.D., M.R. Vafa, D.J. Haile, et al., *Iron loading and erythrophagocytosis increase ferroportin 1 (FPN1) expression in J774 macrophages.* Blood, 2003. **102**(12): p. 4191-7.
37. Gardenghi, S., M.F. Marongiu, P. Ramos, et al., *Ineffective erythropoiesis in beta-thalassemia is characterized by increased iron absorption mediated by down-regulation of hepcidin and up-regulation of ferroportin.* Blood, 2007. **109**(11): p. 5027-35.

38. De Domenico, I., D.M. Ward, C. Langelier, et al., *The molecular mechanism of hepcidin-mediated ferroportin down-regulation.* Mol Biol Cell, 2007. **18**(7): p. 2569-78.
39. Pigeon, C., G. Ilyin, B. Courselaud, et al., *A new mouse liver-specific gene, encoding a protein homologous to human antimicrobial peptide hepcidin, is overexpressed during iron overload.* J Biol Chem, 2001. **276**(11): p. 7811-9.
40. Nemeth, E., E.V. Valore, M. Territo, et al., *Hepcidin, a putative mediator of anemia of inflammation, is a type II acute-phase protein.* Blood, 2003. **101**(7): p. 2461-3.
41. Hunter, H.N., D.B. Fulton, T. Ganz, et al., *The solution structure of human hepcidin, a peptide hormone with antimicrobial activity that is involved in iron uptake and hereditary hemochromatosis.* J Biol Chem, 2002. **277**(40): p. 37597-603.
42. Nicolas, G., C. Chauvet, L. Viatte, et al., *The gene encoding the iron regulatory peptide hepcidin is regulated by anemia, hypoxia, and inflammation.* J Clin Invest, 2002. **110**(7): p. 1037-44.
43. Krause, A., S. Neitz, H.J. Magert, et al., *LEAP-1, a novel highly disulfide-bonded human peptide, exhibits antimicrobial activity.* FEBS Lett, 2000. **480**(2-3): p. 147-50.
44. Ganz, T., *Hepcidin, a key regulator of iron metabolism and mediator of anemia of inflammation.* Blood, 2003. **102**(3): p. 783-8.
45. Shike, H., X. Lauth, M.E. Westerman, et al., *Bass hepcidin is a novel antimicrobial peptide induced by bacterial challenge.* Eur J Biochem, 2002. **269**(8): p. 2232-7.
46. Anderson, G.J., D.M. Frazer, S.J. Wilkins, et al., *Relationship between intestinal iron-transporter expression, hepatic hepcidin levels and the control of iron absorption.* Biochem Soc Trans, 2002. **30**(4): p. 724-6.
47. King, D.S., C.G. Fields, and G.B. Fields, *A cleavage method which minimizes side reactions following Fmoc solid phase peptide synthesis.* Int J Pept Protein Res, 1990. **36**(3): p. 255-66.

48. Lesser CF, M.S., *Salmonellosis*, in *Harrison´s Principles of Internal Medicine*, F. Braunwald, Kasper, Hauser, Longo, Jameson, Editor. 2001, McGraw-Hill. p. 970-975.
49. House, D., A. Bishop, C. Parry, et al., *Typhoid fever: pathogenesis and disease.* Curr Opin Infect Dis, 2001. **14**(5): p. 573-8.
50. Wain, J., T.T. Hien, P. Connerton, et al., *Molecular typing of multiple-antibiotic-resistant Salmonella enterica serovar Typhi from Vietnam: application to acute and relapse cases of typhoid fever.* J Clin Microbiol, 1999. **37**(8): p. 2466-72.
51. Bakken, K. and T.M. Vogelsang, *The pathogenesis of Salmonella typhimurium infection in mice.* Acta Pathol Microbiol Scand, 1950. **27**(1): p. 41-50.
52. Rabsch, W., H. Tschape, and A.J. Baumler, *Non-typhoidal salmonellosis: emerging problems.* Microbes Infect, 2001. **3**(3): p. 237-47.
53. Glynn, M.K., C. Bopp, W. Dewitt, et al., *Emergence of multidrug-resistant Salmonella enterica serotype typhimurium DT104 infections in the United States.* N Engl J Med, 1998. **338**(19): p. 1333-8.
54. Cherayil, B.J., *How not to get bugged by bugs: mechanisms of cellular tolerance to microorganisms.* Curr Opin Gastroenterol, 2003. **19**(6): p. 572-7.
55. Day, D.W., B.K. Mandal, and B.C. Morson, *The rectal biopsy appearances in Salmonella colitis.* Histopathology, 1978. **2**(2): p. 117-31.
56. McGovern, V.J. and L.J. Slavutin, *Pathology of salmonella colitis.* Am J Surg Pathol, 1979. **3**(6): p. 483-90.
57. Rout, W.R., S.B. Formal, G.J. Dammin, et al., *Pathophysiology of Salmonella diarrhea in the Rhesus monkey: Intestinal transport, morphological and bacteriological studies.* Gastroenterology, 1974. **67**(1): p. 59-70.
58. Galan, J.E., *Salmonella interactions with host cells: type III secretion at work.* Annu Rev Cell Dev Biol, 2001. **17**: p. 53-86.

