DEVELOPING AND IMPLEMENTING GENETIC TOOLS DESIGNED TO UNDERSTAND HOST TAKEOVER BY CHLAMYDIA TRACHOMATIS.

by

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A thesis submitted in partial fulfillment
of the requirements for the degree

of

Master of Science

in

Immunology and Infectious Diseases

MONTANA STATE UNIVERSITY
Bozeman, Montana

May 2019

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DEDICATION

I thank God for his blessings that comes in so many different forms. This Master's thesis is dedicated to my wife Sia Mbishi for her non-fading support through my program. Through my highs and lows, she has always been there encouraging me and helping me to push through. Secondly, my friends and colleague from the MBI Department, Ben, Murat, Tanner, Theary, Tats, Calvin and John just to mention a few. My wonderful friends and family from Bozeman, the Steeves, Philip Eykelbosch and family, Lonnie and the whole Bos family, the Josephs, and Shaban Humaj, you guys made Bozeman feel like home.

"Bozeman you have been kind"

ACKNOWLEDGEMENTS

First and foremost, I wish to express my deepest gratitude and appreciation to thank Dr. Blake Wiedenheft for giving me a chance to do my Master's at Montana State University. I would like to thank him for helping me take my first steps in science, and for his tireless efforts to guide, equip and prepare me as a scientist. I have been amazed by his dedication to training and developing young scientists, and it was a great privilege to train under his mentorship.

I am very grateful to my committee members, Dr. Ed Schmidt, Dr. Michael Franklin, and Dr. Scott Grieshaber for their readiness, guidance and their material and moral support throughout my program. They turned the scariest meetings into friendly environments to learn and develop.

I would like to thank Dr. Emma Loveday, Dr. Royce Wilkinson, Dr. Artem Nemudryi and Dr. Anna Nemudraia for their guidance and technical support. Also, all other senior scientists in the Wiedenheft lab, Maryclare Rollins, Sarah Golden, Dr. Heini Mietiinen-granger for their untiring readiness to help and answer my endless questions. Lots of thanks to Myndi Holbrook for being such a valuable member for the Chlamydia team.

Many thanks to the MBI department staff, Ileana, Caitlin, Dana for all the administrative help, you were always welcoming and always happy to answer my never-ending requests and questions.

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Abstract

Chlamydia are gram negative obligate intracellular parasites that are responsible for millions of new infections in humans and animals every year. C. trachomatis is the number one cause of bacterial sexually transmitted infections in the United States, the number one cause of infectious blindness worldwide. Since 2001, there has been a steady increase in the number of new cases of C. trachomatis infections each year. Despite the prevalence and medical importance of C. trachomatis, we still know relatively little about the lifecycle of this parasite and the host factors that are essential for the lifecycle of C. trachomatis. To address this critical gap in our knowledge, my thesis work aimed to develop and implement genetic tools to understand host takeover by C. trachomatis. In this thesis I present results suggesting that I have transformed C. trachomatis with a plasmid carrying the Cas9 gene from Campylobacter jejuni. Additional experiments are necessary to determine if the CjCas9 is expressed, nuclease active, and functional for programable editing in C. trachomatis. In addition to my work aimed at developing a CRISPR-Cas9-based genetic engineering system in C. trachomatis, I also participated in a genome wide knockout screen aimed at identifying human genes necessary for completion of the C. trachomatis lifecycle. The CRISPR-Cas9 genome wide knockout screen identified 103 genes as critical factors for C. trachomatis. To validate results for the screen I have been involved in creating clonal cell lines with deletions in three of the genes that form the Adaptor Protein (AP) Complex (i.e., AP3S2, AP1B2 and AP1G2). The genes have been deleted and future experiments are aimed at measuring the impact of these genes on the C. trachomatis lifecycle.

CHAPTER ONE

CHLAMYDIA AND THE CHALLENGES OF GENETIC MANIPULATION

Abstract

Chlamydia are obligate intracellular parasites infecting a range of hosts from humans to animals. Chlamydia cause millions of new infections every year that results in debilitating disease and significant economic loss. Efforts to develop vaccines against these infections have not been successful and there has been a steady increase in annually reported infections. Here we review progress in studying and understanding Chlamydia-host interactions, as well as the challenges and opportunities for genetic manipulation of Chlamydia.

Introduction

Chlamydia is a genus of gram negative obligate intracellular parasites infecting both humans and animals (Bachmann, Polkinghorne, & Timms, 2014). *C. trachomatis* and *C. pneumoniae* represent the two main human pathogens from this genus. While *C. pneumonia* is known to cause upper and lower respiratory infections, *C. trachomatis* causes a wide spectrum of serotype dependent pathologies. Serotypes A, B, and C cause trachoma, an eye infection that is the leading cause of preventable blindness. Serotypes D-K are the leading cause of bacterial sexually transmitted infections worldwide, while serotypes L1-L3 cause a chronic systemic infection of the lymphatic system known as lymphogranuloma Venereum (Elwell, Mirrashidi, & Engel, 2016; Sixt & Valdivia, 2016). According to CDC, more than 1.5 million new cases of *C. trachomatis* infections were reported in the United State alone in 2016, while the World Health Organization (WHO) reported around 131 million new cases worldwide in the same year making it number one sexually transmitted bacterial infection worldwide (Centers for Disease, 2017).

The *Chlamydia* lifecycle

Chlamydia rely on a unique biphasic lifecycle alternating between two distinct forms: the infectious extracellular form (Elementary Body) and the intracellular replicating form (Reticulate Body). The infection starts by attachment of the small (~0.2µm) Elementary Body (EB) to its host cells through multiple host and bacterial factors interactions. The first interactions involves the bacterial Outer Membrane Complex protein B (OmcB) and the host cell heparan sulfate proteoglycans (Moelleken

& Hegemann, 2008). In addition, the bacterial lipopolysaccharide interacts with the host cystic fibrosis transmembrane conductance regulator (CFTR) and the bacterial major outer membrane protein binds to the host mannose receptor (Elwell et al., 2016). Internalization of the EB coincides with the injection of several type 3 secretion system (T3SS) effectors, leading to cytoskeletal rearrangements and internalization of the EB into an intracytoplasmic vesical (inclusion). Four main effectors facilitate inclusion formation (TarP, CT166, CT694 and TepP) (Elwell et al., 2016). Translocated actin-recruiting phosphoprotein (TarP), nucleates and bundles actin through its globular actin (G-actin) and filamentous actin (F-actin) domains. This is followed by acting rearrangement and membrane remodeling, which leads to internalization of the EB (Jiwani et al., 2013). Some TarP needs to be phosphorylated first before inducing the actin rearrangement (Jiwani et al., 2013). Translocated early phosphoprotein (TepP), is first phosphorylated by host tyrosine kinases then recruits the eukaryotic adaptor proteins CRKI and CRKII to the inclusion and is mainly associated with immune signaling (Chen et al., 2014). CT694 is thought to play a role in disrupting the actin dynamics though an interact with actin-binding protein AHNAK (Bullock, Hower, & Fields, 2012). The cytotoxin CT166, is particularly important after entry where it is used to reverse actin polymerization by inactivating the RHO GTPase RAC1 by glycosylation (Bastidas, Elwell, Engel, & Valdivia, 2013). Six to eight hours post infection, the EB transitions into the Reticulate Body (RB). The RBs replicate through binary fission and the inclusion expands to accommodate the growing number of cells. The RBs start to differentiate back to EB at around 30 hours post infection and the cells lyse 46 to 72 hours post infection,

releasing 200–300 EBs (**Fig. 1.1**) (Bastidas & Valdivia, 2016; Mathews, Volp, & Timms, 1999; Nguyen & Valdivia, 2012). Alternatively, EBs are released by extrusion of the inclusion from the host cell. This exocytosis like mechanism leaves an intact infected cell and is thought to one of the mechanisms that leads to persistence of *Chlamydia* infections (Hybiske & Stephens, 2007).

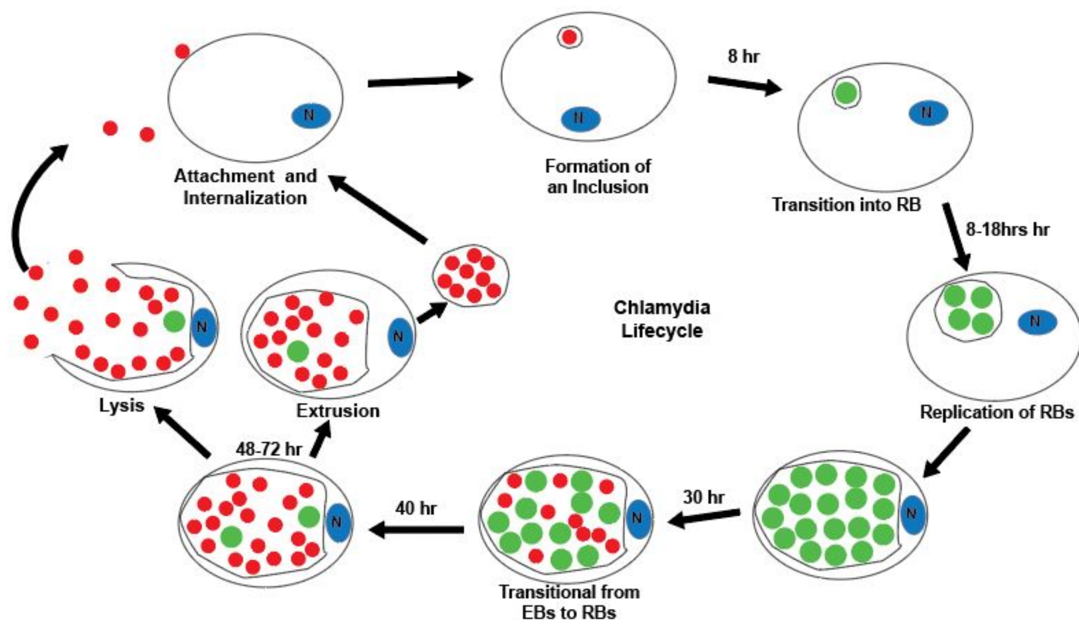


Figure1.1. Chlamydia lifecycle. The cycle starts with the EB attaching to the host and then internalized into the inclusion via receptor mediated endocytosis. Eight hours post infection (HPI) the EB differentiates into RB, which start to multiply. Around 30 HPI the RBs start to differentiate back to EBs, which is followed by exit from the host by lysis or extrusion.

