MECHANISM OF INTRACELLULAR COMMUNICATION DURING YERSINIA PESTIS INFECTION

by

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DEDICATION

This thesis is dedicated to my loving family for all their love, support and help throughout the years. A special thanks to my mother for all the sacrifices she has made for my brother and I and for always being there when I needed her. I would also like to thank my wife for her love and support for putting up with me when I had to spend long hours in the lab, and last but not least to my friends for their support and always being available to grab a drink with me when my experiments didn't work.

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ABSTRACT

MECHANISM OF INTRACELLULAR COMMUNICATION DURING YERSINIA PESTIS INFECTION

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Yersinia pestis (Yp) causes the re-emerging disease plague, and is classified by the CDC and NIAID as a highest priority (Category A) pathogen. Currently, there is no approved human vaccine available and advances in early diagnostics and effective therapeutics are urgently needed. A deep understanding of the mechanisms of host response to Yp infection can significantly advance these three areas. We employed the Reverse Phase Protein Microarray (RPMA) technology to reveal the dynamic states of either protein level changes or phosphorylation changes associated with kinase-driven signaling pathways during host cell response to Yp infection. RPMA allowed quantitative profiling of changes in the intracellular communication network of human lung epithelial cells at different times post infection and in response to different treatment conditions, which included infection with the virulent Yp strain CO92, infection with a derivative avirulent strain CO92 (Pgm-, Pst-), treatment with heat inactivated CO92, and treatment

with LPS. Responses to a total of 111 validated antibodies were profiled, leading to discovery of 12 novel protein hits. The RPMA analysis also identified several protein hits previously reported in the context of Yp infection. Furthermore, the results validated several proteins previously reported in the context of infection with other Yersinia species or implicated for potential relevance through recombinant protein and cell transfection studies. The RPMA results point to strong modulation of survival/apoptosis and cell growth pathways during early host response and also suggest a model of negative regulation of the autophagy pathway. We find significant cytoplasmic localization of p53 and reduced LC3-I to LC3-II conversion in response to Yp infection, consistent with negative regulation of autophagy. These studies allow for a deeper understanding of the pathogenesis mechanisms and the discovery of innovative approaches for prevention, early diagnosis, and treatment of plague.

CHAPTER ONE - INTRODUCTION

History:

Yersinia pestis (Yp) is a gram negative bacteria that is the causative agent of the plague. It is a member of the enterobacteriaceae family, which consists of 11 species 3 of which are pathogenic to humans, including Y*ersinia pestis*. It is responsible for three major outbreaks throughout history. The first major outbreak was the Justinian plague, which lasted over 200 years. It began in Egypt and spread throughout the Middle East and the Mediterranean and eventually affected the known world at that time. Exact death tolls are hard to estimate but it is approximated that about 50-60% of the population perished(R. D. Perry & Fetherston, 1997) .

The second outbreak was the famous Black Death, which started in Asia and spread to Europe through trade routes. It was originally thought that the cause of the Black Death was bubonic plague but recent evidence would suggest that it was in fact spread through an airborne route (pneumonic plague). It killed approximately 60% of the European population and is considered one of the greatest pandemics in history. The third outbreak started in the 1860s in Hong Kong and spread across the globe over the next 50 years killing close to 10 million people. More recent outbreaks of *Yersinia pestis* have been reported all around the world ranging from eastern and southern Africa to South America, Asia and even the United States (R. D. Perry & Fetherston, 1997; Raoult,

Mouffok, Bitam, Piarroux, & Drancourt, 2013). Just during last year, endemic outbreaks in the island of Madagascar were reported (Rasoamanana, Coulanges, Michel, & Rasolofonirina, 1989; Ratovonjato, Rajerison, Rahelinirina, & Boyer, 2014; Richard et al., 2015) Also, the Chinese city of Yumen was sealed off after death due to bubonic plague (Smart News | Smithsonian," n.d.; Ge et al., 2014) . The mortality rates associated with plague have greatly declined (to around 7%) mainly due to public health measures and antibiotics treatment. However, it is believed that the cases of death associated with plague are vastly under reported in underdeveloped and poor areas. In addition, multi-resistant strains of Yp have been reported in recent years (Udani & Levy, 2006), and given the documented history of using plague in biowarfare there still exists grave concern about the potential use of Yp for bioterrorist or biowarfare purposes. There are still at least about 2000 deaths a year associated with *Yersinia pestis* infection around the world, and given the re-emergence of plague during the past two decades in certain parts of Asia and Africa, the CDC has classified the bacteria as an emerging pathogen ("CDC - Plague," n.d.; G R Cornelis, 2000).

The Bacteria:

Yersinia pestis is a facultative anaerobic coccobacillus that is non-motile and does not form spores. Optimum growth of the bacteria occurs between the temperatures of 28°C and 30° C (R. Perry & Fetherston, 1997). It is highly pathogenic in humans and has been the source of major outbreaks throughout history.

Plague manifests itself in humans in 3 forms; bubonic, septicemic and pneumonic. The bacteria are normally transmitted to humans through the flea vector (Spinner & Hinnebusch, 2012). When the flea bites a person, the bacteria spread to the draining lymph nodes where they proliferate and cause inflammation of the lymph nodes, referred to as buboes. The bacteria eventually spread throughout the body at which point it causes septicemic plague. Near the late stage of infection, the bacteria spread to the lungs, leading to secondary pneumonic plague, and from there it can be spread from person to person, as primary pneumonic plague, which presents with a very high mortality rate (Lathem, Price, Miller, & Goldman, 2007; Plano & Schesser, 2013).

Virulence:

Yersinia pestis uses a Type III secretion system (TTSS) to deliver bacterial virulence factors called Yersinia Outer Proteins (YOP) into host cells. The YOP effectors disrupt host cell functions to allow the bacteria to survive, replicate and evade the host immune system. This is achieved through functions such as blocking phagocytosis, inhibiting pro-inflammatory cytokine production, and altering host cell signaling pathways. During the early stages of infection, the bacteria have been known to inhibit the inflammatory response by suppressing INF-γ production, to allow the bacteria to survive intracellularly. However, in later stages of infection, the bacteria can prevent phagocytosis and replicate extracellularly (Meijer, Schesser, Wolf-Watz, Sassone-Corsi, & Pettersson, 2000; Patel & Anderson, 2012). The bacteria also evade phagocytosis by Macrophages and polymorphonuclear leukocytes (PMNs), further allowing them to

replicate extracellularly. In a report by Spinner et al. it was demonstrated that *Yersinia pestis* bacteria that were transmitted through a flea vector were able to evade phagocytosis better compared to bacteria that were grown in Heart Infusion Broth. They suggested that some aspect of proliferating in the fleas produced a phenotype that allow the bacteria to better evade phagocytosis (Spinner & Hinnebusch, 2012).

Type III Secretion System:

The Type III Secretion System (TTSS) of *Yersinia pestis* plays an important role in its ability to circumvent the host immune response to cause disease. Expression of the TTSS is both temperature dependent and Ca^{2+} dependent. This regulation allows the bacteria to express the virulence factors only when inside the mammalian host and not in the flea vector (Spinner & Hinnebusch, 2012). Genes for the TTSS, including for expression of the YOP virulence factors, are located on the 70 kD pCD1 plasmid of Yp. Along with the pCD1 plasmid, which is important for the expression of the TTSS, *Yersinia pestis* carries two other plasmids; pMT1 (also called pFra) and pPla. The MT1 plasmid encodes for Ymt, a phospholipase D (PLD) protein. In the flea, Ymt produces a blockage in the proventriculus that prevents the fleas from feeding normally, causing them to regurgitate while taking a blood meal and thus releasing the bacteria into the host (Hinnebusch et al., 2002). The Pla plasmid encodes for the plasminogen activator protein (Pla). It acts as a surface protease that promotes plasmin degradation of fibrin clots, and is thought to play a role in helping the bacteria invade the lymphatic system from a subcutaneous site of infection (Lathem et al., 2007).

Expression of the pCD1 plasmid is under the control of the Low Calcium Response (Lcr) of Yp (Dewoody, Merritt, & Marketon, 2013). The Lcr is regulated by the protein LcrF. LcrF is a DNA binding protein that regulates expression of TTSS proteins (Hoe & Goguen, 1993; King, Schesser Bartra, Plano, & Yahr, 2013; Li, Hu, Francis, & Chen, 2014). LrcF is further regulated by a Yersinia modulator Protein A (ymoA). At temperatures below 30˚C, the ymoA protein is stable and has inhibitory functions on the LrcF operon. However, at 37˚C, the ymoA protein is degraded and its inhibitory functions are lost, allowing for transcription of the LcrF operon and production of the LcrF protein. The LcrF protein can then form a dimer and bind to bacterial DNA to facilitate expression of TTSS components (R. Perry & Fetherston, 1997; Starke & Fuchs, 2014).

Assembly of the TTSS is a highly regulated and complex process. There are over 20 proteins that come together to form the TTSS structure, which consists of a transmembrane region with inner and outer membrane ring structures and is topped off with a rod and needle structure that has ancestral similarities with the flagellum (Figure 1) (Guy R Cornelis, 2002b). YscC proteins form the outer membrane ring structure, referred to as the OM Ring, and YscD and YscJ form an inner membrane ring structure, referred to as the MS Ring (Figure 1). Together YscC, D and J form the scaffolding for anchoring the other proteins within the TTSS apparatus (Dewoody et al., 2013). Five proteins, YscR, YscS, YscT, YscU and YscV, oligomerize to form the inner membrane export apparatus used to move TTSS substrates through the bacterial membrane with the help of a ATPase complex composed of YscN (the ATPase), YscL and YscK. The YscL

protein acts as a negative regulator of YscN's ATPase activity (Dewoody et al., 2013; Yip et al., 2005). The extracellular region of the TTSS is composed of YscI and YscF, which respectively form a rod and needle structure used to inject bacterial effectors into the target cell (Figure 1) (Guy R Cornelis, 2002a; Plano & Schesser, 2013). The length of the needle structure is regulated by another Ysc protein; YscP. In a study by Dewoody *et al.* (Dewoody et al., 2013), it was shown that deletion of YscP results in over secretion of YscF, causing variable needle lengths, whereas in the presence of YscP uniformed needle lengths of about 40nm are observed (Agrain et al., 2005; Dewoody et al., 2013; Journet, Agrain, Broz, & Cornelis, 2003). The TTSS assembly is characterized by different stages of protein secretion. During the early stage, the proteins that are being secreted are components such as the rod and needle structure proteins. During the middle stages of secretion, proteins that are involved in forming a pore within the membrane of the target cell are secreted. These proteins are YopB and YopD and LcrV (Dewoody et al., 2013). YopB and YopD are able to insert themselves into target host proteins and form a pore for the TTSS needle structure to enter through. The LcrV complex serves as a platform for YopB and YopD to insert themselves into the host membrane.