59. Hansen-Wester, I. and M. Hensel, *Salmonella pathogenicity islands encoding type III secretion systems.* Microbes Infect, 2001. **3**(7): p. 549-59.
60. Waterman, S.R. and D.W. Holden, *Functions and effectors of the Salmonella pathogenicity island 2 type III secretion system.* Cell Microbiol, 2003. **5**(8): p. 501-11.
61. Francis, C.L., T.A. Ryan, B.D. Jones, et al., *Ruffles induced by Salmonella and other stimuli direct macropinocytosis of bacteria.* Nature, 1993. **364**(6438): p. 639-42.
62. Salcedo, S.P., M. Noursadeghi, J. Cohen, et al., *Intracellular replication of Salmonella typhimurium strains in specific subsets of splenic macrophages in vivo.* Cell Microbiol, 2001. **3**(9): p. 587-97.
63. Young, D., T. Hussell, and G. Dougan, *Chronic bacterial infections: living with unwanted guests.* Nat Immunol, 2002. **3**(11): p. 1026-32.
64. Gorvel, J.P. and S. Meresse, *Maturation steps of the Salmonella-containing vacuole.* Microbes Infect, 2001. **3**(14-15): p. 1299-303.
65. Holden, D.W., *Trafficking of the Salmonella vacuole in macrophages.* Traffic, 2002. **3**(3): p. 161-9.
66. Knodler, L.A. and O. Steele-Mortimer, *Taking possession: biogenesis of the Salmonella-containing vacuole.* Traffic, 2003. **4**(9): p. 587-99.
67. Brumell, J.H. and S. Grinstein, *Salmonella redirects phagosomal maturation.* Curr Opin Microbiol, 2004. **7**(1): p. 78-84.
68. Steele-Mortimer, O., J.H. Brumell, L.A. Knodler, et al., *The invasion-associated type III secretion system of Salmonella enterica serovar Typhimurium is necessary for intracellular proliferation and vacuole biogenesis in epithelial cells.* Cell Microbiol, 2002. **4**(1): p. 43-54.
69. Weiss, G., G. Werner-Felmayer, E.R. Werner, et al., *Iron regulates nitric oxide synthase activity by controlling nuclear transcription.* J Exp Med, 1994. **180**(3): p. 969-76.