Host evasion

Despite the significant health burden caused by *Chlamydia* infections, we still know relatively little about the life-cycle and pathogenesis (Kokes et al., 2015). To successfully complete their life, *Chlamydia* have evolved mechanisms to evade host defenses. Some examples include, prevention of apoptosis and evasion of the autonomous immunity. Unlike *Salmonella*, *Shigella* and *Yersinia* which induce apoptosis to evade macrophages, *Chlamydia* prevents apoptosis which bides time for the pathogens to complete its development in the host cell (Sharma & Rudel, 2009). It has been shown that *Chlamydia* species can block apoptosis by mechanisms such as, ubiquitylation and sequestration of apoptotic factors and kinases, and upregulation and/or stabilization of anti-apoptotic proteins, but the *Chlamydia* factors and mechanisms responsible for suppression of apoptosis remain unclear (Elwell et al., 2016). *C. pneumonia* has been shown to evade the host immunity through the suppression of type 1 IFNs. *C. pneumonia* achieves this by cleavage of the tumor necrosis factor (TNF) receptor-associated factor 3 (TRAF3) necessary in the production of IFNβ. To date, the *Chlamydia* protease responsible for this activity and its mechanism(s) of degradation are yet to be elucidated (Elwell et al., 2016).

The challenges of genome engineering in *Chlamydia*

Genetic manipulations in *Chlamydia* are inefficient, due in part to low transformation efficiencies (Bastidas & Valdivia, 2016). EBs have a tough cell wall made up of tight cross-linked proteins and highly condensed DNA. The cell wall is refractory

to penetration by macromolecules such as exogenous DNA and the chromosome is protected by two DNA binding proteins (Hc1 and Hc2) that homologous to eukaryotic histone H1 (Hackstadt, Baehr, & Ying, 1991; Pedersen, Birkelund, & Christiansen, 1996). The reticulate body on the other hand is more labile and has a more porous cell wall with a narrower peptidoglycan. RBs are also actively dividing and they express DNA repair enzymes that are necessary for integration of DNA into the chromosomes by homologous recombination. These characteristics would have made them the ideal *Chlamydia* form to target for genetic manipulation but RB are surrounded by four lipid bilayers membranes, the host cell membrane, the inclusion membrane, and the *Chlamydia* outer and inner membranes (**Fig. 1.2**) (Bastidas & Valdivia, 2016; Sixt & Valdivia, 2016). These surrounding membranes makes delivery of exogenous genetic material into these cells a challenging task.

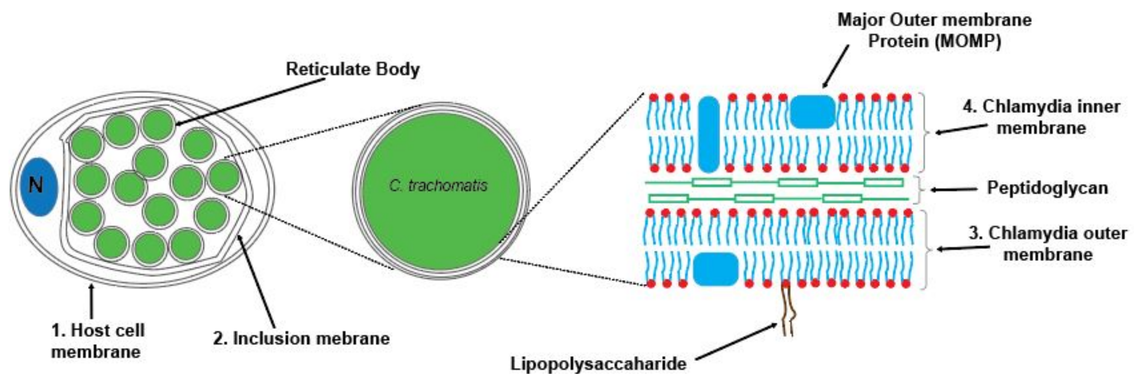


Figure 1.2. Four lipid bilayers encase the RBs**. 1)** The host membrane, **2)** Inclusion membrane, **3)** *Chlamydia* outer membrane, and **4)** *Chlamydia* Inner membrane.

Sixty six years after Fred Griffith first transformed *S. pneumoniae* (Griffith, 1928), until Tam *et al* transformed *C. trachomatis* by electroporation (Tam, Davis, & Wyrick, 1994). The success in *C. trachomatis* transformation only came after identification and characterization of the *C. trachomatis* indigenous 7.5kb plasmid which allowed for the creation of a *C. trachomatis-E.coli* shuttle vector (Hatt, Ward, & Clarke, 1988; Tam et al., 1994). Though the resulting transformants were only transient this opened the door towards stable transformation of *Chlamydia* (Binet & Maurelli, 2009; Y. Wang et al., 2011). The lack of efficient transformation method for *Chlamydia* is mainly attributed to the obligate intracellular lifestyle of these parasites with their unique biphasic lifecycle. As a result of this inability to transform *Chlamydia*, genetic methods have primarily relied on the use of chemical mutagenesis coupled to forward and reverse genetics (Bastidas & Valdivia, 2016; Sixt & Valdivia, 2016). In forward genetics, a phenotype is linked to a genotype while in reverse genetics the genotype is determined first then identification/studying the resulting phenotype (C. B. Gurumurthy et al., 2016).

Methods for Genetic Manipulation of *Chlamydia*

Chemical Mutagenesis.

Several genetic manipulation methods have been employed to study *Chlamydia*. Chemical mutagens such as ethyl methyl sulfonate (EMS) and N-ethyl-N-nitrosourea (ENU) have been used to generate *Chlamydia* mutant libraries can be then studied by forward and reverse genetics approaches (Kari et al., 2011; Kokes et al., 2015). Kokes *et al* used chemical mutagenesis coupled to forward genetics, to determine how *C.*

trachomatous hijacks the Golgi apparatuses. InaC was identified as the bacterial factor required in modulating the assembly of F-actin for redistribution of Golgi around the bacterial inclusion. InaC achieves this by binding the host ADP-ribosylation factor (ARF) which are responsible for host cell vesicular trafficking (Kokes et al., 2015). Using reverse-genetics, another group was able to isolate tryptophan synthase gene (trpB) null mutant that was incapable of surviving IFN-γ–induced tryptophan starvation (Kari et al., 2011).

In reverse genetics approach, *Chlamydia* are first exposed to a chemical mutagen, to select for a mutant carrying mutations in a gene of interest, pools of approximately 10 *Chlamydia* cells are prepared on a 96-well plate. Then using specific primers, the gene of interest is amplified by PCR. This is followed by identification of mutated targets by using Targeting Induced Local Lesions in Genomes (Tilling) method and sequencing to determine the genotypes of the identified mutants. Finally, plaque assays are used to isolated individual strains carrying the mutant is interest (Bastidas & Valdivia, 2016; Kari et al., 2011).

In forward genetic approach, a rifampin-resistant *C. trachomatis strain* was subjected to chemical mutagenesis, which was followed by isolation of individual mutant by plaque assay. These plaque assays also provide an opportunity for screening strains with interesting phenotypes (e.g., aberrant plaque morphologies), and this is followed by sequencing of the genomes of the isolates to identify genetic lesions. Since multiple mutations exists in the same isolate, a wildtype Rifampicin resistant strain is co-infected with the mutant strain which allows development of recombinants strains by lateral gene

transfers and from these recombinants strains the specific mutations that results in the phenotype of interest can be identified since it will always be found in recombinants that display the phenotype of interest (Bastidas & Valdivia, 2016; Nguyen & Valdivia, 2012).

While random mutagenesis has proven useful in certain contexts, this approach has intrinsic limitations. Due to the random nature of the mutations introduced by chemical mutagenesis, and sometimes presence of multiple mutations in the same cell, the process of linking phenotypes to specific genotypes is tedious, costly and time-consuming (Bastidas & Valdivia, 2016; Kari et al., 2011; Nguyen & Valdivia, 2012). *Chlamydia* have reduced genomes with *C. trachomatis* L2 strain possessing a 1.04Mb genome. For this reason many genes expected to be essential, thus most loss of function mutations are eliminated from these screens (Elwell et al., 2016; Ouellette, 2018). This highlights the need of a targeted genetic manipulation tool that can allow studying of both essential and non-essential genes.

Chlamydia Transformations

Programmed genetic manipulation requires access to the cell. To date, three different methods have been employed to transform *Chlamydia*. These include, electroporation, dendrimer (e.g., polyamidoamine) delivery, and chemical transformation (Calcium Chloride transformation) (Bastidas & Valdivia, 2016; Kannan et al., 2013). Irrespective of this progress, *Chlamydia* manipulation has proved to be a challenging task since these methods have either been difficult to replicate or have very low transforming efficiencies (Binet & Maurelli, 2009; Kannan et al., 2013; Mueller, Wolf, & Fields,

2017). Currently, CaCl2 transformation method is the gold standard method of *Chlamydia* transformation, but the approach require multiple replicates, large amounts of DNA (12ug per experiment) and sometimes several transformations (Bastidas & Valdivia, 2016; Ouellette, 2018).