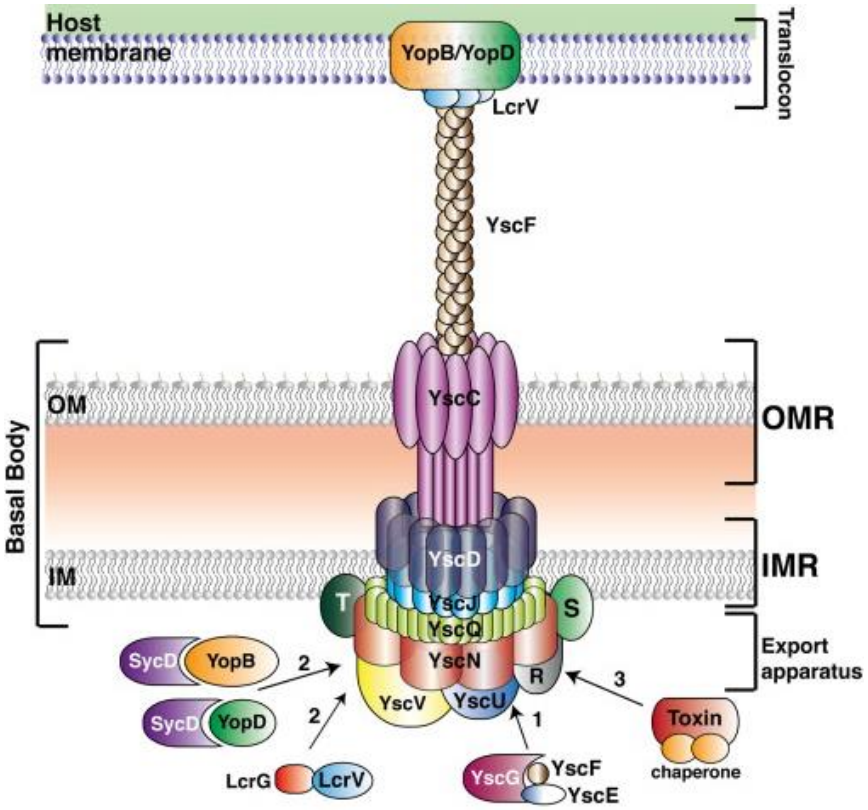


Figure 1. Schematic of the Yersinia pestis Type Three Secretion System.

Late stage secretins are classified as the YOP effectors. These are the proteins that are injected into the host target cells to disrupt host cellular pathways, thus allowing the bacteria to survive and replicate within the host. (Dewoody et al., 2013)

YOP Effectors:

There are 6 YOP effectors (YopE, YopH, YopM, YpkA, YopJ and YopT) that are injected into the target cells to help evade the host immune response.

YopH and YpkA play important roles in altering host signaling pathways in order to subvert host defenses (de la Puerta et al., 2009; Dukuzumuremyi et al., 2000a; Navarro et al., 2007; Rolán, Durand, & Mecsas, 2013). YopH is a phosphotyrosine phosphatase

that has been shown to interact with host proteins such as Lck, FAK and $p30^{cas}$ in order to subvert phagocytosis by the host cell (Alonso et al., 2004; Black & Bliska, 1997; G R Cornelis, 2000). YopH has also been shown to modulate host immune responses by suppressing B cell, T cell and neutrophil activation (Cantwell, Bubeck, & Dube, 2010; Sauvonnet, Lambermont, van der Bruggen, & Cornelis, 2002; Yao, Mecsas, Healy, Falkow, & Chien, 1999). YopH has also been shown to interact with Gab1,Gab2 and p85(de la Puerta et al., 2009). YpkA is a serine/threonie kinase and has been shown to play a role in modulating the host cytoskeleton through direct interactions with number of proteins important in preventing bacteria phagocytosis, including Rho GTPases, Actin and vasodilator-stimulated phosphoprotein (VASP) (Barz, Abahji, Trülzsch, & Heesemann, 2000; Dukuzumuremyi et al., 2000a, 2000b; Ke et al., 2015; Lee, Grimes, & Robinson, 2015). Interaction with Galphaq-mediated signaling has also been reported which could be involved in the excess bleeding seen in Yp infected persons (Laskowski-Arce & Orth, 2007)

Two other Yops effectors that can regulate the host cytoskeleton are YopT and YopE. Both have been shown to associate with Rho GTPases (RhoA, Rac-1 and Cdc42), to prevent actin filament polymerization and formation of stress fibers (Aepfelbacher, Roppenser, Hentschke, & Ruckdeschel, 2011a, 2011b; G R Cornelis, 2000; Rosqvist, Forsberg, & Wolf-Watz, 1991).

YopJ has been shown to act as an acetyltransferase and can block host MAPK and NFκB pathways, which result in reduced secretion of TNFα and IL-8 by the cells. These

effects ultimately result in reduction of the pro-inflammatory response and cell death (Mittal, Peak-Chew, & McMahon, 2006; Orth, 2002; Orth et al., 2000).

The last Yop effector, YopM, is the least characterized. Some studies have shown it to contain an E3 ubiquitin domain and it is believed that it could function as an ES ubiquitin ligase (Soundararajan, Patel, Subramanian, Sasisekharan, & Sasisekharan, 2011). Others have shown that YopM translocates into the nucleus of the host cell to act as a transcription factor (Benabdillah, Mota, Lützelschwab, Demoinet, & Cornelis, 2004). YopM has also been shown to interact with the RSK family of kinases and prevent dephosphorylation of RSK, leading to over activation of the RSK family of kinases (Hentschke et al., 2010). Whatever role YopM plays in the cell, its contribution to virulence has been demonstrated (Trülzsch, Sporleder, Igwe, Rüssmann, & Heesemann, 2004).

Identification of new host-pathogen protein interactions have helped shed light on pathways that might be important for the pathogenesis of Yp. However, despite these advances much still remains to be discovered. In particular, a comprehensive analysis of changes in intracellular communication of host protein pathways that are involved in important signaling functions has not yet been performed for the Yp infection and could yield highly significant information. In this regard, new approaches to study host pathogen interactions in the context of whole infection, as well as comparative analysis of different infection parameters would greatly improve our understanding of the mechanisms of Yp pathogenesis. Using the RPMA platform, we addressed the need to

identify and shed light on new mechanisms of host-pathogen interactions as they relate to intracellular communication mechanisms within the host.

Reverse Phase Protein Microarray:

Protein arrays can be classified into three main categories; Forward phase, Reverse Phase and Sandwich arrays. The difference between forward phase and reverse phase has to do with whether the antigen or antibody is immobilized onto a surface, usually nitrocellulose. In a Forward phase array, the antibody is immobilized and the antigen or analyte is passed over for binding to the antibody, whereas in the reverse phase arrays the analyte is immobilized and the antibody is passed over for binding to the analyte. One major benefit of the reverse phase approach over sandwich arrays is that in sandwich arrays you need to have two specific antibodies that bind to two distinct and separate regions of the antigen, as a primary antibody is immobilized and after the analyte is passed over to bind the primary antibody a secondary antibody is used to bind to the captured analyte.

Unlike DNA and RNA arrays that limit analysis to gene mutations and protein expression levels, protein arrays allow analysis of protein functionality within cells by identifying, in a quantitative manner, the proteins that have undergone post translational modifications such as phosphorylation, acetylation, glycosylation, and ubiquitination. With highly specific antibodies increasingly becoming commercially available, this makes the protein array approach an essential tool for studying post translationally modified proteins and cell communication pathways. The ability to analyze different

sample types (heterogeneous tissue samples, enriched cell populations from micro dissected samples, cell lines, serum/plasma samples and bodily fluids) is another important advantage of the protein array technology.

The reverse phase protein microarray (RPMA) platform is a new and fully validated technology that is gaining recognition for its ability to study complex cellular signaling networks through quantitative analysis of total protein levels and post translationally modified proteins. In the RPMA methodology, the analytes are robotically spotted onto nitrocellulose coated glass slides as capture molecules, and, after blocking, the slides are probed with specific primary antibodies that have been validated for single-band specificity by Western immunoblotting and for use with RPMA (Figure 2). Primary antibody binding is detected using appropriate biotinylated secondary antibody followed by streptavidin-conjugated fluorophore treatment for detection. Each microarray consists of a self-contained assay comprised of triplicate samples, controls and calibrators that are analyzed with one class of antibody and amplification chemistry. RPMA has been widely used in cancer research, leading to discovery of changes in cellular signaling pathways in comparative studies of normal vs. cancer cells (Wulfkuhle et al., 2012).More recently, it has also been applied for the studies of a variety of other diseases, such as neurodegenerative diseases, cardiovascular disease, and infectious diseases (Ahmed et al., 2014; Dernick & Obermuller, 2011; Mueller, Zhou, et al., 2010; Popova et al., 2010; Younossi et al., 2011) The RPMA technology affords the use of a quantitative and high throughput platform for simultaneous analysis of multiple signaling pathways within the cell, in order to provide an overall picture of the signaling architecture of the cell for any

given state, abilities that are not practically afforded by more traditional platforms such as DOT blots or western analysis (Mueller, Liotta, & Espina, 2010).

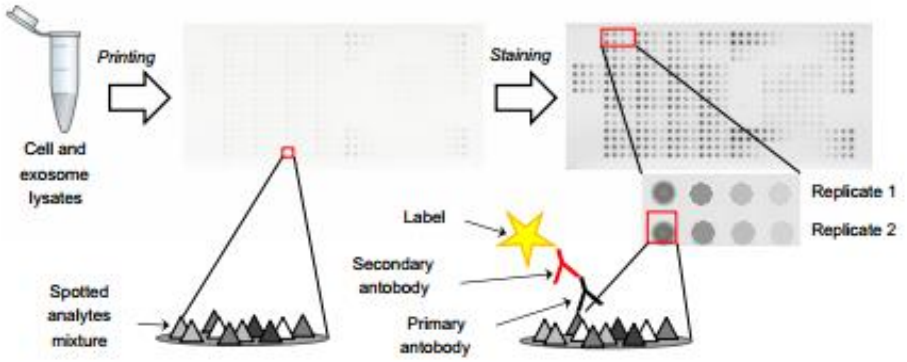


Figure 2: RPMA work flow:

Analytes are robotically spotted onto nitrocellulose coated glass slides, the slides are then probed with specific primary antibodies that have been validated for single-band specificity by Western immunoblotting and for use with RPMA and probed using an appropriate biotinylated secondary antibody followed by streptavidin-conjugated fluorophore treatment for detection. (Image taken and edited from (Biasutto, Chiechi, Couch, Liotta, & Espina, 2013).

The studies of how host pathways are altered during the course of infection with Yp have been limited. While some host targets of specific YOPs have been identified (Black & Bliska, 1997; de la Puerta et al., 2009; Mittal et al., 2006) a comprehensive analysis of how the intracellular communication mechanisms of the host are modulated in

the context of whole infection has been lacking. Furthermore, an analysis of how the specific parameters of the infection process such as the multiplicity of infection (MOI), times post infection, or host cell type, may alter the modulation of host pathways has not been yet reported. Our laboratory has previously performed the first quantitative phosphoproteomic analysis of host response to Yp infection that led to the identification of a number of altered host signaling pathways and demonstrated the functional relevance of the AKT pathway during Yp infection of primary human monocytes. In this study, we have used the RPMA technology to perform a comparative quantitative analysis of the modulation of host phosphorylation under a variety of different infection parameters, including different times post infection (p.i.), different cell types, and infection of host cells with both a fully virulent and a derivative avirulent Yp strain. Because *Yersinia pestis* has been shown to target macrophages, and since infection through an aerosol route is of the greatest concern, we used a human monocytic cell line (U937) along with a human lung epithelial cell line (16HBE14o-) in our studies.

In addition to validation of published reports on host signaling in response to Yp, we report here the identification of a number of novel proteins that show significant change in either phosphorylation levels or total protein levels and that have not been previously reported within the context of Yp infection. Furthermore, since several previously reported host response findings that we have validated here had been performed in the context of recombinant protein expression and transfections, our results provide evidence that these host proteins are indeed involved in the context of whole infection. We also provide a comparative profiling analysis between changes observed

during infection with the fully virulent Yp strain and a derivative avirulent Yp strain and also changes that are observed in response to heat-killed bacteria or LPS treatment, and are therefore independent of TTSS. Finally, based on our RPMA analysis of the human lung epithelial and monocytic cells, and also analyses of the localization of p53 (S15) protein and LC3-I to LC3-II conversion during infection with Yp, we propose and discuss a model of negative regulation of the autophagy pathway as part of the host response to Yp infection.