70. Zhang, Y., R.R. Crichton, J.R. Boelaert, et al., *Decreased release of nitric oxide (NO) by alveolar macrophages after in vivo loading of rats with either iron or ethanol.* Biochem Pharmacol, 1998. **55**(1): p. 21-5.
71. Ratledge, C. and L.G. Dover, *Iron metabolism in pathogenic bacteria.* Annu Rev Microbiol, 2000. **54**: p. 881-941.
72. Drechsel, H. and G. Jung, *Peptide siderophores.* J Pept Sci, 1998. **4**(3): p. 147-81.
73. Schryvers, A.B. and I. Stojiljkovic, *Iron acquisition systems in the pathogenic Neisseria.* Mol Microbiol, 1999. **32**(6): p. 1117-23.
74. Wandersman, C. and I. Stojiljkovic, *Bacterial heme sources: the role of heme, hemoprotein receptors and hemophores.* Curr Opin Microbiol, 2000. **3**(2): p. 215-20.
75. Wandersman, C. and P. Delepelaire, *Bacterial iron sources: from siderophores to hemophores.* Annu Rev Microbiol, 2004. **58**: p. 611-47.
76. Braun, V., *Iron uptake mechanisms and their regulation in pathogenic bacteria.* Int J Med Microbiol, 2001. **291**(2): p. 67-79.
77. Braun, V., *Avoidance of iron toxicity through regulation of bacterial iron transport.* Biol Chem, 1997. **378**(8): p. 779-86.
78. Andrews, N.C., *Disorders of iron metabolism.* N Engl J Med, 1999. **341**(26): p. 1986-95.
79. Brittenham, G., *The red cell cycle*, in *Iron Metabolism in Health and Disease*, J.H.B.e. al, Editor. 1994, W.B. Saunders Company Ltd: London. p. 31-62.
80. Kondo, H., K. Saito, J.P. Grasso, et al., *Iron metabolism in the erythrophagocytosing Kupffer cell.* Hepatology, 1988. **8**(1): p. 32-8.
81. Hentze, M.W., M.U. Muckenthaler, and N.C. Andrews, *Balancing acts: molecular control of mammalian iron metabolism.* Cell, 2004. **117**(3): p. 285-97.
82. Dunstan, S.J., V.A. Ho, C.M. Duc, et al., *Typhoid fever and genetic polymorphisms at the natural resistance-associated macrophage protein 1.* J Infect Dis, 2001. **183**(7): p. 1156-60.

83. Vidal, S.M., D. Malo, K. Vogan, et al., *Natural resistance to infection with intracellular parasites: isolation of a candidate for Bcg.* Cell, 1993. **73**(3): p. 469-85.
84. Blackwell, J.M., *The macrophage resistance gene Lsh/Ity/Bcg.* Res Immunol, 1989. **140**(8): p. 767-9.
85. Schurr, E., D. Malo, D. Radzioch, et al., *Genetic control of innate resistance to mycobacterial infections.* Immunol Today, 1991. **12**(3): p. A42-5.
86. Vidal, S., P. Gros, and E. Skamene, *Natural resistance to infection with intracellular parasites: molecular genetics identifies Nramp1 as the Bcg/Ity/Lsh locus.* J Leukoc Biol, 1995. **58**(4): p. 382-90.
87. Forbes, J.R. and P. Gros, *Divalent-metal transport by NRAMP proteins at the interface of host-pathogen interactions.* Trends Microbiol, 2001. **9**(8): p. 397-403.
88. Gruenheid, S., E. Pinner, M. Desjardins, et al., *Natural resistance to infection with intracellular pathogens: the Nramp1 protein is recruited to the membrane of the phagosome.* J Exp Med, 1997. **185**(4): p. 717-30.
89. Blackwell, J.M. and S. Searle, *Genetic regulation of macrophage activation: understanding the function of Nramp1 (=Ity/Lsh/Bcg).* Immunol Lett, 1999. **65**(1-2): p. 73-80.
90. Jurado, R.L., *Iron, infections, and anemia of inflammation.* Clin Infect Dis, 1997. **25**(4): p. 888-95.
91. Collins, H.L., *The role of iron in infections with intracellular bacteria.* Immunol Lett, 2003. **85**(2): p. 193-5.
92. Gangaidzo, I.T., V.M. Moyo, E. Mvundura, et al., *Association of pulmonary tuberculosis with increased dietary iron.* J Infect Dis, 2001. **184**(7): p. 936-9.
93. Weinberg, E.D., *Iron loading and disease surveillance.* Emerg Infect Dis, 1999. **5**(3): p. 346-52.
94. Knutson, M.D., M. Oukka, L.M. Koss, et al., *Iron release from macrophages after erythrophagocytosis is up-regulated by ferroportin 1*

overexpression and down-regulated by hepcidin. Proc Natl Acad Sci U S A, 2005. **102**(5): p. 1324-8.