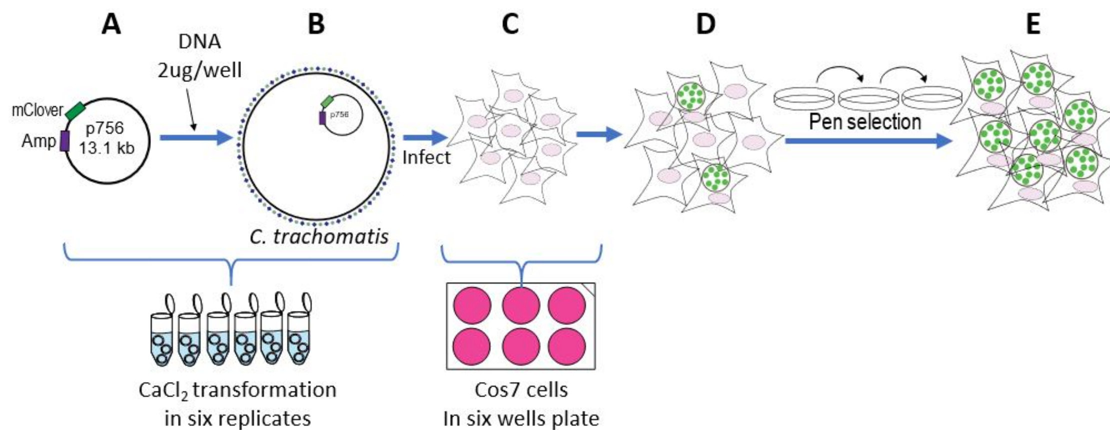


Figure 1.3. Calcium Chloride-based *Chlamydia* transformation**. A and B)** $CaCl_2$ incubation of Chlamydia with plasmid DNA done in six replicates. **B**) Infection of Cos7 cells using Chlamydia from A and B. **D and E**) Development and enrichment of transformant expressing the green fluorescent protein mClover.

Targeted Mutation Using Targetron

Group II introns are ribozymes that rely on an intron encoded protein (IEP) LtrA (also known as maturase) to perform its own retrotransposition (**Fig 1.4**) (Cousineau et al., 1998). The maturase encoded in the RNA sequence has three activities; first it acts as an endonuclease where it mediates the excision of the intron (**Fig.1. 4A and 1.4B),** and second, it facilitates homing of this excised intron into a gene in a sequence specific manner and the third function is the reverse transcription of the inserted RNA into DNA

(**Fig. 1.4C**). The intron target recognition depends on Watson-Crick pair interaction between three regions on the intron designated as EBS2, EBS1, and δ which recognize three regions on the target designated as IBS2, IBS1, and δ', respectively. The intron usually gets spliced out after transcription of the gene into which it was inserted which results in formation of a wildtype transcript. However, the maturase can be expressed in trans allowing for integration of the intron into a gene of interest and removal of the maturase to prevent the intron from being spliced out, which results in perturbation of the target gene. By changing the EBS1, EBS2, and δ the intron can be targeted to different genes and the removal of the maturase from the intron allows it to carry cassettes of antibiotic resistance genes and fluorescent protein markers (Enyeart et al., 2013; Enyeart, Mohr, Ellington, & Lambowitz, 2014; Zimmerly & Semper, 2015).

The group II intron system was repurposed as a genome editing tool known as Targetron and has been used to perform targeted genetic manipulation in *Chlamydia* (Johnson & Fisher, 2013). Targetron was first used to perform inactivation of IncA an inclusion membrane protein required for homotypic fusion of *Chlamydia* inclusions (Johnson & Fisher, 2013; Suchland, Rockey, Bannantine, & Stamm, 2000). Another

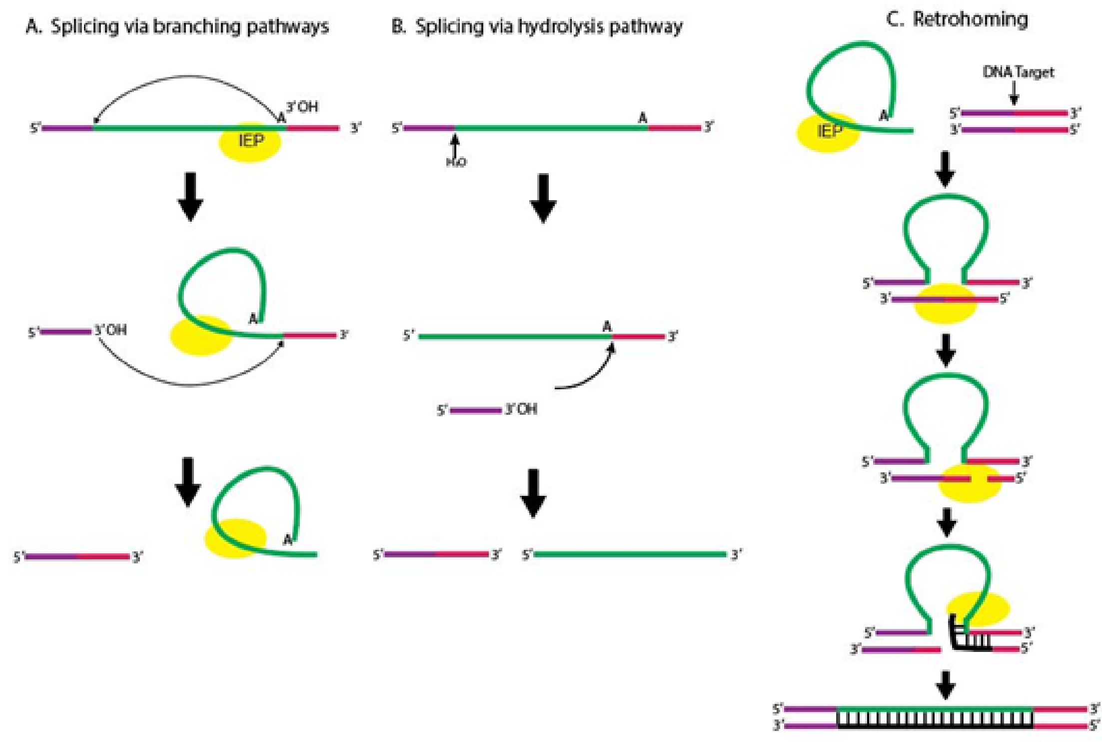


Figure 1.4. Group II intron splicing and retrohoming. **A)** Intron-encoded protein (IEP) facilitated splicing **B)** *In vitro* self-splicing of the intron that is independent of the IEP **C)** Group II intron insertion and reverse transcription into a target site (retrohoming)

study used Targetron for inactivation of CT228, which resulted in shift to extrusion as a means of exit for *C. trachomatis* as opposed to lysis of the host cell. This phenotype was attributed to myosin phosphatase (MYPT1) recruitment to the inclusion. Extrusion was further demonstrated to elongate the duration of *C. trachomatis* infection *in vivo* due to delay in detection of the infection by the immune system (Shaw et al., 2018). The feasibility of Targetron as tool for developing gene knockouts in *Chlamydia* was further

demonstrated by another group which used Targetron to knockout *rsbV1* gene. The knockout of this gene which codes for an anti-anti-sigma factor that is associated with σ^{66}regulation, resulted in the repression of σ^{66}-dependent genes transcription (Thompson et al., 2015).

Fluorescence Reported Allelic Exchange Mutagenesis

Fluorescence Reported Allelic exchange (FRAEM) is a gene deletion technique that relies on replacement of a gene sequence with a selection marker such as antibiotic resistance genes and a fluorescent protein gene. The replacement is achieved by homologous recombination of the gene on the chromosome with DNA sequence on a plasmid that contains the cassette flanked with homology arms for the exchange of nucleotides between the plasmid and genome (**Fig. 1.5**) (Binet & Maurelli, 2009; Mueller, Wolf, & Fields, 2016). In one study, this system was used to delete 4 genes in *C. trachomatis*. Tryptophan Synthase Alpha Chain (*trpA*) deletion by this system resulted in inability of the mutant strain to synthesize tryptophan from indole. In this study, they were also able to delete effectors transported by the type III secretion system (i.e., CTL0063, CTL0064, and CTL0065) (Mueller et al., 2016).

Targetrons and FRAEM are important genetic tools, but delivery of these systems is limited by low transformation efficiencies. In addition, identifying target sequence for the group II intron insertion can be challenging and integration is inefficient in some locations (Bastidas & Valdivia, 2016; Johnson & Fisher, 2013). FRAEM on the other

hand, requires large amounts of DNA of up to 20ug and recombination events happened at a frequency of 10^{-6} (Binet & Maurelli, 2009). As a result, these methods have not been widely adopted. To date, roughly eighteen genes have been inactivated/deleted by using either Targetron or FRAEM (Bastidas & Valdivia, 2016; Johnson & Fisher, 2013; Mueller et al., 2016, 2017; Shaw et al., 2018; Weber et al., 2017)

Manipulating the Human Host to Understand *Chlamydia* Biology

Due to the inherent difficulty in manipulating *Chlamydia* cells, there have been ongoing efforts to dissect *Chlamydia* pathogenesis by studying host factors. A gene trap, loss of function screen, was performed on human haploid-cells host factors necessary for *C. trachomatis* replication. Genes identified in this screen include; *B3GAT3*, *B4GALT7*, and *SLC35B2*, which encode glucuronosyltransferase I, galactosyltransferase I, and the 3′-phosphoadenosine 5′-phosphosulfate (PAPS) transporter 1 (PAPST1), respectively. Each of these genes are involved in host cell sulfation and are critical for attachment and entry (Rosmarin et al., 2012). A genome wide knockdown study performed using RNA-interference (RNAi) in drosophila cells showed that *C. caviae* infection requires the mitochondrial Tom complex (Derre, Pypaert, Dautry-Varsat, & Agaisse, 2007). Another RNAi-based screen showed that the MEK-ERK pathway is necessary for *C. trachomatis* replication (R. K. Gurumurthy et al., 2010). Recently, a FACS-based genome-wide knockout screen performed using CRISPR-Cas9, revealed the requirement of Coatomer Complex I (COPI) in *Chlamydia trachomatis* invasion (J. S. Park et al., 2019). Overall all, these 4 studies have revealed some signification insight on host factors associated

with *Chlamydia* infection but do not provide direct identification and interrogation of the bacterial factors involved in *Chlamydia* pathogenesis.