CHAPTER TWO: METHODS

Bacterial Strains and Reagents:

Bacterial strains used in this study were virulent *Yersinia pestis* CO92 and a derivative avirulent strain, *Yersinia pestis* CO92 (Pgm-, Pst-) (a gift from Drs. Susan Welkos and Christopher Cote, USAMRIID), that is pigmentation (pgm)-deficient and cured of the plasminogen-activator-encoding pPst plasmid ((Jenkins, Worsham, & Welkos, 2009; Kota et al., 2013; Welkos et al., 2002). Treatment with the heat-killed version of *Y. pestis* CO92 strain (heat-killed at 65˚C for 30 min) was also performed. For infections, bacterial strains were streaked onto Sheep Blood Agar (SBA) plates from a frozen stock and grown at 28° C. A single colony was isolated and used to inoculate cation-adjusted Mueller-Hinton broth (CAMHB) and grown overnight at 28°C to use for infecting cells. Overnight cultures were enumerated using OD_{600} readings (an OD_{600} reading of 1 is equivalent to 5.8 X 10^8 CFU). Antibodies used for the RPMA analysis are listed in Table 1, along with dilution factors used and vendor information. All the antibodies had been previously validated for RPMA use. For each Western blot validation, and also the LC3 Western analysis, the identical antibody used for RPMA was utilized.

16HBE14o- Cell Infections:

Immortalized human airway epithelial cells (16HBE14o-) were purchased from Dr. D.C. Greunert (California Pacific Medical Center Research Institute, San Francisco, CA).16HBE14o- (HBE) cells were grown in Bronchial Epithelial Cell Basal Medium (Clonetics™ BEGM™ BulletKit™ (CC-3170) supplemented with: BPE, 2 ml; Hydrocortisone, 0.5 ml; hEGF, 0.5 ml; Epinephrine, 0.5 ml; Transferrin, 0.5 ml; Insulin, 0.5 ml; Retinoic Acid, 0.5 ml; Triiodothyronine, 0.5 ml (Lonza, Walkersville, MD). Cells were cultured in 6 well plates and 2×10^6 cells per well were infected at a multiplicity of infection (MOI) of 10 with either fully virulent strain of *Y. pestis*, CO92, or a derivative avirulent *Y. pestis* strain CO92 (Pgm- , Pst-), or were treated with heat-killed *Y. pestis* CO92. Untreated, and *E.coli* lipopolysaccharide (LPS)-treated cells (100 ng/ml), were also included as controls. Cells were harvested at 30 min, 1 hour, 4 hours and 8 hours post infection, washed with 1 x PBS and then lysed using lysis buffer: 30 ml 2x Novex Tris-Glycine Sample Loading Buffer SDS (Invitrogen), 20ml T-PER Tissue Protein Extraction Reagent (Thermo Scientific), 200µl 0.5M EDTA pH 8.0, 1X Complete Protease Inhibitor Cocktail (Roche), 80µl 0.1M Na3VO4, 400µl 0.1M NaF, 1.3ml 1M DTT. Samples were then stored at -20 ºC.

Bacterial Uptake and Intracellular Growth Measurements:

1 X 10^5 16HBE14o-cells were infected with CO92 (Pgm- , Pst-) strain at MOI of 10 and incubated at 37° C for 2 hours. The cells were subsequently incubated with 50 µg/ml gentamycin for 1 hour at 37° C to eliminate the extracellular bacteria. The cells were then washed and resuspended in BEGM media containing 8 µg/ml Gentamycin. At designated time points post infection (0, 8, and 24 hours p.i.) cells were washed and then lysed by

incubating in 0.2% Triton-X 100 for 5 min at 37° C. Dilutions of the cell lysates were spread onto SBA plates and incubated at 37° C to allow bacterial colony growth. Bacterial colonies were enumerated and CFU calculations were used to measure the levels of bacterial uptake and intracellular growth.

RPMA Analysis:

Sample protein levels were first determined using pre-printed slides containing all the samples as well as a BSA curve and stained with Sypro to calculate total protein levels in the samples. Equivalent total protein amounts for samples were arrayed onto nitrocellulose coated glass slides by direct contact printing using a high resolution 2470 arrayer (Aushon Biosystems, Brillerica, MA). RPMA analysis was performed as previously described (Federici et al., 2013). Samples were printed in triplicate and averages were calculated for analysis. Sample slides were probed with 111 different antibodies against either total or phosphorylated forms of proteins that are involved in various cell signaling pathways. A complete list of all the antibodies used for this analysis and categorized by functional relevance is provided in Table 1.

RPMA Data Analysis:

For each specific post-infection time point, the RPMA results for the different infection or treatment conditions were normalized to the control group (uninfected and untreated) in order to calculate relative fold-change levels. For total protein levels, a 2 fold increase/decrease was set to be significant and for phosphorylation levels the significant fold increase/decrease was set to 1.5. Proteins that showed significant fold change were then separated into four main categories based on the respective infection or

treatment condition that elicited the observed changes (Table 2). Extensive literature searches were performed using Pubmed for all the proteins that showed significant fold change, in order to identify novel proteins that have not been previously reported to play a role during *Y. pestis*-host interactions (Table 4).

Western Blot Analysis:

RPMA findings were validated by Western blot analysis for 6 selected proteins that displayed significant fold change on RPMA arrays, including cleaved PARP (D214) and p53 (S15). 15ul of cell lysate in lysis buffer was boiled for 10 minutes at 96 ºC and loaded onto a 4-20% bis-tris polyacrylamide gel (Invitrogen). The gel was run at 200 V for 40 min, transferred to nitrocellulose membrane using the iBlot Gel Transfer Device (Invitrogen), and blocked overnight in 5% dry milk in PBS-T. The same primary antibodies used for the RPMA analysis (Table 1) were added at appropriate dilutions in 5% dry milk/PBS-T for 1 hour at room temperature, followed by a one hour incubation with an appropriate secondary antibody (goat-anti Rabbit or goat-anti Mouse) in PBS-T. The protein bands were visualized using SuperSignal West-Femto Maximum Sensitivity Substrate (Pierce). The blots were imaged using Chemidoc XRS System (BioRad, CA). Actin controls were used to normalize total protein levels for different samples relative to one another.

LC3-B Western blots were performed as follows: 20 µl of total cell extract from uninfected/untreated cells, the cells infected with Yp CO92, and LPS-treated cells were run on a 4-12% bis-tris polyacrylamide gel (Invitrogen) at 200 V for 40 min, and

transferred to nitrocellulose membrane using the iBlot Gel Transfer Device (Invitrogen). The membrane was blocked overnight in 5% dry milk in PBS-T, and then washed 3 times with PBS-T and incubated with the LC3B primary antibody (1:1000) for 1 hour at room temperature. Following 3 more washes with PBS-T, the secondary antibody was added at 1:5000 dilution and the blot was incubated at room temperature for 1 hour. After three washes with PBS-T to remove excess secondary antibody, the protein bands were visualized using SuperSignal West-Femto Maximum Sensitivity Substrate (Pierce). The blots were imaged using Chemidoc XRS System (BioRad, CA). Actin controls were used to normalize total protein levels for different samples relative to one another.

p53 (S15) Immunofluorescence Staining:

Immunofluorescence staining was performed to analyze the cellular localization of activated p53 protein (phosphorylated at residue S15). Eight well chamber slides (EW-01838; Thermo Scientific) were seeded with HeLa cells at a cell density of 10,000 cells per well. The following day, the cells were either left untreated and uninfected (control), or were infected with *Y. pestis* CO92 (Pgm-, Pst-) at MOI of 10 for 8 hours to match the exact RPMA conditions that showed an effect, or were treated with Doxorubicin as described previously (Kurz, Douglas, & Lees-Miller, 2004). Cells were then fixed with 1% paraformaldehyde for 15 min at 37˚C, and then rehydrated with PBS for 5 min at room temperature. The cells were subsequently permeabilized using 0.1% Triton X-100 (Sigma) for 10min at room temperature and blocked with 2% BSA (Sigma) for 1 hour at room temperature. The cells were then washed with PBS three times and incubated with the same anti-p53 (S15) primary antibody used in the RPMA study (Cell signaling;

catalog number 9282) at 1:500 dilution for 1 hour at room temperature. Subsequent PBS washes were performed, followed by incubation with Alexa-Fluor 488 Goat-anti Mouse secondary antibody at 1:1000 dilution for 1 hour at room temperature. The cells were then washed three times with PBS and slides were mounted using Vectashield with DAPI (Vector Laboratories: Burlingame, Ca). Control conditions were also included side by side to account for any background signal; for this, cells received an identical treatment except that addition of either the primary antibody or the secondary antibody was omitted and an equivalent volume of PBS was added instead. The slides were imaged using a Nikon Eclipse TE2000-U confocal microscope.

CHAPTER THREE: RESULTS

RPMA Analysis of HBE Cells Infected with *Yersinia pestis*

In order to study host cell signaling pathways that are altered during *Y. pestis* infection, including pathways that are modulated independently of the TTSS mechanism, we compared host response to infection of 16HBE14o- cells (human bronchial epithelial cells) with the fully virulent strain CO92 with the response to infection with a derivative avirulent strain CO92 (Pgm- , Pst-), and also the host response to treatment with heat-inactivated CO92 or LPS was analyzed side by side. Untreated cells were also included as controls. For the avirulent strain CO92 (Pgm- , Pst-), we found that about 5% of the bacteria were taken up by the 16HBE14o- cells during infection at MOI of 10. While a 4 fold increase in the number of intracellular bacteria was observed by 8 hours post infection, the bacteria stopped growing after this time and the same number of intracellular bacteria as the 8 hour time point was observed at 24 hours post infection. For our RPMA analysis, 30 min, 1 hour, 4 hours and 8 hours post infection times were analyzed using 111 different host-specific pre-validated antibodies, in order to quantify changes in protein phosphorylation levels or total protein levels (Table 1). The antibodies were selected to allow a wide range functional analysis of host cellular pathways (Table 1), and also included those that are known or suggested from previous

studies to be affected during Yp infection, such as apoptosis, cytoskeletal modulation, inflammation, immune response, and autophagy.

The heat map in Figure 3 presents an unsupervised hierarchical 2-way clustering analysis of the RPMA data. According to this analysis, similar host response profiles were observed between the virulent and avirulent *Y. pestis* strains, particularly at the later time points (4 and 8 hours post infection). On the other hand, samples treated with heat-killed bacteria group with the untreated and LPS-treated controls group. While the host response profile to live bacteria appears distinct from that of the control samples as a whole, there is no discernable clustering of the proteins according to functional categories or pathways with regard to treatment with live bacteria versus the control samples.

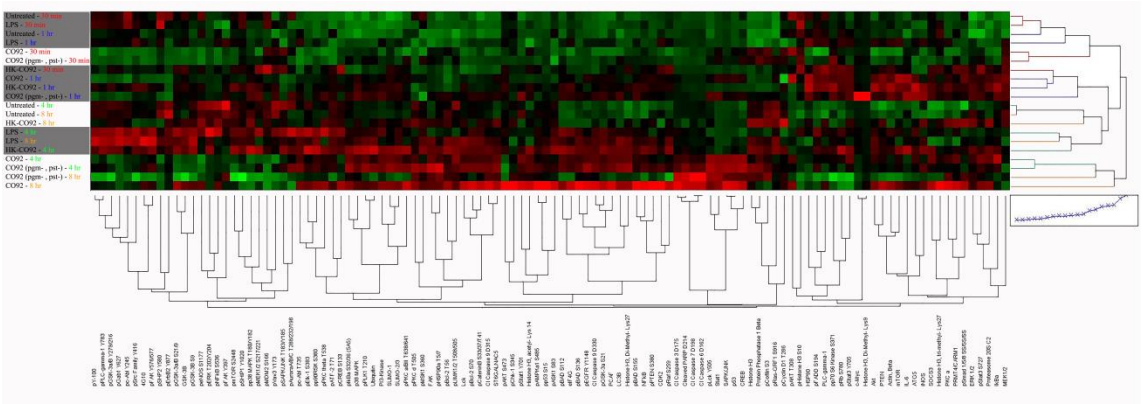


Figure 3 Heat map of RPMA results for HBE cells.