95. Meresse, S., O. Steele-Mortimer, E. Moreno, et al., *Controlling the maturation of pathogen-containing vacuoles: a matter of life and death.* Nat Cell Biol, 1999. **1**(7): p. E183-8.
96. Vidal, S.M., E. Pinner, P. Lepage, et al., *Natural resistance to intracellular infections: Nramp1 encodes a membrane phosphoglycoprotein absent in macrophages from susceptible (Nramp1 D169) mouse strains.* J Immunol, 1996. **157**(8): p. 3559-68.
97. Hueck, C.J., M.J. Hantman, V. Bajaj, et al., *Salmonella typhimurium secreted invasion determinants are homologous to Shigella Ipa proteins.* Mol Microbiol, 1995. **18**(3): p. 479-90.
98. Kakuta, K., K. Orino, S. Yamamoto, et al., *High levels of ferritin and its iron in fetal bovine serum.* Comp Biochem Physiol A Physiol, 1997. **118**(1): p. 165-9.
99. Chaudhuri, A., S. Orme, T. Vo, et al., *Phosphorylation of TRAF2 inhibits binding to the CD40 cytoplasmic domain.* Biochem Biophys Res Commun, 1999. **256**(3): p. 620-5.
100. Hemmi, H., O. Takeuchi, T. Kawai, et al., *A Toll-like receptor recognizes bacterial DNA.* Nature, 2000. **408**(6813): p. 740-5.
101. Totemeyer, S., P. Kaiser, D.J. Maskell, et al., *Sublethal infection of C57BL/6 mice with Salmonella enterica Serovar Typhimurium leads to an increase in levels of Toll-like receptor 1 (TLR1), TLR2, and TLR9 mRNA as well as a decrease in levels of TLR6 mRNA in infected organs.* Infect Immun, 2005. **73**(3): p. 1873-8.
102. Lauth, X., J.J. Babon, J.A. Stannard, et al., *Bass hepcidin synthesis, solution structure, antimicrobial activities and synergism, and in vivo hepatic response to bacterial infections.* J Biol Chem, 2005. **280**(10): p. 9272-82.
103. Cherayil, B.J. and D. Antos, *Inducible nitric oxide synthase and Salmonella infection.* Microbes Infect, 2001. **3**(9): p. 771-6.

104. Goswami, T., A. Bhattacharjee, P. Babal, et al., *Natural-resistance-associated macrophage protein 1 is an H+/bivalent cation antiporter.* Biochem J, 2001. **354**(Pt 3): p. 511-9.
105. Jabado, N., P. Cuellar-Mata, S. Grinstein, et al., *Iron chelators modulate the fusogenic properties of Salmonella-containing phagosomes.* Proc Natl Acad Sci U S A, 2003. **100**(10): p. 6127-32.
106. Govoni, G., F. Canonne-Hergaux, C.G. Pfeifer, et al., *Functional expression of Nramp1 in vitro in the murine macrophage line RAW264.7.* Infect Immun, 1999. **67**(5): p. 2225-32.
107. Puschmann, M. and A.M. Ganzoni, *Increased resistance of iron-deficient mice to salmonella infection.* Infect Immun, 1977. **17**(3): p. 663-4.
108. Gunshin, H., B. Mackenzie, U.V. Berger, et al., *Cloning and characterization of a mammalian proton-coupled metal-ion transporter.* Nature, 1997. **388**(6641): p. 482-8.
109. Fleming, M.D., C.C. Trenor, 3rd, M.A. Su, et al., *Microcytic anaemia mice have a mutation in Nramp2, a candidate iron transporter gene.* Nat Genet, 1997. **16**(4): p. 383-6.
110. Fleming, M.D., M.A. Romano, M.A. Su, et al., *Nramp2 is mutated in the anemic Belgrade (b) rat: evidence of a role for Nramp2 in endosomal iron transport.* Proc Natl Acad Sci U S A, 1998. **95**(3): p. 1148-53.
111. Tabuchi, M., T. Yoshimori, K. Yamaguchi, et al., *Human NRAMP2/DMT1, which mediates iron transport across endosomal membranes, is localized to late endosomes and lysosomes in HEp-2 cells.* J Biol Chem, 2000. **275**(29): p. 22220-8.
112. Gruenheid, S., F. Canonne-Hergaux, S. Gauthier, et al., *The iron transport protein NRAMP2 is an integral membrane glycoprotein that colocalizes with transferrin in recycling endosomes.* J Exp Med, 1999. **189**(5): p. 831-41.
113. McKie, A.T. and D.J. Barlow, *The SLC40 basolateral iron transporter family (IREG1/ferroportin/MTP1).* Pflugers Arch, 2004. **447**(5): p. 801-6.