CRISPR-Cas9 as a Genome Editing Tool

Cas9 is an RNA guided nuclease that protect bacteria and archaea from infection by invading genetic elements (**Fig. 1.6A**). This nuclease has recently been repurposed for sequence specific gene editing in a wide variety of cell types and multi-cellular organisms (**Fig. 1.6B**) (Ding, Li, Chen, & Xie, 2016; Wilkinson & Wiedenheft, 2014).

There are more than 32 CRISPR system subtypes reported to date and CRISPR-Cas9 is the most used type for genetic engineering of all the different types (Koonin, Makarova, & Zhang, 2017). Cas9 specificity and programmability relies on the sgRNA complementary base paring to target sequence (protospacer) and the presence of a Protospacer Adjacent Motif (PAM) (Kim et al., 2017; Wilkinson & Wiedenheft, 2014). As a genetic engineering tool, all the CRISPR-Cas9 nuclease does, is introduce a double-stranded DNA break at a specified sequence. Changes to the target sequence are introduced by the cellular DNA repair machinery (Gori et al., 2015). Double-stranded breaks (DBS) introduced by CRISPR-Cas9 can be repaired by one of two major pathways; Non-Homologous End Joining (NHEJ) or Homologous Recombination (HR). There is a third pathway known as Microhomology-Mediated End Joining (MMEJ). This system is sometimes referred to as alternative NHEJ because is less active and usually suppressed by NHEJ factors (Chiruvella, Liang, & Wilson, 2013). In this review I will focus on NHEJ and HR (Chiruvella et al., 2013).

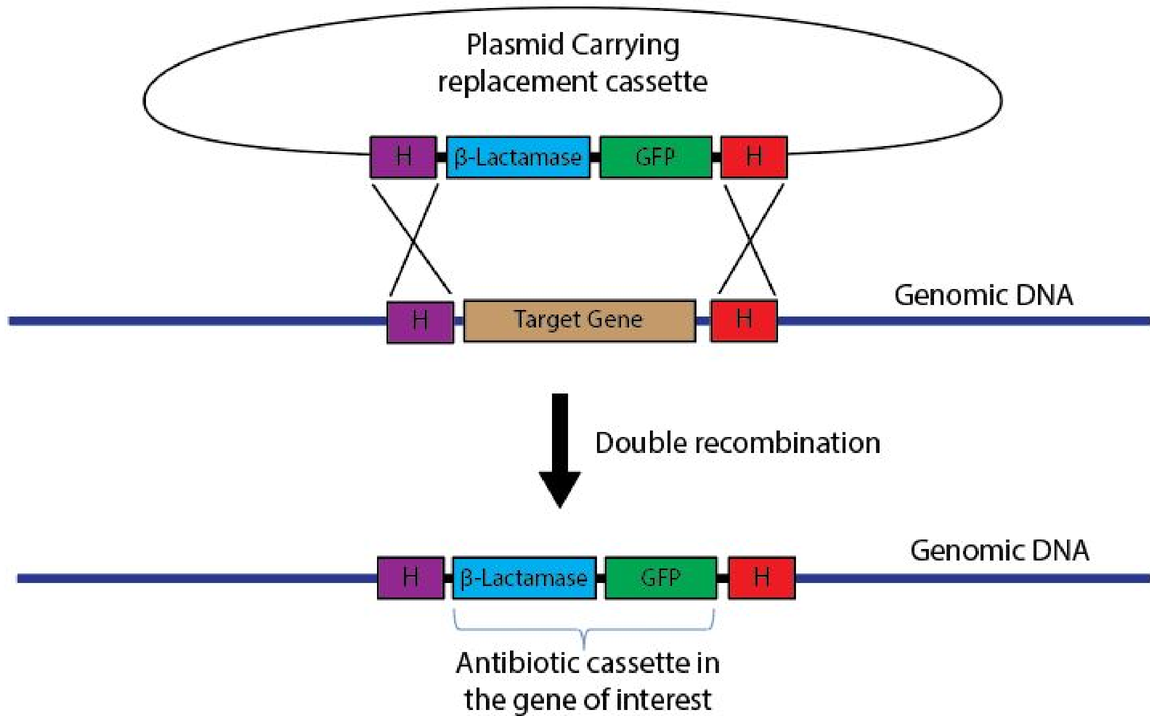


Figure 1.5. Gene deletion by allelic exchange**.** A suicide plasmid carrying a cassette with antibiotic and fluorescent protein genes flanked by two homology arms double recombines with genomic DNA replacing the target gene. The Antibiotic resistance gene is used for selection and the fluorescent proteins allows for easy observation of recombinants mutants.

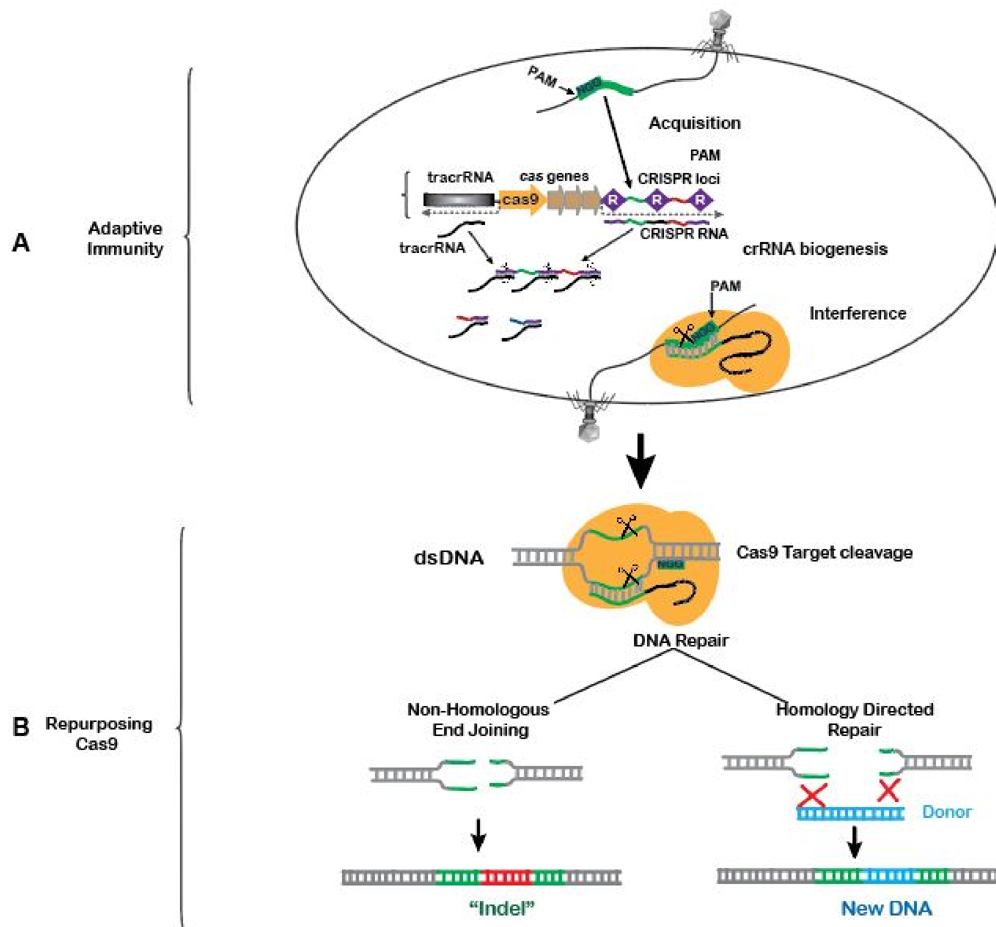


Figure 1.6. Repurposing the CRISPR-Cas immune system for applications in genome engineering. **A)** CRISPR immunity functions in three stages, acquisition of foreign DNA, CRISPR RNA (crRNA) biogenesis, and target interference. First, Bacteria acquire resistance against foreign nucleic acid by cleaving and integrating short fragments of foreign nucleic acid (called protospacers) into CRISPR loci. Protospacers are adjacent a protospacer adjacent motif (PAM). The CRISPR loci consist of a series of short repeats (R, purple diamonds) and unique spacers (red and green lines). CRISPR loci are transcribed and the RNA is processed into a library of small CRISPR-derived RNAs (crRNAs) that are recognized and associate with Cas9. crRNA loaded Cas9 recognize, binds and cleaves dsDNA target using its two nuclease domains. **B)** Cas9 targeting relies on PAM recognition and base pairing between the crRNA and the target DNA. The Cas9 nuclease can be programmed to target any DNA sequence with an adjacent PAM by designing a crRNA complementary to the target sequence. Genomic double-stranded DNA breaks are repaired by the error-prone non-homologous end joining (NHEJ) which cause indels and disrupts genes or homology directed repair (HDR) which relies on a

NHEJ is an error-prone pathway that efficiently joins of two DNA ends of a double-stranded DNA break without the use of a homologous template (**Fig. 1.7A**). NHEJ usually results in non-templated insertions or deletions (indels), which frequently result in frameshift mutations. In mammalian cells, NHEJ predominates in G_0 and G_1 during the cell cycle. Several proteins have been shown to be involved in NHEJ pathway (**Fig. 1.7A**). First is the DNA-dependent protein kinase (DNA-PK), this is composed of a large catalytic subunit and Ku which is a protein with high affinity to double-stranded ends of DNA. The second component is a complex of proteins called XRCC4 required during ligation where it is thought to facilitate alignment or gap filling right before ligation. Third component is the DNA ligase IV, an ATP-depend DNA ligase which exist in complex with XRCC4 and is responsible for ligation of the DNA ends (Chiruvella et al., 2013; Lees-Miller & Meek, 2003).