The Heat map shows unsupervised hierarchical 2-way clustering analysis of all the samples analyzed by RPMA. Rows represent the different samples used in the RPMA

analysis and the columns represent the different antibodies that were tested. Relative signal intensities were assigned based on comparison with the lowest signals on the arrays. Red color depicts higher signal values and green color depicts lower signal values; color intensity indicates the strength of signal.

Identification and Categorization of Proteins with Significant Fold Change

To identify changes in the protein expression levels or specific protein phosphorylation during Yp infection, relative fold changes were calculated by normalizing the samples with respect to the uninfected and untreated control samples for each time point. The thresholds for significant fold change was set to 1.5 fold or higher for phosphorylation events and 2 fold or higher for total protein levels. The identified hits were then categorized into 4 distinct categories that allow distinguishing the changes that are unique to infection with the fully virulent strain (CO92) from those that occur in response to infection with the avirulent strain, or significant changes that are observed during LPS and heat-killed bacterial treatments (Table 2). This categorization also highlights significant changes that are not unique to any particular treatment and are observed during two or more of the treatment conditions that we have tested.

Table 1. List of RPMA antibodies and the cellular pathways of their target proteins. All **t**he RPMA antibodies that were evaluated are listed and separated based on the

specific pathways in which their target proteins are involved. For each antibody, the name of the vendor company, vendor catalog number, and protein band sizes in kilodalton (kDa), are indicated. Antibody dilutions for Western blot validation are also listed.

Cell Cycle Regulation and Growth	Catalog #	Company	MW (kDa)	Dilutions
Akt	9272	Cell Signaling	60	1:1000
Akt (S473)	9271	Cell Signaling	60	1:1000
Akt (T308)	9275	Cell Signaling	60	1:1000
AMPKα1 (S485)	4184	Cell Signaling	62	1:1000
ATF-2 (T71)	9221	Cell Signaling	70	1:1000
ATF-2 (T69/T71)	9225	Cell Signaling	70	1:500
Aurora A (T288)/B (T232)/C (T198)	2914	Cell Signaling	35, 40, 48	1:500
c-Abl (T735)	2864	Cell Signaling	120	1:1000
c-Abl (Y245)	2861	Cell Signaling	135	1:1000
CDK2 (78B2)	2546	Cell Signaling	33	1:1000
Chk1 (S345)	2341	Cell Signaling	56	1:1000
cMyc	9402	Cell Signaling	57-70	1:500
Cyclin D1 (T286)	2921	Cell Signaling	36	1:1000
CREB	9192	Cell Signaling	43	1:1000
CREB (S133)	9191	Cell Signaling	43	1:1000
EGFR (Y1148)	4404	Cell Signaling	175	1:500
eIF4G	2498	Cell Signaling	220	1:1000
Elk-1 (S383)	9828	Cell Signaling	62	1:1000
eNOS (S1177)	9571	Cell Signaling	140	1:1000

ERK 1/2	9102	Cell Signaling	42, 44	1:1000
ERK 1/2 (T202/Y204)	9101	Cell Signaling	42, 44	1:1000
FAK (Y397) (18)	611806	BD	125	1:1000
FAK (Y576/577)	3281	Cell Signaling	125	1:1000
GAB1 (Y627)	3231	Cell Signaling	110	1:1000
GSK-3α (S21) (46H12)	9337	Cell Signaling	51	1:2000
GSK-3α (Y279)/beta (Y216)	44-604	Bio Source	47, 51	1:1000
GSK-3α/β (S21/9)	9331	Cell Signaling	46, 51	1:1000
GSK-3β (S9)	9336	Cell Signaling	46	1:1000
GSK3-β	9332	Cell Signaling	46	1:1000
MEK1/2	9122	Cell Signaling	45	1:1000
MEK1/2 (S217/221)	9121	Cell Signaling	45	1:1000
mTOR	2972	Cell Signaling	289	1:1000
p53	9282	Cell Signal	53	1:1000
p53 (S15)	9284	Cell Signaling	53	1:1000
p70 S6 Kinase (S371)	9208	Cell Signaling	70, 85	1:1000
p90RSK (S380)	9341	Cell Signaling	90	1:1000
PI3-Kinase	610045	BD	85	1:2500
PKC (pan) (betaII S660)	9371	Cell Signaling	78, 80, 82, 85	1:1000
PKC delta (T505)	9374	Cell Signaling	78	1:1000
PLK1 (T210)	558400	BD	68	1:10,000
Raf (S259)	9421	Cell Signaling	74	1:500
Ras-GRF1 (S916)	3321	Cell Signaling	155	1:1000
Rb (S780)	8180	Cell Signaling	110	1:1000
Ribosomal protein L13a	2765	Cell Signaling	110	1:1000
SAPK/JNK	9252	Cell Signaling	46, 54	1:1000

SAPK/JNK (T183/Y185)	9251	Cell Signaling	46, 54	1:1000
SHIP1 (Y1020)	3941	Cell Signaling	145	1:1000
SHP2 (Y580)	44-558	Biosource	70	1:500
Smad1/5 (S463/S465) /Smad9 (S465/S467)	9511	Cell Signaling	60	1:500
Src Family (Y416)	2101	Cell Signaling	60	1:1000
Ubiquitin (P4D1)	3936	Cell Signaling	Many	1:500
Cell Survival and Apoptosis	**Catalog #**	**Company**	**MW (kDa)**	**Dilutions**
Akt	9272	Cell Signaling	60	1:1000
Akt (S473)	9271	Cell Signaling	60	1:1000
Akt (T308)	9275	Cell Signaling	60	1:1000
AMPKα1 (S485)	4184	Cell Signaling	62	1:1000
ASK1 (S83)	3761	Cell Signaling	155	1:1000
ATF-2 (T71)	9221	Cell Signaling	70	1:1000
ATF-2 (T69/T71)	9225	Cell Signaling	70	1:500
Bad (S112)	9291	Cell Signaling	23	1:1000
Bad (S136)	9295	Cell Signaling	23	1:500
Bad (S155)	9297	Cell Signaling	23	1:1000
Bcl-2 (S70) (5H2)	2827	Cell Signaling	28	1:1000
Bcl-2 (T56)	2875	Cell Signaling	28	1:1000
c-Abl (T735)	2864	Cell Signaling	120	1:1000
c-Abl (Y245)	2861	Cell Signaling	135	1:1000
Caspase-3, cleaved (D175)	9661	Cell Signaling	17, 19	1:1000
Caspase-3, cleaved (D175) (5A1)	9664	Cell Signaling	17, 19	1:1000
Caspase-6, cleaved (D162)	9761	Cell Signaling	18	1:1000

Caspase-7, cleaved (D198)	9491	Cell Signaling	20	1:1000
Caspase-9, cleaved (D315)	9505	Cell Signaling	35	1:1000
Chk1 (S345)	2341	Cell Signaling	56	1:1000
cMyc	9402	Cell Signaling	57-70	1:500
CREB	9192	Cell Signaling	43	1:1000
CREB (S133)	9191	Cell Signaling	43	1:1000
ERK 1/2	9102	Cell Signaling	42, 44	1:1000
Erb-B2 (Y877)	2245	Cell Signaling	185	1:1000
FADD (S194)	2781	Cell Signaling	28	1:1000
FAK (Y397) (18)	611806	BD	125	1:1000
FAK (Y576/577)	3281	Cell Signaling	125	1:1000
GSK-3α (S21) (46H12)	9337	Cell Signaling	51	1:2000
GSK-3α (Y279)/beta (Y216)	44-604	Bio Source	47, 51	1:1000
GSK-3α/β (S21/9)	9331	Cell Signaling	46, 51	1:1000
GSK-3β (S9)	9336	Cell Signaling	46	1:1000
GSK3-β	9332	Cell Signaling	46	1:1000
MDM2 (S166)	3521	Cell Signaling	90	1:1000
MSK1 (S360)	9594	Cell Signaling	90	1:1000
mTOR (S2448)	2971	Cell Signaling	289	1:1000
p38 MAP Kinase	9212	Cell Signaling	40	1:1000
p38 MAP Kinase (T180/Y182)	9211	Cell Signaling	40	1:1000
p53	9282	Cell Signal	53	1:1000
p53 (S15)	9284	Cell Signaling	53	1:1000
PARP, cleaved (D214)	9541	Cell Signaling	89	1:1000
PI3-Kinase	610045	BD	85	1:2500
Protein phosphatase 1 β	Ab53315	Abcam	37	1:10,000

PTEN (S380)	9551	Cell Signaling	54	1:1000
PTEN	9188	Cell Signaling	54	1:1000
pY100	9411	Cell Signaling	100	1:1000
Rb (S780)	8180	Cell Signaling	110	1:1000
SAPK/JNK	9252	Cell Signaling	46, 54	1:1000
SAPK/JNK (T183/Y185)	9251	Cell Signaling	46, 54	1:1000
SHIP1 (Y1020)	3941	Cell Signaling	145	1:1000
SHP2 (Y580)	44-558	Biosource	70	1:500
Ubiquitin (P4D1)	3936	Cell Signaling	Many	1:500
Immune Response	**Catalog #**	**Company**	**MW (kDa)**	**Dilutions**
GSK-3α (S21) (46H12)	9337	Cell Signaling	51	1:2000
GSK-3α (Y279)/beta (Y216)	44-604	Bio Source	47, 51	1:1000
GSK-3α/β (S21/9)	9331	Cell Signaling	46, 51	1:1000
GSK-3β (S9)	9336	Cell Signaling	46	1:1000
GSK3-β	9332	Cell Signaling	46	1:1000
IL-6	5143-100	Bio Vision	21-28	1:1000
iNOS	2977	Cell Signaling	130	1:500
IκB-α	551818	BD	42	1:500
IκB-α (S32/36) (5A5)	9246	Cell Signaling	40	1:2000
JAK1 (Y1022/1023)	3331	Cell Signaling	130	1:1000
Lck	2752	Cell Signaling	56	1:1000
Lck (Y505)	44-850	Biosource	56	1:1000
NFkB	3034	Cell Signaling	75	1:1000
NF-κB p65 (S536)	3032	Cell Signaling	65	1:1000
PKC (pan) (betaII S660)	9371	Cell Signaling	78, 80, 82, 85	1:1000

PKC θ (T538)	9377	Cell Signaling	79	1:1000
PKCα	3550	Upstate	82	1:1000
Stat1	9172	Cell Signaling	84, 91	1:1000
Stat1 (Y701)	9171	Cell Signaling	84, 91	1:1000
Stat1 (Y701)	07-307	Upstate	92	1:1000
Stat3 (S727)	9134	Cell Signaling	79, 86	1:1000
Stat3 (Y705) (9E12)	05-485	Upstate	92	1:10,000
Cytoskeletal Rearrangement, Cell Migration, and Vesicle Trafficking	**Catalog #**	**Company**	**MW (kDa)**	**Dilutions**
Actin, β	4967	Cell Signaling	45	1:1000
AMPKa1(S485)	4184	Cell Signaling	62	1:1000
Catenin-Beta (S33/37/T41)	9561	Cell Signaling	85	1:1000
Cofilin (S3) (77G2)	3313	Cell Signaling	19	1:1000
FAK	556368	BD	116	1:1000
FAK (Y397) (18)	611806	BD	125	1:1000
FAK (Y576/577)	3281	Cell Signaling	125	1:1000
GSK-3α (S21) (46H12)	9337	Cell Signaling	51	1:2000
GSK-3α (Y279)/beta (Y216)	44-604	Bio Source	47, 51	1:1000
GSK-3α/β (S21/9)	9331	Cell Signaling	46, 51	1:1000
GSK-3β (S9)	9336	Cell Signaling	46	1:1000
GSK3-β	9332	Cell Signaling	46	1:1000
HSP70 (C92F3A-5)	SPA-810	Stressgen	70	1:2000
HSP90a (T5/7)	3488	Cell Signaling	90	1:500
JAK1 (Y1022/1023)	3331	Cell Signaling	130	1:1000
LIMK1 (T508)/LIMK2 (T505)	3841	Cell Signaling	72	1:1000