114. Droemann, D., D. Albrecht, J. Gerdes, et al., *Human lung cancer cells express functionally active Toll-like receptor 9.* Respir Res, 2005. **6**(1): p. 1.
115. Doerfler, W., *DNA methylation and gene activity.* Annu Rev Biochem, 1983. **52**: p. 93-124.
116. Muiznieks, I. and W. Doerfler, *The impact of 5'-CG-3' methylation on the activity of different eukaryotic promoters: a comparative study.* FEBS Lett, 1994. **344**(2-3): p. 251-4.
117. Holler, M., G. Westin, J. Jiricny, et al., *Sp1 transcription factor binds DNA and activates transcription even when the binding site is CpG methylated.* Genes Dev, 1988. **2**(9): p. 1127-35.
118. Watt, F. and P.L. Molloy, *Cytosine methylation prevents binding to DNA of a HeLa cell transcription factor required for optimal expression of the adenovirus major late promoter.* Genes Dev, 1988. **2**(9): p. 1136-43.
119. Rhodes, K., R.A. Rippe, A. Umezawa, et al., *DNA methylation represses the murine alpha 1(I) collagen promoter by an indirect mechanism.* Mol Cell Biol, 1994. **14**(9): p. 5950-60.
120. Zingg, J.M., G. Pedraza-Alva, and J.P. Jost, *MyoD1 promoter autoregulation is mediated by two proximal E-boxes.* Nucleic Acids Res, 1994. **22**(12): p. 2234-41.
121. Nemeth, E., S. Rivera, V. Gabayan, et al., *IL-6 mediates hypoferremia of inflammation by inducing the synthesis of the iron regulatory hormone hepcidin.* J Clin Invest, 2004. **113**(9): p. 1271-6.
122. Garcia-del Portillo, F. and B.B. Finlay, *Targeting of Salmonella typhimurium to vesicles containing lysosomal membrane glycoproteins bypasses compartments with mannose 6-phosphate receptors.* J Cell Biol, 1995. **129**(1): p. 81-97.
123. Garcia-del Portillo, F., M.G. Pucciarelli, W.A. Jefferies, et al., *Salmonella typhimurium induces selective aggregation and internalization of host cell surface proteins during invasion of epithelial cells.* J Cell Sci, 1994. **107 (Pt 7)**: p. 2005-20.

124. Eriksson, S., S. Lucchini, A. Thompson, et al., *Unravelling the biology of macrophage infection by gene expression profiling of intracellular Salmonella enterica.* Mol Microbiol, 2003. **47**(1): p. 103-18.
125. Tsolis, R.M., A.J. Baumler, I. Stojiljkovic, et al., *Fur regulon of Salmonella typhimurium: identification of new iron-regulated genes.* J Bacteriol, 1995. **177**(16): p. 4628-37.
126. Heithoff, D.M., C.P. Conner, U. Hentschel, et al., *Coordinate intracellular expression of Salmonella genes induced during infection.* J Bacteriol, 1999. **181**(3): p. 799-807.
127. Wosten, M.M., L.F. Kox, S. Chamnongpol, et al., *A signal transduction system that responds to extracellular iron.* Cell, 2000. **103**(1): p. 113-25.
128. Chlosta, S., D.S. Fishman, L. Harrington, et al., *The iron efflux protein ferroportin regulates the intracellular growth of Salmonella enterica.* Infect Immun, 2006. **74**(5): p. 3065-7.
129. Nairz, M., I. Theurl, S. Ludwiczek, et al., *The co-ordinated regulation of iron homeostasis in murine macrophages limits the availability of iron for intracellular Salmonella typhimurium.* Cell Microbiol, 2007.

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„Mein Lebenslauf wird aus Datenschutzgründen in der elektronischen Version meiner Arbeit nicht mit veröffentlicht.“

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„Ich, Sabine Chlosta, erkläre, dass ich die vorgelegte Dissertationsschrift mit dem Thema „Ferroportin 1 expression regulates intracellular growth of *Salmonella enterica serovar Typhimurium*, a link between innate immunity and hereditary hemochromatosis“ selbst verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel benutzt, ohne die (unzulässige) Hilfe Dritter verfasst und auch in Teilen keine Kopien anderer Arbeiten dargestellt habe.“

Sabine Chlosta 16. Oktober 2007