While the NHEJ machinery is found in some prokaryotic genomes, it mainly operates in eukaryotes and is primarily used as a backup in prokaryotes (Dudas & Chovanec, 2004).

Homologous Recombination

Homologous Recombination (HR) relies on a homologous template to repair DNA breaks (**Fig. 1.7B**). HR provides mechanism to repair DNA in a programmable fashion that allows for specific DNA editing through the supply of a donor template with the desired changes (Altenbuchner, 2016; Ding et al., 2016; Jiang, Bikard, Cox, Zhang, & Marraffini, 2013). HR has been demonstrated as the primary pathway in DSB in

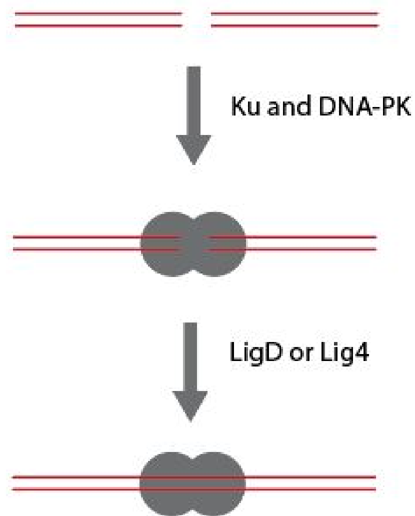
prokaryotes (Altenbuchner, 2016; Dudas & Chovanec, 2004). This system has well been studied in *E. coli*, which uses the RecBCD pathway to perform this process (**Fig. 1.7B**). The first step in HDR involves RecBCD protein complexes which recognizes and process the break to produce linear single-stranded DNA. This is followed by complementary pairing of the produced single stranded DNA with the repair template (homologous DNA template) a process which facilitated by a complex of single-stranded DNA binding protein (SSB), RecA, RecF, RecO, and RecR proteins. The RuvAB facilitates the formation of holiday junction and branch migration, followed by resolution of junctions by the endonuclease RuvC. Branch migration and junction resolution can also be performed by RecG.

In eukaryotes, HR plays slightly different roles between lower and higher eukaryotes. In *S. cerevisiae*, HR is the preferred DSB repair pathway, while NHEJ plays a minor role. Several genes have been implicated in HR, including RAD51 and RAD 52 gene families (Dudas & Chovanec, 2004). It has also been shown that, the breast cancer susceptibility proteins, Brca1 and Brca2 are involved in the HR process in mammalian cells where they play a role as recombination mediators (Wardell et al., 2017). HR in eukaryotic operates during meiosis leading to crossover and exchange of genetic material that results in variation and adaptation of the offspring. HRa is also involved in restoration of somatic cell genomic stability following DNA lesions such as double-stranded breaks (Amunugama & Fishel, 2012).

While the achaea HR pathway is different from both bacterial and eukaryotic pathways, it is more similar to the eukaryotic pathway. Strand exchange in bacteria and

eukaryotes is mediated by RecA-family recombinases and Rad51/Dmc1 respectively, while RadA is the archaeal homology for this process. Bacteria use single strand DNA binding protein (SSB) to bind and protect resected single strand DNA ends but eukaryotes and most archaea use replication protein A (RPA). Recombination mediators assist in recombinase nucleoprotein filament formation by overcoming the inhibition imposed by SSB/RPA. Some of bacterial recombination mediators that facilitate recombinase nucleoprotein filament (RecA ssDNA complex) during HR include RecF RecX and DinI. In humans this process is accomplished by BRCA2 while yeast use

A. Bacterial Non-Homolgous End Joining



B. Homologous Recombination

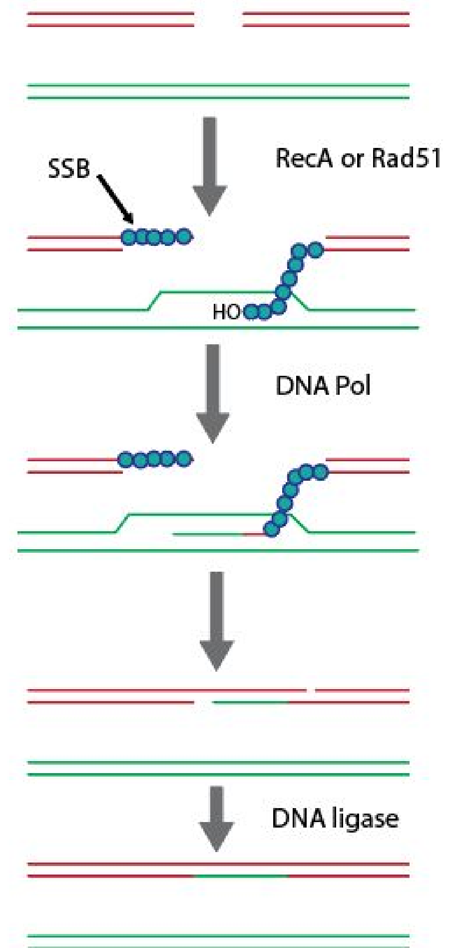


Figure 1.7. Mechanisms of repairing double-stranded DNA breaks. **A)** Non-homologous end joining (NHEJ) Protein Ku (depicted as a pair of green spheres) brings together the ends of double-stranded DNA, which are then then covalently joined by DNA ligase D (LigD) in bacteria and Lig4 in eukarya. **B)** During homologous recombination (HR), the DNA duplex is resected by a 5′ to 3′ exonuclease. The resulting 3′-OH single-stranded extension invade a homologous sister chromatid (green) facilitated by RecA (or eukaryal Rad51) protein. The invading strand is used as a primer for a DNA polymerase that copies the template chromatid information across the break. The recombination intermediates are resolved and the residual single-strand nicks in the repaired duplex are eventually sealed by DNA ligase.

identified; Ral1, which is found in crenarchaeota and RadB, found in members of the phylum Euryarchaeota (Wardell et al., 2017).

DNA Repair in *Chlamydia*

Genomic studies have shown that *Chlamydia* lack in their genomes the genes necessary for NHEJ (Bastidas & Valdivia, 2016), but carries homologs of all the genes necessary for HR based repair of DNA DSB cleavage (CameriniOtero & Hsieh, 1995; Dudas & Chovanec, 2004). *Chlamydia* genome carries all the gene homologs for the RecBCD pathway (Bastidas & Valdivia, 2016). These findings provide evidence for the possibility of application of CRISPR-Cas system as genome editing tool for *Chlamydia* spp. The ability to use the HR pathway in repairing DSB introduced by the CRISPR-Cas will not just enable gene knockouts but gene editing which might be crucial for studying essential genes in *Chlamydia* through generation of hypomorphic mutations (Bastidas & Valdivia, 2016).

CRISPR-Cas9 Versality for engineering the *Chlamydia* genome

CRISPR-Cas9 system has several advantages over previously developed methods of editing. The first advantage is that the RNA-guides are easy to design and inexpensive to synthesize. While targets are restricted to regions adjacent to a PAM, different Cas9 orthologs use a wide variety of different PAMs which increase the flexibility of this system to target virtually any sequence by design. CRISPR-Cas9 doesn't just produce knockouts, the ability to edit genes may allow for development of hypomorphic mutation

(reduced activity of the gene). The wildtype Cas9 cleaves both strands of a DNA using a RuvC and HNH nuclease domains. Mutations in both RuvC and HNH active sites render the enzyme catalytically dead (dCas9). Nuclease dead Cas9s still bind the DNA target, and these programable DNA binding proteins have been used to deliver transcriptional activators and repressors. Alternatively, a mutation in only one of the two active sites result in nickase activity (i.e., cleaving only one strand of the duplex). Cas9 nickases have been used to improve target specific, to create overhangs that facilitate repair by HR, and for the delivery of base editing enzymes that are more efficient on nicked substrates (Wilkinson & Wiedenheft, 2014).

In *Chlamydia*, the wildtype Cas9 from Streptococcus pyogenes is toxic (Ouellette, 2018). The toxicity has been attributed to leaky expression of Cas9 from the tetracycline inducible promoter and possible off-target inhibition of expression of essential gene(s). To overcome this limitation Ouellete et al recently reported using catalytically dead *Staphylococcus aureus* Cas9 (dSaCas9) to conditionally knockdown IncA, an inclusion protein required for homotypic fusion of *Chlamydia* inclusions (Ouellette, 2018). The use of dCas9 for knockdowns, rather than knockouts, will also provide another advantage in studying *Chlamydia* since many genes in *Chlamydia* are expected to be essential for *Chlamydia* viability (Ding et al., 2016; Elwell et al., 2016). The availability of inducible promoter for *Chlamydia* vectors just adds to the versality of this system. Genes editing/knockdown can be induced at any desirable point during the lifecycle of the bacteria.

Future Directions

Despite the significant health burden caused by *Chlamydia* species, several aspects about the *Chlamydia* lifecycle remain enigmatic. The *Chlamydia* lifecycle and reduced genome pose two significant barriers for the development of a facile genetic manipulation system (Bastidas & Valdivia, 2016). Current transformation methods have low efficiencies and consequentially, this has made development of a facile genetic tools for *Chlamydia* genome engineering a challenging task (Ouellette, 2018). Some progress has been made in recent years, the use of chemical mutagenesis. Fluorescent Reported Allelic Exchange Mutagenesis (FRAEM) and Targetron have all contributed significantly to the genetic dissection of these parasites (Johnson & Fisher, 2013; Kari et al., 2011; Mueller et al., 2016; Shaw et al., 2018). Since most genes in *Chlamydia* are expected to be essential, we anticipate that most CRISPR-Cas9 mediated gene deletion will be toxic. However, double stranded DNA breaks may enhance homologous direct repair and thus CRISPR-Cas9 might be used in combination with DNA donors to make site specific mutations, rather than gene deletions. In addition, Cas9 orthologs have been proposed to have distinct activities (i.e. RNA-guided RNA targeting) (Dugar et al., 2018). Theses Cas9 ortholog may provide an alternative tool to study essential genes in *Chlamydia* by circumventing the toxic effect of complete gene knockouts. Moreover, the use of nuclease inactive Cas9 mutants provide a method for targeted gene repression by blocking transcription (Ouellette, 2018). While these and other genetic engineering tools may accelerate our understanding of Chlymidia biology, the real bottleneck for genetics continues to be an efficient transformation system.