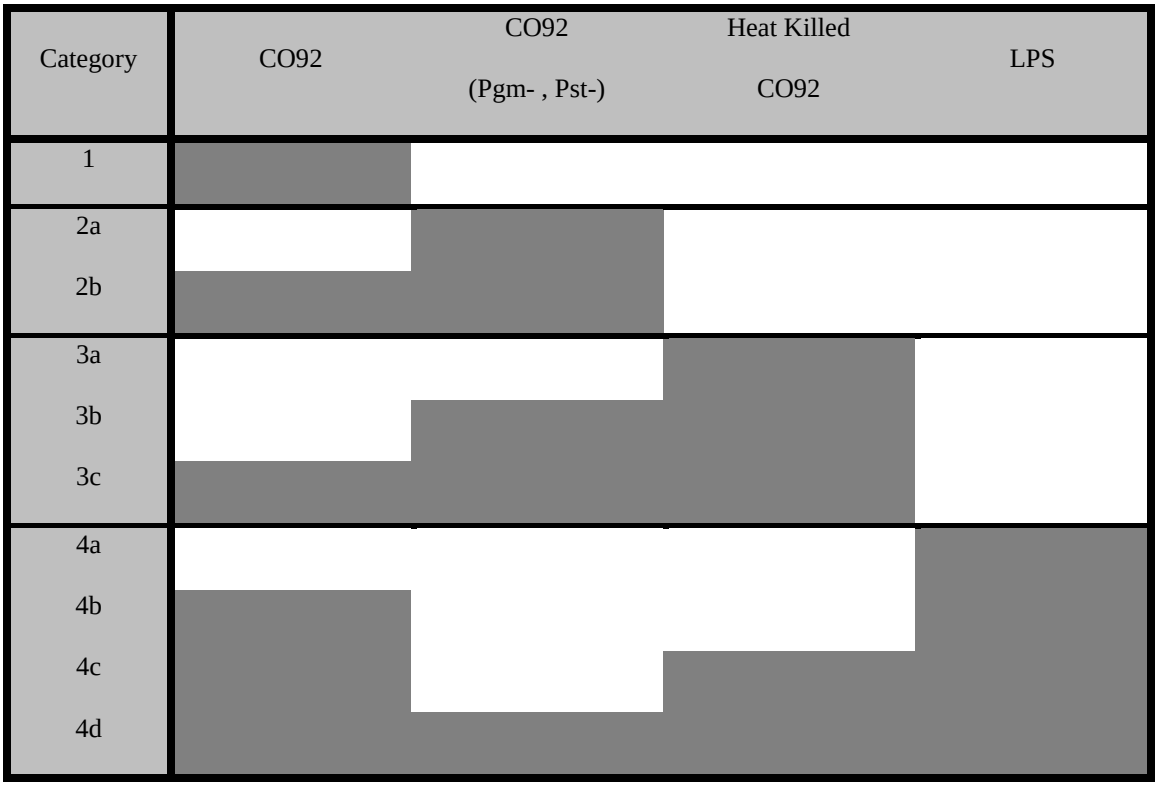
PLC-γ-1	2822	Cell Signaling	155	1:1000
PLC-γ-1 (Y783)	2821	Cell Signaling	155	1:1000
Vav3 (Y173)	44-488	Biosource	95	1:500
Chromatin Modulation	**Catalog #**	**Company**	**MW (kDa)**	**Dilutions**
Histone H3, acetyl- (Lys14)	07-353	Millipore	17	1:1000
Histone H3, Di-Methyl (Lys9)	9753	Cell Signaling	15	1:500
Histone H3, Di-Methyl (Lys27)	9755	Cell Signaling	15	1:500
Histone H3, Pan-Methyl (Lys9)	4069	Cell Signaling	15	1:500
Histone H3, tri-methyl (Lys27)	07-449	Millipore	17	1:5000
Histone H3	Ab1791	Abcam	17	1:500
Histone H3 (S10)	06-570	Upstate	17	1:1000
PCAF (C14G9)	3378	Cell Signaling	93	1:500
SUMO-1	4930	Cell Signaling	Many	1:500
SUMO 2-3	4971	Cell Signaling	Many	1:500
Autophagy	**Catalog #**	**Company**	**MW (kDa)**	**Dilutions**
p53 (S15)	9284	Cell Signaling	53	1:1000
p53	9282	Cell Signaling	53	1:1000
Akt (S473)	9271	Cell Signaling	60	1:1000
Akt (T308)	9275	Cell Signaling	60	1:1000
Akt	9272	Cell Signaling	60	1:1000
c-Abl (Y245)	2861	Cell Signaling	135	1:1000
eNOS (S1177)	9571	Cell Signaling	140	1:1000
p90RSK (S380)	9341	Cell Signaling	90	1:1000
Atg5	2630	Cell Signaling	55	1:500
LC3B	2775	Cell Signaling	14,16	1:500

AMPKα1 (S485)	4184	Cell Signaling	62	1:1000
mTOR	2972	Cell Signaling	289	1:1000

Category 1 proteins are those that showed a significant change only in response to fully virulent Y. *pestis* CO92 strain. Categories 2a and 2b are those that showed a significant change in response to either the attenuated strain alone or in response to both the attenuated and virulent strains, respectively. Category 3 proteins designate those that showed a significant change in response to the heat-inactivated CO92, and are subdivided into subcategories 3a, 3b and 3c. Category 3a refers to significant changes in response to the heat inactivated treatment alone; categories 3b refers to significant changes in response to both the heat inactivated bacteria and the avirulent strain, and category 3c designates significant responses that are observed for heat inactivated bacteria, the avirulent strain, and the virulent strain. Category 4 proteins signify those that showed significant fold changes in response to LPS treatment, and are subdivided into subcategories 4a, 4b, 4c and 4d. Category 4a refers to significant changes in response to LPS treatment only. Category 4b refers to significant changes in response to both the LPS treatment and infection with the virulent strain. Category 4c designates those proteins that showed significant fold change in response to LPS treatment, treatment with the heat-inactivated bacteria, and infection with the virulent strain. Category 4d designates proteins that showed significant change in response to all four treatments conditions. Of

all the proteins analyzed by RPMA, 25 proteins were identified to exhibit significant fold changes, as defined by the assigned threshold values for significance (Table 2).

Table 2. Proteins that showed a fold increase of 1.5 and higher for total protein and 2 fold and higher for phosphorylated protein were divided into 6 categories based on infection model. Gray colored boxes represent significant fold increase for that particular infection model. Category **(1)** proteins only show fold increase with wild type *Y. pestis* CO92 infected cells. Category 2 proteins show a fold increase with the avirulent*Y. pestis* CO92 (*pgm-pst-)* strain alone**(2a)** or with CO92 (*pgm-pst-)* as well as wild type CO92**(2b).** Category 3 proteins show fold change with HK-CO92 only **(3a)**with HK-CO92 as well as CO92 (*pgm-pst-*)**(3b)** or with CO92, CO92 (*pgm-pst-*) and HK-CO92**(3c)**. Category 4 proteins show increased fold change with LPS alone **(4a)** or along with other treatment conditions such as LPS and CO92 only **(4B)**, LPS, CO92, and HK-CO92 **(4c)** or all 4 treatements**(4d).**



Analysis of HBE Cells.

Category 1 consists of 6 proteins that show significant phosphorylation increases (Akt-S473 and-T308, AMPKa1-S485, CHK1-S345, Ras-GRF1-S916, p53-S15, and Bad-S155). These proteins are known to be involved in multiple intracellular signaling pathways, and based on their established roles the consequences of the observed RPMA changes suggest apoptotic/survival and/or autophagy-related functional outcomes (Table 3). Therefore, the RPMA results show that during the early phases of Yp infection the apoptosis/survival pathways and mechanisms involved in negative regulation of autophagy are among the very key signaling mechanisms that become engaged. Within

category 1, with the exception of Ras-GRF1(S916) that was altered within the first hour post infection, the remainder of the proteins showed significant changes later, at 8 hours post infection.

Category 2 contains 15 proteins, 9 of which show significant increases in either protein levels or phosphorylation post infection (Histone H3 Di-methyl (Lys9), SAPK/JNK, Cleaved caspase 3-D175, Cleaved caspase 6-D162, Cleaved caspase 7-D198, Cleaved caspase 9-D330 and -D315, p53, Cleaved PARP-D214, and c-Myc) and 6 of which show a significant decrease in phosphorylation (SHP2-Y580, Gab1-Y627, ERK 1/2-T202/Y204, PLC-γ-1-Y783, p38-T180/Y182, and SHIP1-Y1020). Except for Histone H3 Di-methyl (Lys9), all the other proteins in this category are known to be involved in the apoptosis/survival pathways and the specific phosphorylation changes shown by RPMA for 12 of these proteins point to an overall induction of apoptosis following Yp infection (Table 3). This striking observation suggests that within category 2 (which designates changes elicited by the avirulent Yp strain alone, or by both the avirulent and the virulent strains) a major signaling response of the host is the activation of cell death pathways.

The remaining 6 host proteins from the total of 25 identified hits belong to category 3 (treatment with heat-killed Yp) and/or category 4 (LPS treatment). Because these treatment conditions are devoid of TTSS function, the host protein changes observed in response to these treatments cannot be caused by TTSS-dependent mechanisms, but

clearly extensive future studies are required to identify the mechanisms that cause the observed host changes during these treatment conditions. The live Yp strains also elicited significant changes for some of these proteins (listed for subcategories 3b, 3c, and 4b, 4c, and 4d). The 6 proteins, iNOS, cAbl-T735, CREB-S133, Bad-S112 and -S136, GSK-3α-S21, and P90RSK-S80, all show significant fold increase, with some as early as 30 min post infection. For category 3, the increased phosphorylation of the Bad protein at S112 and S136 residues lead to an inhibition of its apoptotic functions and therefore are predicted to promote cell survival. Furthermore, the observed significant increases in iNOS levels, activation of CREB by phosphorylation at S133, and inhibition of GSK-3α by phosphorylation at S21, are all predicted to lead to survival outcomes based on the known functions of these proteins (Kotliarova et al., 2008; Park et al., 2005; Song, Ouyang, & Bao, 2005)**.** Therefore, our RPMA results point to a coordinated early induction of survival during Yp infection of lung epithelial cells that can occur through TTSS-independent mechanisms.

Table 3. Protein hits identified by RPMA for HBE cells and their proposed functions in the context of Yp infection.

Proteins with significant changes relative to the control condition are listed. For each protein, the observed changes at specific post infection times are indicated. Green color represents significant decrease (≥2-fold decrease for total protein levels and ≥1.5-fold decrease for phosphorylation events) and red color represents significant increase. Light

red represents a 1.5 to 3-fold increase for phosphorylation events and 2 to 3-fold increase for total protein levels. Medium red represent a 3 to 6-fold increase for either phosphorylation or total protein level changes. Dark red color, assigned only to one protein at the 1h time point [HistoneH3,Di-Methyl(Lys9)],representsan11-fold increase in total level of this protein. Category designations for each time point has been indicated based on the categorization scheme of Table 2. The left side columns indicate possible functional outcomes of the observed protein changes based on the known roles of the proteins. Asterisks denote proteins that fall into different categories at different time points.

Possible Functional Outcome		Protein Name	Time Post Infection (h)			
			0.5	1	4	8
Category 1: Virulent *Y. pestis* Strain Only						
Apoptosis	**Inhibition of Autophagy**	p53 (S15)				**1**
Survival		Akt (S473)			**1**	**1**
		Akt (T308)				**1**
		AMPKα-1 (S485)				**1**
		CHK-1 (S345)				**1**
		Ras-GRF1 (S916)		**1**		
		Bad (S155)				**1**
Category 2: Avirulent *Y. pestis* Strain Only OR Both Avirulent and Virulent *Y. pestis* Strains						
Chromatin Effect		Histone H3, Di-Methyl (Lys9)*		**2a**	**2b**	**1**

Apoptosis		SAPK/JNK*		**1**	**2b**	**2b**
		Cleaved Caspase 3 (D175)				**2b**
		Cleaved Caspase 6 (D162)				**2b**
		Cleaved Caspase 7 (D198)			**2a**	**2b**
		Cleaved Caspase 9 (D330)				**2b**
		Cleaved Caspase 9 (D315)				**2b**
		p53	**2b**	**2b**	**2b**	**2b**
		Cleaved PARP (D214)			**2a**	**2b**
		cMyc		**2a**		
		SHP2 (Y580)*			**1**	**2b**
		Gab1 (Y627)	**2b**	**2b**	**2b**	**2b**
		ERK 1/2 (T202/Y204)			**2b**	**2b**
		PLC-γ-1 (Y783)*	**1**			**2a**
	Survival	p38 (T180/Y182)				**2a**
		SHIP1 (Y1020)				**2a**
Category 3 and 4: TTSS Independent						
Immune Response		iNOS		**3b**		**4c**
Survival		c-Abl (T735)			**4a**	**4b**
		CREB (S133)	**3a**	**3a**		
		Bad (S112)	**3a**	**4d**	**4d**	**4b**
		Bad (S136)			**3b**	**4b**
		GSK-3α (S21) *****		**3c**	**3a**	**1**
		P90RSK (S380)			**4d**	**4b**

Validation of RPMA Results by Western Blots

Even though RPMA antibodies have already been pre-validated and reported in previous RPMA publications (Popova et al., 2010; Sereni, Pierobon, Angioli, Petricoin, & Frederick, 2013; Wilson, Liotta, & Petricoin, 2010; Younossi et al., 2011), we nevertheless performed another tier of validation of our RPMA results by Western blot analysis of selected hits, testing both the same sample set that was used for the RPMA analysis and a biological replicate sample set. Multiple RPMA hits were validated by Western blot and two representative examples are presented in Figure 4, for cleaved PARP (D214) and p53 (S15) proteins. Our Western analysis confirms the RPMA findings of an increase in PARP (D214) levels and activation of p53 by increased phosphorylation at S15 residue during Yp infection (Figures 4A and 4D). We also observed a faint band for p53 (S15) with the Yp CO92 (pgm-,pst-) strain that was not seen in the RPMA analysis (Figure 4D). For different treatment conditions, fold changes in protein levels or phosphorylation levels were measured by comparing the band intensities to untreated controls, and the comparisons between the RPMA and Western fold changes for Cleaved PARP (D214) and p53 (S15) are presented (Figures 2B and 2E). Two-way scatter plot analysis shows strong positive correlation between the western

blot and RPMA results, with a R^2 value of 0.9 for cleaved PARP (D214) and a R^2 value of 0.98 for p53 (S15) (Figure 4C and 4F).

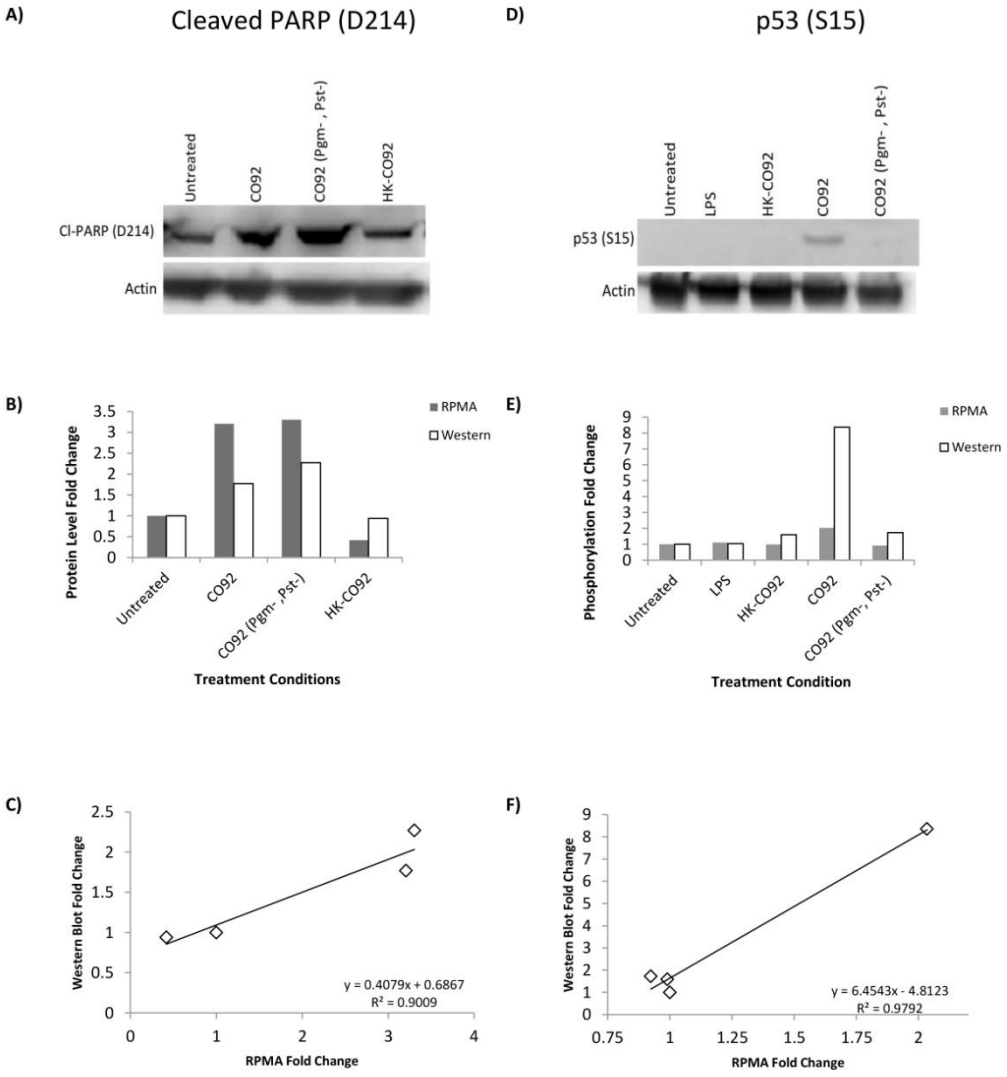


Figure 4 Western blot validation of RPMA results.

(A,D) Western blot analysis of human bronchial epithelial (HBE) cells for control condition (uninfected and untreated), treatment with heat-killed Yp CO92 (HK-CO92) or with LPS, and infection with Yp CO92 or Yp CO92 (Pgm-, Pst-). The blot in (A) was probed with the same antibody that was used for the RPMA analysis of cleaved PARP (D214), and the blot in (D) was probed with the same antibody against p53 (S15) that was used for RPMA analysis. Actin levels were also probed for all samples to serve as loading control and allow normalization of the signals for measuring fold changes relative to the control condition. (B,E) Bar graphs showing protein level fold changes of cleaved-PARP (D214) in (B), and phosphorylation fold changes of p53 (S15) in (E), for both the RPMA and Western blot analyses of the treatment conditions. For comparison purposes, the value for the control condition (uninfected and untreated) was set at 1. (C,F) Two-way scatter plot showing positive correlation between the RPMA results and the Western blot results for Cleaved-PARP (D214) and p53 (S15).

Discovery of Novel Proteins and Confirmation of Previous Findings within the Context of *Y. pestis* Infection

Our RPMA analysis of the human lung epithelial cells yielded 25 proteins that displayed significant changes in either site-specific phosphorylation or total protein levels in response to Yp infections; a complete list of these proteins as well as the pathways engaged by the novel hits are shown in Table 4. After a comprehensive literature search, 12 of the 25 hits were identified to be novel findings, and have not been previously

reported for Yp infection. Our study also led to the identification of several hits that have been previously reported, although the majority of them have been reported either in the context of infection with other Yersinia species or within the context of using recombinant proteins and transfections with specific Yp components (Table 4). Thus, our identification of several previously reported changes not only provides an important level of validation for our RPMA findings but also places a number of the previous reports within the more relevant context of whole infection with *Y. pestis*, which we have performed using a relevant model cell type and under both fully virulent and avirulent infection conditions.

Two of the main pathways that are highlighted by our novel hits are the autophagy and survival-related pathways. In addition, cell cycle and growth regulation, and regulation of chromatin structure, are implicated. The identification of these novel hits by RPMA helps to fill in the current gap of knowledge with regard to the overall signaling network changes that occur during early response to infection with *Y. pestis*. For instance, as discussed in the next section, our discovery of specific changes in several proteins that regulate autophagy, together with initial mechanistic results, leads to a model of negative regulation of autophagy during early response of human lung epithelial cells to Yp infection.

Table 4 Discovery of novel hits and validation of previous findings in the context of Yp infection.

Novel protein hits discovered through the RPMA analysis and protein hits that have been previously implicated for relevance to the process of Yersinia infections are listed. For the novel hits, the affected pathways in which they are known to be involved are indicated; the bold type highlights those pathways that are implicated by the specific RPMA changes observed for these proteins during Yp infection. Previously reported proteins are separated based on whether the studies had been performed in the context of Yp infection or infection with other Yersinia species, as well as studies that were based on treatment with recombinant Yp proteins or cell transfections.

Novel Proteins	Functional Relevance	Category
Bad (S112)	Apoptosis/**Survival**	3,4
Bad (S136)	Apoptosis/**Survival**	3,4
Bad (S155)	Apoptosis/**Survival**	1
Cl-Caspase-6 (D162)	**Apoptosis**/Survival	2
cMyc	**Apoptosis**/Survival, Growth	2
c-Abl (T735)	**Apoptosis**/Survival, Growth	4
p53	**Apoptosis**/Survival, **Autophagy Regulation**, Cell Cycle	2
p53 (S15)	**Apoptosis**/Survival, **Autophagy Regulation**, Cell Cycle	1
AMPK-α1 (S485)	Apoptosis/ **Survival**, **Autophagy Regulation**, Homeostasis	1
SHIP1 (Y1020)	Apoptosis/Survival, Growth	2
SHP2 (Y580)	**Apoptosis**/Survival, Growth	1,2
Chk-1 (S345)	Apoptosis/**Survival**, Growth, Cell Cycle	1

iNOS	Immune Response	3,4
Histone H3, Di-methyl (Lys9)	Chromatin Structure	1,2
Ras-GRF1 (S916)	Cytoskeleton Modulation	1
Reported Proteins	**Functional Relevance**	**Reference(s)**
Reported for *Yersinia pestis*		
Cl-Caspase-3 (D175)	Caspase activity low with Yp Kim YopJ Pla degrades FasL, which decreases Caspase-3 activation YopK contributes to Caspase-3 cleavage	(Zheng et al., 2012) (Caulfield et al., 2014) (Peters et al., 2013)
Cl-Caspase-7 (D198)	Caspase-7 activity low with Yp Kim YopJ Pla degrades FasL, which decreases Caspase-7 activation	(Zheng et al., 2012) (Caulfield et al., 2014)
Cl-PARP (D214)	Cleaved-PARP indicates caspase pathway activation by Yp	(Zheng et al., 2012)
Reported for other Yersinia species		
Akt (S473), Akt (S308)	*Y. enterocolitica* YopH inactivates Akt pathway	(Sauvonnet et al., 2002)
Cl-Caspase-9 (D330) Cl-Caspase-9 (D315)	*Y. enterocolitica* YopP activates Caspase-9 through Bid	(Denecker et al., 2001)
CREB (S133)	YopJ of *Y. pseudotuberculosis* can block CREB activation	(Meijer et al., 2000)
GSK-3α (S21)	*Y. enterocolitica* YopH modulates PI3K/Akt/GSK-3α pathway	(Sauvonnet et al., 2002)
P90RSK (S380)	*Y. enterocolitica* YopM induces sustained RSK activation	(Hentschke et al., 2010)