Acknowledgments

E.J.K. B.W wrote this review as part of E.J.K's thesis project. Research in the Wiedenheft lab is supported by the National Institutes of Health (P20GM103500, P30GM110732-03, R01GM110270, R21AI130670-02, and R01GM108888), the M. J. Murdock Charitable Trust, a young investigator award from Amgen, Montana University System Research Initiative, Gordon and Betty Moore Foundation, and the Montana State University Agricultural Experimental Station.

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CHAPTER TWO

DEVELOPMENT OF A CRISPR-CAS9 GENOME EDITING TOOL FOR *CHLAMYDIA TRACHOMATIS*

Abstract

Chlamydia trachomatis is a medically important obligate intracellular parasite. The intracellular lifestyle has made genetic manipulation of these parasites challenging and we currently lack a facile and versatile to perform genetic engineering of Chlamydia. Here we set out to repurpose the Cas9 nuclease for genome engineering in C. trachomatis. To achieve this goal, we designed a series of dual expression vectors containing the cas9 gene from Streptococcus pyogenes, an antibiotic selection cassette, and a fluorescent reporter. Repeated efforts to transform these plasmids using various protocols failed, so we designed a new plasmid containing a smaller cas9 gene from Campylobacter jejuni. The progress and next steps for implementing CjCas9 for sequence specific editing of the C. trachomatis genome are discussed.

Introduction

Chlamydia trachomatis is an obligate intracellular parasite (Bachmann et al., 2014). *C. trachomatis* causes a range of serovar-specific infections in humans ranging from blinding trachoma caused by serovars A-C, sexually transmitted infections caused D-K, and the systemic lymphogranuloma venereum (LGV) caused by serotype L1-L3. (Elwell et al., 2016; Sixt & Valdivia, 2016). According to the CDC, more than 1.5 million new cases of *C. trachomatis* infections were reported in the United State alone in 2016, while the World Health Organization (WHO) reported around 131 million new cases worldwide in the same year making it number 1 sexually transmitted bacterial infection worldwide (Centers for Disease, 2017). *Chlamydia* have a unique biphasic lifecycle that involves two alternating forms. The infection starts with attachment of the infectious but non-replicating form of the bacteria, called an Elementary Body (EB). Upon internalization, the EB resides in a vacuole-like structure termed an inclusion. The inclusion is composed of the host cell membrane and modified by inclusion proteins (Incs) (Elwell et al., 2016). Six to eight hours post infection, the EB's transition into the vegetative form termed as the reticulate Bodies (RB). This non-infectious form then multiplies through binary fission until the inclusion fills up the entire cell. At around 30 hrs post infection, the RBs start to differentiate back to EBs and infected cells typically lysis 46 to 72 hrs post infection (Bastidas & Valdivia, 2016; Nguyen & Valdivia, 2012).

This unique lifecycle has presented a major challenge for development of genetic methods in *Chlamydia*. The EB has a tough cell wall and a low metabolic rate, both of which render them intractable by many of common manipulation techniques. RBs, on the

other hand, are metabolically more active and less resistant to manipulation compared to the EB, but RBs are not infectious so manipulated cells cannot be propagated (Bastidas & Valdivia, 2016; Sixt & Valdivia, 2016). These features have restricted development of efficient methods for genetic manipulation. In addition, *Chlamydia* also have significantly reduced genomes (Elwell et al., 2016). The genome reduction in *Chlamydia* is reflective of their intracellular lifestyle which has resulted in dependency of *Chlamydia* on their hosts for various metabolic needs. With reduced genome, most genes in *Chlamydia* genomes are expected to be essential which makes it difficult to study *Chlamydia* factors involved in pathogen-host interactions using knockouts (Ding et al., 2016; Elwell et al., 2016).

Despite the substantial health and economic impact of *Chlamydia* infections, our understanding of *Chlamydia* lifecycle and pathogen-host interactions remains limited (Kokes et al., 2015). We still don't know the mechanism by which *C. trachomatis* evades different host defense mechanisms. To date, no effective vaccine against *Chlamydia* has been developed and this can largely be attributed to our limited understanding of the *Chlamydia* lifecycle (Elwell et al., 2016). Genetic manipulation of *Chlamydia* have relied chemical mutagenesis coupled to forward and reverse genetics (Kari et al., 2011; Kokes et al., 2015; Nguyen & Valdivia, 2012). The random nature of mutations introduced by chemical mutagenesis and the presence of multiple mutations on the same cell, make this approach costly, labor intensive, and time consuming (Kari et al., 2011). Recent advances have enabled targeted mutations in *Chlamydia* using fluorescence reported allelic exchange (FRAEM) and group II intron (Targetron) based techniques (Johnson & Fisher,

2013; Mueller et al., 2016). Despite this significant progress, there are challenges associated with these approaches.

Targetron relies on group II introns and finding a target sequences for efficient insertional inactivation of genes can be a challenging (Bastidas & Valdivia, 2016; Johnson & Fisher, 2013). Alternatively, FRAEM requires large amounts of DNA and recombination events have been reported to be very low. As a result, there has not been an extensive application for these systems in *Chlamydia* genome engineering. (Bastidas & Valdivia, 2016; Johnson & Fisher, 2013; Mueller et al., 2016, 2017; Shaw et al., 2018). Since many genes in *Chlamydia* are expected to be essential, there is need for a tool that will not only enable gene knockouts but also generation of hypomorphic mutations and targeted gene knockdown that will enable the manipulation essential genes in *Chlamydia* (Kari et al., 2011).

CRISPR is an abbreviation for Clusters of Regularly Interspaced Short Palindromic Repeats. CRISPRs are part of an adaptive defense mechanism in prokaryotes (Altenbuchner, 2016). They rely on RNA-guided endonucleases for targeted degradation of foreign nucleic acids (Altenbuchner, 2016). These systems have been repurposed for genome engineering in a wide variety of different organisms (Feng et al., 2013; Jiang et al., 2013; Kistler, Vosshall, & Matthews, 2015; Mali, Yang, et al., 2013). The most frequently used enzyme used for genome engineering is CRISPR-associated protein 9 (Cas9). Different variants of Cas9 have been used for applications such as generation of gene knockouts, knockdowns, knock-ups, knock-ins and base modifications (Bikard et al., 2013; Ding et al., 2016; Ouellette, 2018).

Here we report efforts to develop a CRISPR-Cas9 for site specific gene editing in *C. trachomatis*. To achieve this goal, we designed a plasmid containing the *Streptococcus pyogenes* Cas9 (SpyCas9) endonuclease, single guide RNA (sgRNA), and a DNA template for repairing of the DNA break introduced by Cas9. The plasmid also contains a β-lactamase gene for selection of transformants and an mCherry gene for conducive observation of transformants. This system was designed to perform gene editing by converting mClover encoding gene to Cyan Fluorescent Protein (CFP) encoding gene (**Fig. 2.1**). However, we were unable to transform *C. trachomatis* these plasmids. We created another plasmid carrying a smaller Cas9 ortholog from *Campylobacter jejuni*, which was successfully delivered into *C. trachomatis* and we discuss possible application for this smaller Cas9 for Chlamydia genome engineering.

Material and Method

Plasmid Constructions

First, the TetR-CPAF region was cut from the pBomb vector by restriction digestion with BamHI and SalI (New England Biolabs). This fragment was cloned into pUC19 by ligation using T4 DNA ligase (In house made) by using the BamHI and SalI restriction sites. Then performed a mutagenesis PCR to remove the CPAF gene from the pUC19 using primers RW_19July16_1 (tctactagtggttaaccgcggtag**GTCGAC**ctgcaggca) and RW_19July16_2 (cttggccggccttgaccggtcatcta**GCGGCCGC**agatc) adding SalI and NotI restriction sites respective (Capitalized and bolded letters)The *cas9* gene from *Streptococcus pyogenes* was PCR amplified from the plasmid pMJ806 (Addgene 39312),

using primers that were designed to add a 5' NotI and a 3'AgeI restriction sites Primer RW_27July16_1 (ctagtt**GCGGCCGC**tcgatggataagaaatactcaataggcttag) and primer RW_27July16_2 (ctg**ACCGGT**ttagtcacctcctagctgactca). Used Q5 polymerase (NewEngland Biolabs), PCR conditions; Ta=65C (20sec), extension 72C (2min), 25 cycles. The PCR product was then ligated using T4 DNA ligase (in house made) into pUC19 shuttle vector using AgeI and NotI (New England Biolabs). The gel purified vector was mixed with the Cas9 insert at ratio of 2.5:1 ratio insert:vector and T4 DNA ligase then incubated at room temperature for 1h. Then Cas9 variants were cloned into the pBomb4R-Tet-mCherry vector (a kind gift from Dr Grieshaber at university of Idaho) by restriction endonuclease digestion of the Cas9 genes from pUC19 and ligation into the pBomb4R vector using BamHI and SalI (Vector and insert were gel purified then ligate at 3:1 ratio of insert:vector, at RT 1h using T4 DNA ligase. Three Different sgRNAs targeting the mClover encoding gene at different places and two donor DNA template of different homology arm lengths were cloned into the multiple cloning site of the pUC19 vector. The cassettes carrying the sgRNAs under the IhtA promoter and donor templates were ordered as geneblock. These cassettes were then cloned into the pBomb4R-Tet-mCherry carrying Cas9 gene by restriction digest and ligation. Using this approach, we created 12 plasmids carrying either Cas9-WT or Cas9n, three different sgRNAs and three different donor templates. These plasmids were used in *C. trachomatis* transformations. The plasmid p259 (pBomb4R carrying wildtype *Campylobacter jejuni* cas9) was created by ligation of the *Cj*Cas9-WT in place of the *Spy*Cas9 gene by using the AgeI and NotI sites. The *Cj*Cas9 gene was PCR amplified from the pET-*Cj*Cas9 vector purchased from

Addgene while adding the NotI and AgeI restriction enzyme sites at both ends of the gene.