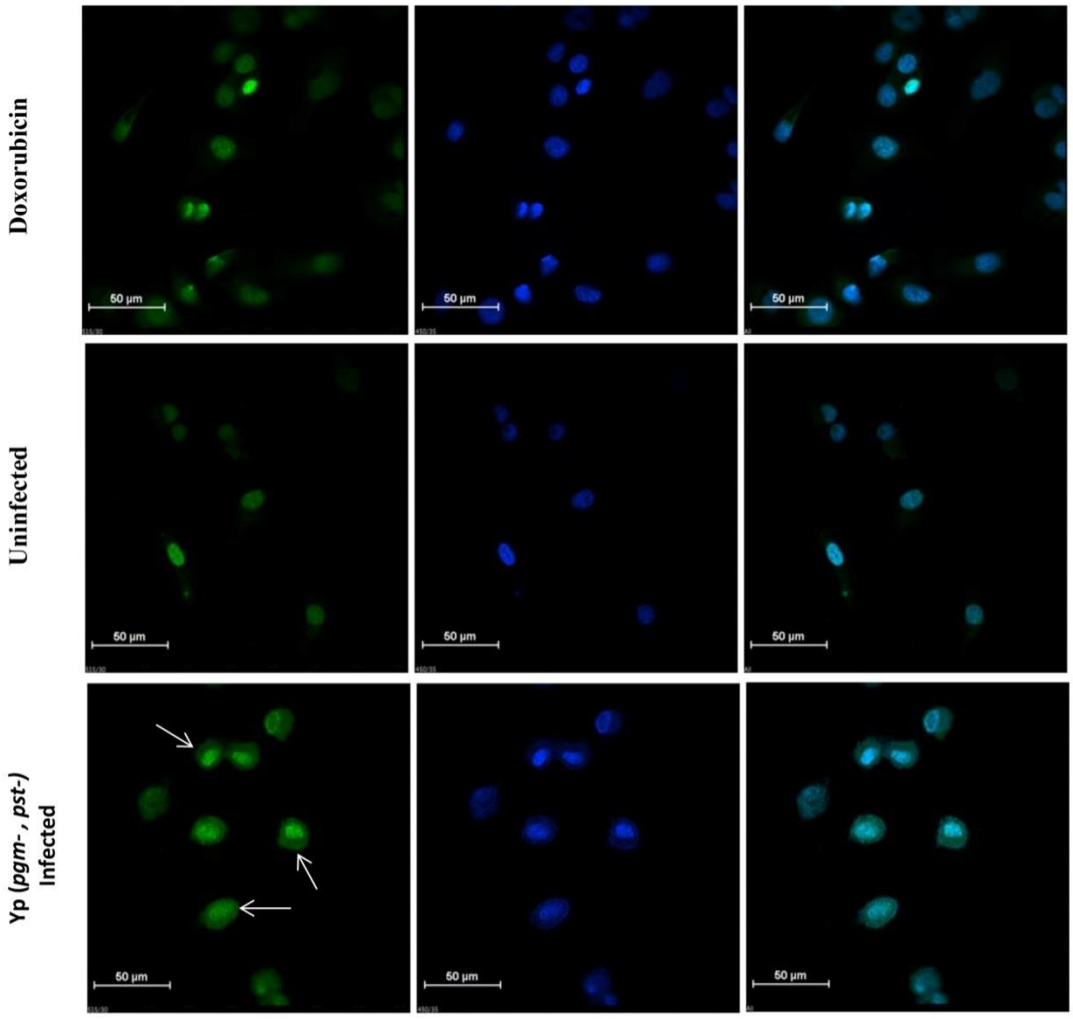
Reported using recombinant proteins or transfected cells		
Akt (S473), Akt(S308)	Akt is involved in *Y. pseudotuberculosis* entry into host cells	(Uliczka et al., 2009)
ERK 1/2 (T202/Y204)	rF1 induces phosphorylation of ERK1/2 in macrophages rYopB and rLcrV inhibit expression of phospho-ERK 1/2	(Sharma et al., 2005) (Sodhi et al., 2004)
PLC-γ-1 (Y783)	PLC-γ-1 plays a role in *Y. pseudotuberculosis* cell entry	(Uliczka et al., 2009)
Gab1 (Y627)	Transfected YopH of Yp associates with Gab1	(de la Puerta et al., 2009)
SAPK/JNK	JNK plays a role in rF1 induced activation of macrophages	(Sharma et al., 2005)

Evidence for Negative Regulation of Autophagy

Several identified hits are proteins that are known to regulate the autophagy process; pAKT (S473 and T308), AMPKalpha-1 (S485), and p53 (S15). Based on our RPMA analysis, the AKT kinase is activated during infection with Yp through phosphorylation of its S473 residue whereas AMPK activity is lowered through phosphorylation of its S485 residue, which is known to lead to AMPK inactivation (Ning, Xi, & Clemmons, 2011). Because activated AKT is an inhibitor of autophagy whereas activated AMPK is an inducer, these results suggest that autophagy is down-regulated during the first hours

post Yp infection. Our results also show that the levels of p53, another known regulator of autophagy, are significantly increased during Yp infection. Because studies have shown that cytoplasmic localization of p53 down-regulates autophagy (Liang & Clarke, 2001; Tasdemir, Maiuri, et al., 2008), we analyzed the nuclear versus cytoplasmic localization of p53 post Yp infection. Confocal microscopy analysis showed that while prior to infection p53 shows nuclear localization, subsequent to *Y. pestis* infection activated p53 (S15) shows significantly increased localization in the cytoplasm compared to uninfected controls (Figure 5A). Specifically, 91% of infected cells examined showed a clear presence of p53 in the cytoplasm, compared to only 15% for the uninfected cells (Figure 5B), consistent with a negative regulation of autophagy. We also analyzed the LC3 conversion from LC3-I to LC3-II during Yp infection. Conversion from LC3-I to LC3-II occurs through conjugation of phosphatidylethanolamine (PE) to LC3-I and is a marker for induction of autophagy (Tanida, Ueno, & Kominami, 2008). This conjugation leads to the recruitment of LC3-II to the phagosomal membrane, and as autophagy is induced the ratio of LC3-II to LC3-I increases. Our data shows that after infection with Yp the ratio of LC3-II/LC3-I decreases approximately two fold in comparison to the uninfected/untreated condition and also the LPS treatment condition (Figure 6), indicating an inhibition of autophagy. Together, the LC3 and p53 results are consistent with a model of negative regulation of autophagy following Yp infection and suggest a coordinated down-regulation through activation of AKT, inhibition of AMPK, and cytoplasmic localization of p53 (Figure 5).

A)



B)

Treatment	Total Cell Count	Nuclear	Cytoplasmic	% Cytoplasmic
Doxorubicin	200	130	70	35
Uninfected	200	170	30	15
Yp Infected	200	18	182	91

Figure 5 p53 localization in the cytoplasm of Yp-infected cells.

(A) Confocal microscopy image of HeLa cells for analysis of p53 localization. Top panel shows cells treated with Doxorubicin at 1µM for 1h at 37◦C to induce p53 expression and serve as positive control. Middle panel shows uninfected cells and

bottom panel shows cells infected with Yp CO92 (Pgm-, Pst-) for 8h. Green fluorescence (left column) indicates activated p53 signal, obtained by probing with p53 (S15) antibody, and blue signal (middle column) corresponds to DAPI nuclear stain. The right column shows superimpositions of the two signals. White arrows show p53 localization in the cytoplasm of the infected cells compared to uninfected and Doxorubicin-treated cells. (B) For each treatment condition, cells were scored for cytoplasmic or nuclear localization of p53 (S15) and percentages of cells showing cytoplasmic signal were calculated.

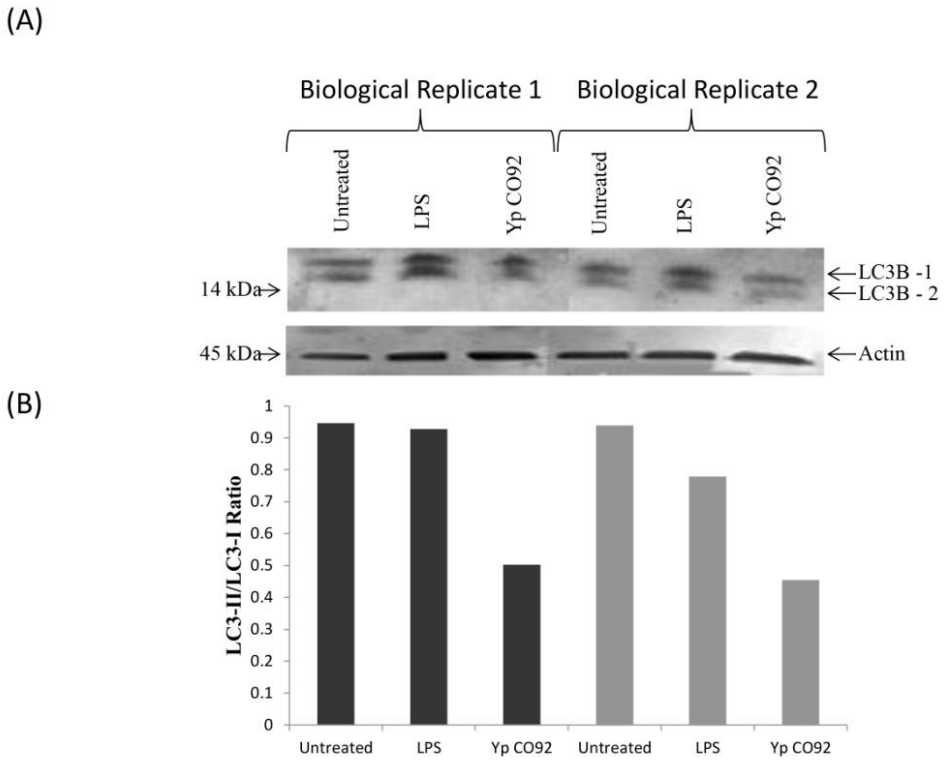


Figure 6 Decreased conversion of LC3B-I to LC3B-II during Yp infection.

(A) LC3-B Western blot analysis of total cell extracts obtained from HBE cells at 8h post infection with CO92, or post treatment with LPS, compared to the uninfected and untreated control. The results for two sets of biological replicates are shown. Actin levels were also probed for all samples to serve as loading control and allow normalization of the signals. (B) Bar graph showing LC3II/LC3I ratios for all samples based on the densitometry analysis for the LC3I and LC3II protein bands.

We have observed similar protein changes that point towards a down regulation of autophagy through RPMA analysis of U937 cells. Levels of p53, Akt (S473) and

AMPKα-1 were all upregulated after infection with both the virulent and avirulent strains of Yp.