Plasmid Preparation

Plasmids were prepared in a dam-/dcm- K12 strain of *E. coli* from New England Biolabs. The K12 cells were transformed with the different plasmids by heat-shock and then grown in Lysogenic Broth (LB) medium (Fisher Bioreagent, Cat no: BP9722-500) supplemented with 1 I.U./mL of penicillin for selection and maintenance of the plasmid. The cultures were incubated overnight at 37°C in a shaker. Plasmid DNA was extracted the following day using the Miniprep kit from Qiagen. Plasmid DNA was concentrated to 1ug/ul before transformation into *C. trachomatis*.

Cell culture, *C. trachomatis* transformation and propagation

C. trachomatis L2 (LGV 434/Bu) cells were a kind gift from Dr. Scot Grieshaber at the University of Idaho. *C. trachomatis* L2 cells were propagation in Cos7 cells. The Cos7 cells were cultured in RPMI medium (Corning, Cat no: 10-040-CV) at 37^0C and 5% CO_2, the medium was supplemented with 10% Fetal Bovine Serum (Atlas Biologicals, Cat no: F-0500-D) and 10 I.U./mL Penicillin and 100 µg/mL Penicillin and Streptomycin for *C. trachomatis* transformants isolation and Cos7 cells propagation respectively.

We followed the Mueller protocol for *C. trachomatis* transformation. Briefly, *C. trachomatis* cells were transformed with unmethylated DNA by CaCl2 based chemical

transformation (Mueller et al., 2017). A day before the transformation, a monolayer of Cos7 in a 6-well plate was prepared by plating 5×10^5 to 6×10^5 cells/well. The transformation was performed in six replicates per plasmid. 2.5×10^6 inclusion forming units *C. trachomatis* serovar L2 was added to 50ul of Tris-CaCl2 buffer (0.5g/mL) then 2ug of unmethylated plasmid DNA was added into this mixture and incubated for 30 min at room temperature. After the incubation, 1ml of Hank's Balanced Salt Solution (HBSS, Corning) was added into each of the tubes containing the transformation mixture. The medium was removed from the plates prepared the day before, then the cells were washed with PBS and the transformation mixtures were added on to the cells followed by an addition 1ml of HBSS. The infection was performed by spinning the plates for 1hour at 900×g and 20°C. After the centrifugation the transformation mixture is removed from the plates and 2ml of antibiotic-free RPMI supplemented with 10% FBS media is added and the cells area incubated for 8 hours before addition of medium containing 1 I.U./mL of penicillin for selection of transformants. At 48 hours post infection, the infected cells were harvested by scraping, centrifuged at 20 k ×g for 30 min at 4°C, and resuspended in 1mL of HBSS. The resuspended mixture was then centrifuged for 5 min at 200×g and 4°C, and the supernatant was used to infect a new monolayer of Cos7 cells with an additional 1mL of HBSS. This process of infecting and harvesting infected cells was repeated until wild-type inclusions were visible.

Microscopy

Development of *Chlamydia* wildtype inclusion and expression of the encoded fluorescent protein which indicate acquisition of the plasmid was monitored by microscopic examination using the Olympus CKX53 inverted microscope. Plates were examined 20 to 48 hours post infection (HPI) during each passage to check for inclusion development. The cells were imaged using a Nikon TiEclipse inverted fluorescence microscope and the appropriate wells were passaged on for expansion of the observed transformants and subsequent purification of EBs and storage at -80°C

C. trachomatis Purification

C. trachomatis elementary bodies (EBs) were harvested by the renografin-based protocol (Caldwell, Kromhout, & Schachter, 1981). Briefly, *C. trachomatis* were used to infect a monolayer of Hela cells at a multiplicity of infection (MOI) of 5, and this was done in five T-150 flasks. 48 HPI the supernatant was removed from the flasks and 5ml of HBSS was added per flask of infected cells, and cells were scraped and collected into 50 ml conical tubes. The scraped flasks were rinsed with another 5ml of HBSS per flask of infected cells and this was collected into the 50 ml conical tube. The cells were then sonicated in the 50 ml conical tubes three times (20 second each at 500Watts and 20kHz) followed by centrifugation for 15 min at 500xg, 4°C. The supernatant was transferred into a 250 ml autoclaved centrifuge tube and the pellet was resuspended in 25 ml of HBSS per 50 ml conical flask. This was sonicated again three times followed by centrifugation for 15 min at 500xg, 4°C. The supernatant was added into the 250 ml

autoclaved tube above and the pellet was discarded. The supernatants were then aliquoted into 4 centrifuge tubes (40ml) and were spun for 30 min, 30,000xg, 4°C, the supernatants were discarded, and the pellet was resuspended serially in 15ml of sterile SPG (3ml per T-150 flask) using a 18G syringe (Blunt needle) and the resuspended pellet collected into a 15ml conical tube. The cell suspension was sonicated again 3 times and kept on ice, 8ml of 30% renografin solution was added into ultracentrifuge tubes (for 5 T-150 flask you need 2 tubes) followed by 7.5 ml of the cell suspension then the tubes were topped off with SPG. This was then centrifuged for 30 min, 40,000xg, 4°C with brakes off. Then the ultracentrifuge tubes were cut in half and the supernatant was decanted and the pellets were resuspended with 1ml SPG/T-150 flask using 18G syringe (2.5ml per tube). The suspension was kept on ice while being aliquoted into 1.5 ml tubes (100 to 200 ul per tube) then stored at -80°C

Results

Demonstration of mClover to CFP editing in HEK 293 Cells

Fluorescent proteins are routinely used as simple visual reports for diverse biological activities. Here we set out to convert one fluorescent protein (enhanced Green Fluorescent Protein) to another (Cyan Fluorescent Protein) as a way to measure the efficiency of our gene editing methods (**Fig. 2.1**). The amino acid sequence of enhanced Green fluorescent protein eGFP and Cyan Fluorescent Protein (CFP) are 98.33% identical. Switching only oneresidue (i.e., Y66W) converts the emission spectrum of eGFP from an emission peak at 507 nm to CFP, which has an emission peak at 480 nm.

To test the feasibility of our approach, we used two plasmids, pEGFP-N1carrying eGFP encoding gene and another version of pEGFP-N1 with the eGFP gene converted to CFP encoding gene using PCR mutagenesis. We expressed these fluorescent proteins in HEK293 cells using these two plasmids. We detected the expression of these fluorescent proteins on both green and cyan channels. But due to the closeness of the emission wavelengths of eGFP (510 nm) and CFP (480 nm), we observed a high background of the eGFP in the cyan channel (**Fig. 2.2A**). To reduce the spectral overlap, we switched from eGFP (510 nm) to a related green fluorescent protein (mClover) with an emission spectrum that is shifted further right (515 nm). This reduced the mClover background in the cyan channel (**Fig 2.2C**) and the CFP background in the green channel (**Fig. 2.2D**). by

Next, we sought to convert mClover to CFP in HEK293 cells. Switching four amino acids (i.e., G65T, Y66W, A69Q and S72A) converts the emission spectrum of mClover to (515 nm) to 480 nm. These four residues are proximal in the primary structure (located between amino acids 66 to 72), making it possible for four amino acids to be swapped a single recombination event. To achieve this goal, we PCR amplified the mClover gene from a HctAp-mKate2_Ihta-cloverLVA plasmid. This was cloned into the pEGFP-N1 vector and the resulting vector pRAW 219. The pRAW219, purified Cas9 protein complexed with in vitro transcribed sgRNA and a PCR amplified donor template were transfected into HEK293 cells. This experiment was done using a 826 or 1080 nt long donor template for switching four residues (G65T, Y66W, A69Q and S72A).

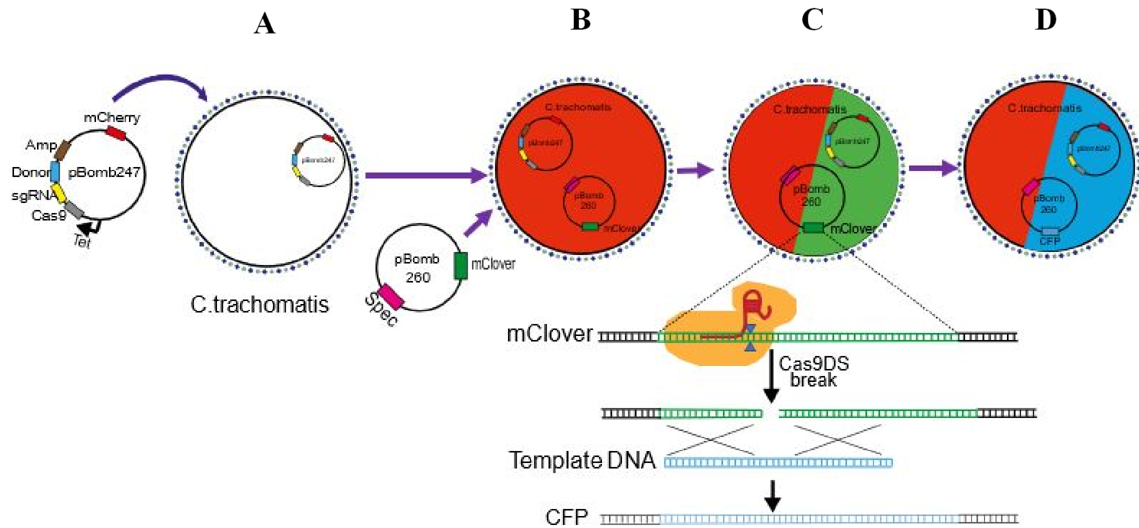

Figure 2.1. Schematic of experiments designed to convert mClover to Cyan Fluorescent Protein **A)** *C. trachomatis* transformation with a plasmid containing CRISPR-Cas9 system and an mCherry encoding gene. **B)** mCherry expressing *C. trachomatis* is transformed with a second plasmid carrying an mClover encoding gene. **C)** Cas9 induction and CRISPR-Cas9 targeting leading to mClover encoding gene editing to CFP encoding gene. **D)** CFP expressing cells indicative of a successful editing of mClover to CFP encoding gene.