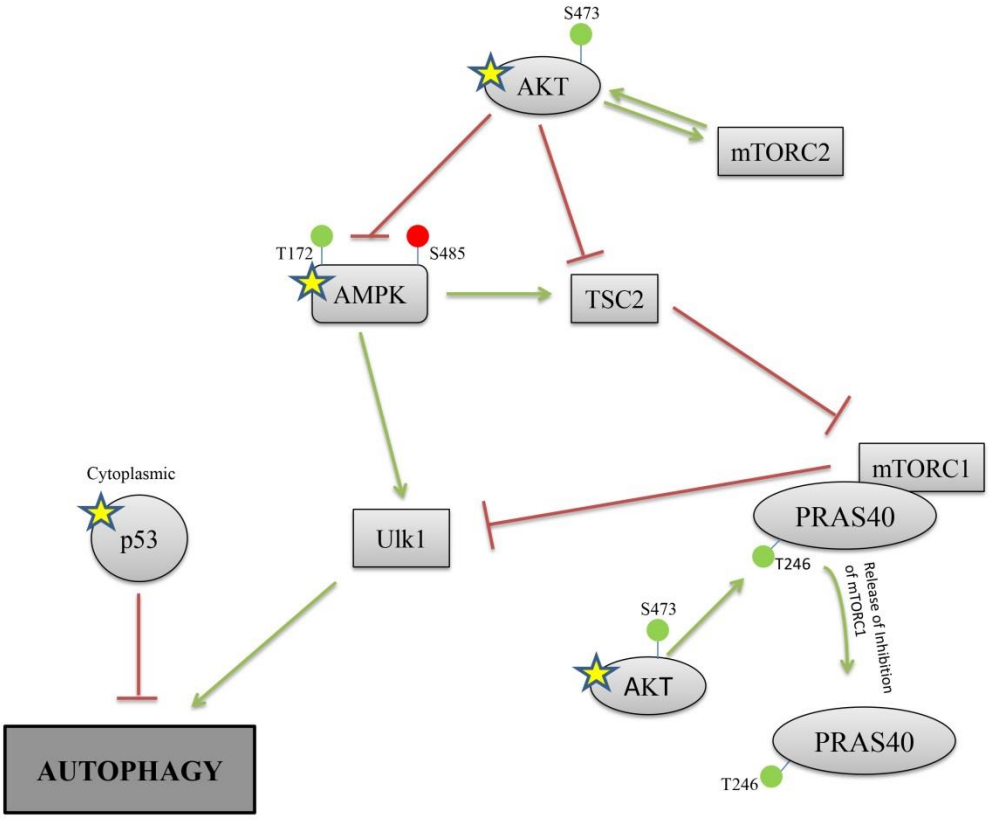


Figure 7. Proposed model of negative regulation of autophagy during Yp infection. Pathway map for regulation of the autophagy process highlights proteins that were identified by the RPMA analysis (Yellow star). Red lines depict inhibitor signals and green lines depict activation. The RPMA results demonstrate activation of AKT, p53, proteins, and inhibition of AMPKα-1, during Yp. Based on reported studies, all the observed changes are consistent with a model of down regulation of autophagy as part of the host response to Yp infection.

CHAPTER FOUR: DISCUSSION

We took advantage of the high throughput assay format and high sensitivity and reliability of the RPMA platform to quantitatively profile the host response to *Y. pestis* infection, particularly the signaling pathways that become modulated. Because the RPMA platform is amenable to simultaneous analysis of numerous pathways, it affords the possibility of measuring the overall signaling network changes that occur in the cell. For measuring changes in phosphorylation states or total protein levels, we probed with a total of 111 antibodies that span multiple functional categories relevant to infection processes, including apoptosis and survival, autophagy, cell cycle regulation and cellular growth, cytoskeleton modulation and cellular migration, immune response, and chromatin modulation. One of the cell types that we analyzed was the human bronchial epithelial cell line 16HBE14o-, which has been demonstrated to serve as a model system of the airways (Forbes, Shah, Martin, & Lansley, 2003). We used both a fully virulent strain of Yp, CO92, and a derivative avirulent strain for the infections and also profiled changes that can be elicited in a TTSS-independent manner.

Implication of Autophagy, Cell Cycle and Growth, and Survival-related Pathways

Compared to the control condition (untreated and uninfected cells), a total of 25 proteins in 16HBE14o- cells showed significant fold change in response to the treatment conditions. Survival-related pathways, and pathways involved in regulation of autophagy

or cell cycle and growth, are strongly highlighted by the RPMA results. Interestingly, the functional consequences of the RPMA changes observed for category 1 hits, which represent responses observed only for infection with the virulent Yp strain, point to an overall activation of survival functions. Thus, activated Akt (S743), phosphorylated Ras-GRF1 (S916) (activator of Ras), and phosphorylated BAD (S155), are all involved in cell survival pathways (Brunet et al., 1999; DATTA, 1997; Downward, 2004; Jeon, Chandel, & Hay, 2012). In addition, the increased levels of active CHK-1 (S345) (DNA damage cell cycle checkpoint) also points towards activation of survival pathways (Liou et al., 2014; Sarmento et al., 2014; Wang et al., 2014)**.**

Category 2 proteins also strongly point to regulation of survival-related pathways, although unlike category 1 proteins the observed RPMA changes highlight more the activation of cell death pathways. In addition, inhibition of cell growth and proliferation is suggested by some of the observed changes. Thus, the specific modulations observed for 12 of the 15 proteins in this category suggest activation of apoptotic functions (Allsopp et al., 2000; Amaral, Xavier, Steer, & Rodrigues, 2010; Boulares et al., 1999; Evan et al., 1992; Hakem et al., 1998; Janicke, 1998; Marsden et al., 2002; Packham & Cleveland, 1995; Porter & Jänicke, 1999; Ruiter, Zerp, Bartelink, van Blitterswijk, & Verheij, 1999; Slee, Adrain, & Martin, 2001; Verheij et al., 1996). From this group of proteins, ERK (T202/Y204), Gab1 (Y627), SHP 2 (Y580) and SHIP1 (Y1020) have also been implicated for cell growth (Araki, Nawa, & Neel, 2003; Balmanno & Cook, 2009; Eulenfeld & Schaper, 2009; Gloire et al., 2006; Hakak, Hsu, & Martin, 2000; Ivins Zito, Kontaridis, Fornaro, Feng, & Bennett, 2004; Lu & Xu, 2006; Mattoon, Lamothe, Lax, &

Schlessinger, 2004; Mood et al., 2006). Since all of these 4 proteins show down-regulation in response to Yp infection, these results also provide evidence for a potential down-regulation of cell growth as part of the host response to Yp. Since both categories 1 and 2 profile significant host responses under functional TTSS conditions, the observed changes may be related to the functions of the Yop effector proteins although clearly further mechanistic studies are needed to address this question. In addition, it remains to be investigated which of the host pathway changes that we have observed by our RPMA analysis reflect Yp manipulation of the host machinery and which are reflections of direct host response.

Treatments with heat-killed CO92 strain and with LPS allowed profiling host responses that can occur independently of the TTSS machinery. Similar to the findings for category 1 and category 2, changes observed in response to treatment with heat killed Yp or LPS suggest activation of survival-related pathways. For example, BAD (Bcl-2 Associated Death) is a protein that induces apoptosis by blocking BAX from binding to Bcl-2 or Bcl-XL, and allowing cytochrome C release from mitochondria to activate the intrinsic death pathway. Phosphorylation of the BAD protein at Ser 136, Ser 112, or Ser 155, which are observed in our RPMA analysis, has been shown to inactivate BAD, and therefore promote cell survival (Brunet et al., 1999; DATTA, 1997; Peso, 1997). Similarly, the observed increased levels of activated CREB (S133) is known to result in increased cell proliferation and survival (Mayr & Montminy, 2001; Shaywitz & Greenberg, 1999). GSK-3α, a serine-threonine kinase, which influences multiple downstream pathways ranging from cell survival/apoptosis to growth and immune

response (Downward, 2004; Kotliarova et al., 2008; Ohteki, 2000; Tseng, Engel, & Keating, 2006)**,** can also be modulated through TTSS-independent mechanisms. Thus, in response to heat-killed Yp treatment, GSK-3α shows increased phosphorylation at S21 residue during early post infection times (1 and 4 hours post infection), which is known to inactivate GSK-3α (Kotliarova et al., 2008). Consistent with our interpretations for the observed changes for the BAD and CREB proteins, the inactivation of GSK-3α may also be pointing to promotion of survival.

Model for Negative Regulation of the Autophagy pathway

Several of our identified hits are known to modulate the autophagy process. Autophagy is a cellular mechanism that can be utilized by the host to eliminate invading pathogens but it can also be manipulated by the pathogen to survive and replicate (Owen et al., 2014). *Yersinia pestis* has previously been shown to reside in autophagosomes of mouse macrophages in order to evade host immune responses (Pujol et al., 2009). In our study, we observe that multiple proteins that are known to modulate the autophagy pathway are altered in infected cells; pAKT (S743 and T380), AMPKα-1 (S485), and p53 (S15) all showed increased levels at 8 hours post infection. In active form, AMPK is a known inducer of autophagy through its activation of ULK1 as well as its role in blocking inhibition of autophagy by mTOR (Xu et al., 2014). Activated AKT (phosphorylated at S473) can phosphorylate AMPK at its S485 residue, leading to AMPK inactivation and therefore inhibition of autophagy (Ning et al., 2011). In addition, AKT directly blocks activation of TSC2 (an inhibitor of mTOR and inducer of autophagy) to provide further regulation of the autophagy process (Cai et al., 2006; Inoki,

Li, Zhu, Wu, & Guan, 2002)**.** The p53 protein is another RPMA hit that has also been shown to play a role in autophagy. Cytoplasmic p53 has been shown to repress autophagy (Mariño, Niso-Santano, Baehrecke, & Kroemer, 2014; O'Brate & Giannakakou, 2003) whereas nuclear p53 induces autophagy through transactivation of genes that suppress mTOR (Tasdemir, Chiara Maiuri, et al., 2008). Together, our RPMA results (AKT and p53 activation, and AMPKα-1 inactivation), and our demonstration of the cytoplasmic localization of p53 and reduced LC3-I to LC3-II conversion during Yp infection provide evidence of negative regulation of autophagy as part of the host response to Yp (Figure 7). It is of significance that the RPMA changes are mostly observed in response to the virulent Yp strain, and therefore negative regulation of autophagy may represent an important mechanism in determination or regulation of virulence. Further mechanistic studies are required to strengthen our autophagy model and to investigate whether it plays a role in regulation of Yp virulence, in particular given the role of autophagy in bacterially infected cells (Yuk, Yoshimori, & Jo, 2012) and the reported findings that intracellular Yp bacteria residing in autophagosomes can avoid xenophagy (Pujol et al., 2009).

Discovery of Novel Hits and Validation of Previous Studies in the Context of Yp Infection

Our RPMA analysis led to the identification of 12 novel hits with respect to Yp infection. Consistent with the reported importance of autophagy and apoptosis/survival pathways during Yp infection (Caulfield et al., 2014; Ke, Chen, & Yang, 2013; Peters et al., 2013; Pujol et al., 2009), many of the novel hits implicate these pathways.

Furthermore, the cell cycle and growth control pathways are implicated through modulation of proteins such as Chk-1 (S345), c-Myc, SHIP1 (Y1020), or SHP2 (Y580) given the reported functions of these proteins (Gloire et al., 2006; Liu et al., 2000; Niida, Katsuno, Banerjee, Hande, & Nakanishi, 2007; Schmidt, 1999; Tsang, Han, Man, Lee, & Poon, 2012; Yang et al., 2006)**.** A level of validation of our RPMA results comes from identification of several hits that have been previously reported to be relevant (Table 4). For instance, the RPMA hits Gab1, Gab2, and Lck have been shown to be targeted by YopH in order to regulate the host immune response (de la Puerta et al., 2009). Or, extending previous findings for infection with *Y. pseudotuberculosis* (Uliczka et al., 2009), our RPMA results show that the AKT kinase is also activated during infection with Yp. However, the majority of the previous reports related to our RPMA hits have been either in relation to infection with other Yersinia species or within the context of using recombinant proteins and transfections with specific Yp components (Table 4). Therefore, beyond providing a level of validation, our identification of several reported changes also places a number of the previous findings within the more relevant context of whole infection with Yp.

With continuing significant concern over the rising level of antibiotic resistance for many bacterial infections, focusing on development of host-based therapies founded on an understanding of the host response mechanisms provides a viable alternative for devising effective countermeasures. In addition, a better understanding of the host response networks will allow advances in development of much needed vaccines and early diagnostic measures. The type of high throughput host protein analysis that is

afforded by RPMA and has been presented here provides a unique opportunity to measure protein network changes that occur across the host cell in response to infection. The RPMA results presented here should provide a strong foundation for further mechanistic studies of Yp infection, and help pave the way for devising novel effective countermeasures for treatment and prevention of plague.

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