Upon transfection, we observed development of CFP expression in some of the cells which is indicative of CRISPR-Ca9 editing of the mClover encoding gene (**Fig 2.3**). Using this system, we tested three different sgRNAs and all seem to facilitate conversion of mClover to CFP in three different population of HEK293 cells.

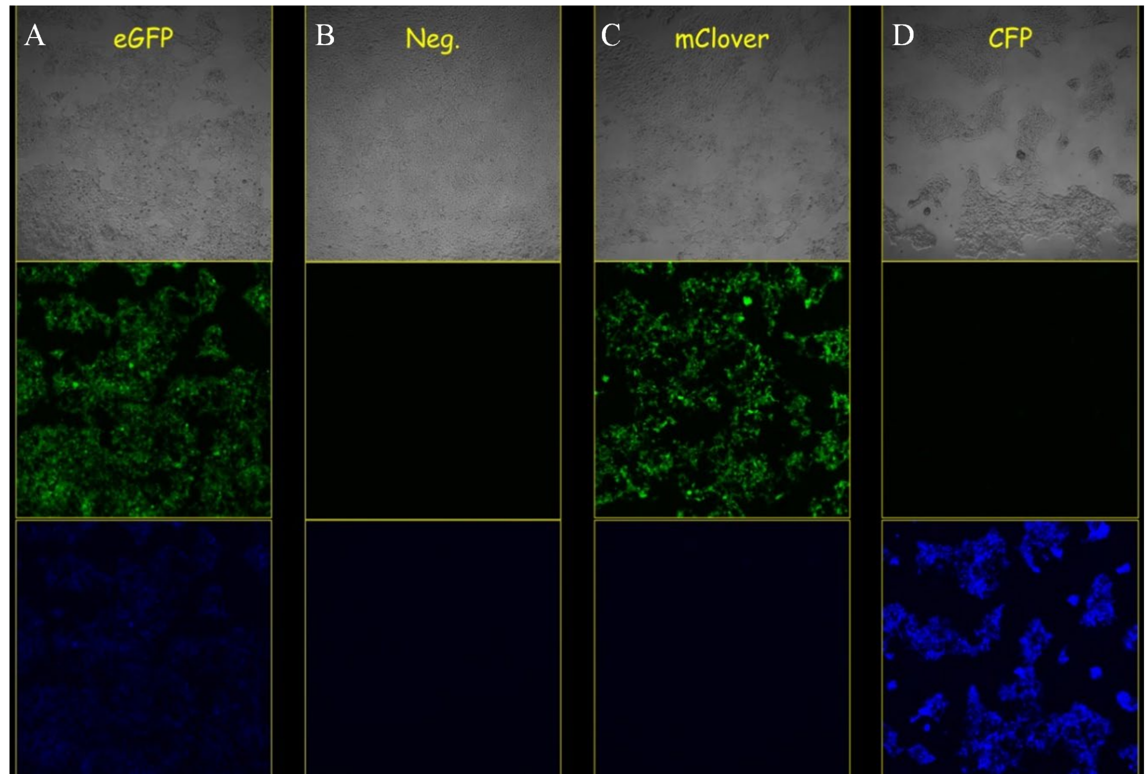

Figure 2.2. CFP and mClover have distinct spectral signatures. Comparison of fluorescence detection in green and blue channels showing the detection of eGFP (Emission wavelength 507nm) in the CFP channel (475nm) and lack of this interference when mClover (Emission wavelength 506nm) is used instead of eGFP. **Column A)** eGFP emission wavelength overlapping with the CFP emission wavelength was significantly detected in the CFP channel. **Column B)** Negative control of non-transfected HEK 293 cells. **Column C)** mClover is well detected in the green channel and no emission

C. trachomatis transformations with plasmid DNA containing the *Streptococcus pyogenes* Cas9 gene.

Next, we sought to perform the editing in *C. trachomatis*. Twelve plasmids were created containing either the wildtype *Streptococcus pyogenes cas9* gene (*Spy*Cas9) or the SpyCas9 nickase (SpyCas9n), which contains a mutation in the RuvC-like nuclease domain and thus only cleave the complimentary strand of the DNA target (Mali, Aach, et

al., 2013). *Spy*Cas9n was created since it has been shown that double-stranded breaks can be toxic in some system such as *Clostridium cellulolyticum* (Xu et al., 2015a*).* This toxicity has been shown to be due to low expression of major components of the Non-homologous End Joining (NHEJ) DNA repair system such as the Ku protein, ATP-dependent DNA ligase and DNA polymerase LigD (Xu et al., 2015b). It has been established that the intrinsic potency of sgRNA targeting different sequencing on the same gene have different impacts on the efficiencies of the guides in producing knockouts (Yuen et al., 2017), for this reason we created plasmids carrying different sgRNAs targeting the mCLover encoding gene at different positions. We also cloned donor template DNA with different homology arms lengths anticipating that this will recombine with the cleaved gene at different efficiencies(Song & Stieger, 2017). However, we were unsuccessful in transforming *C. trachomatis* with these plasmids carrying the wildtype SpyCas9.

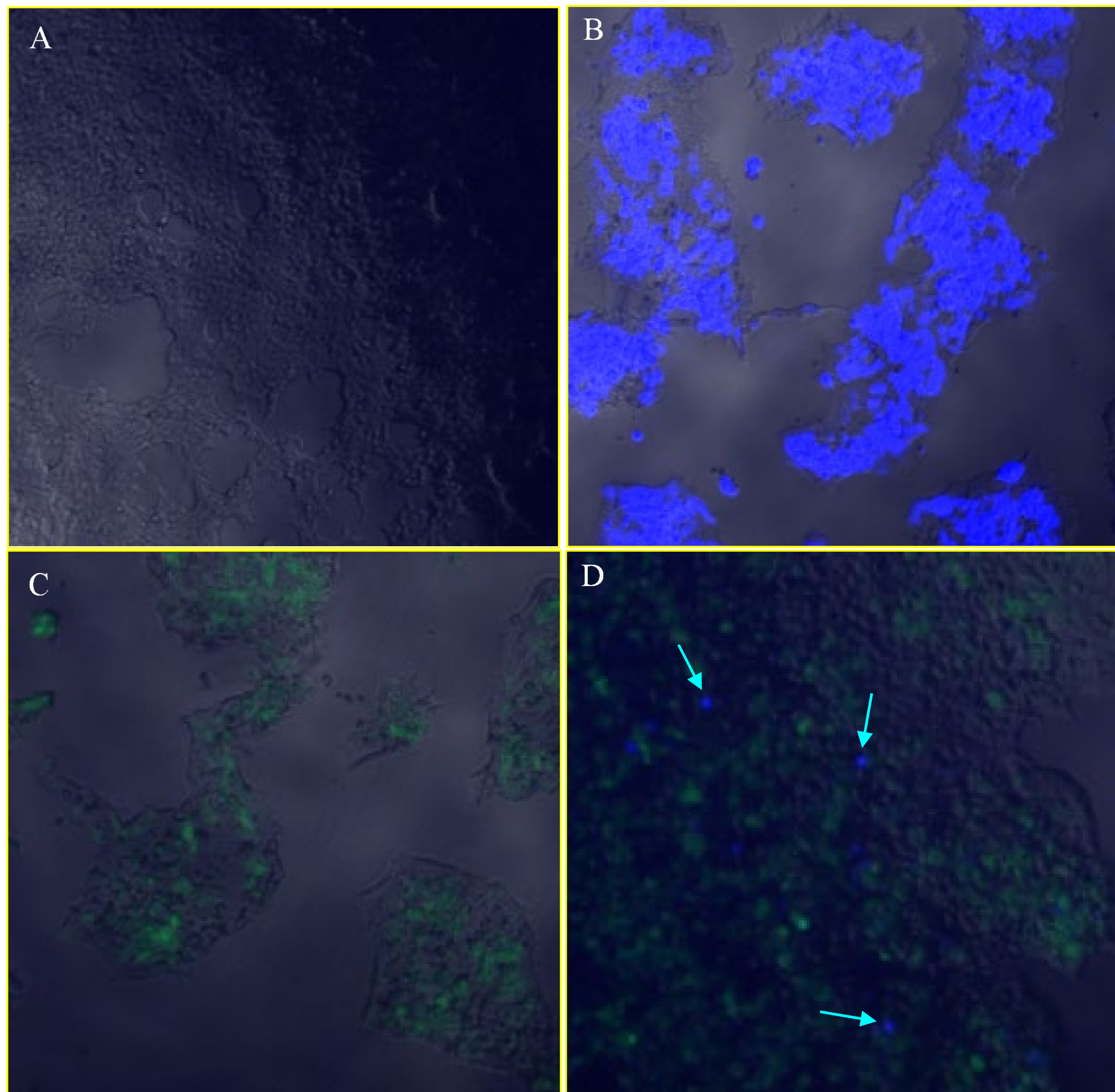

Figure 2.3. Converting mClover to CFP using SpyCas9 and an *in vitro* transcribed sgRNAs **A)** Non-transfected HEK 293 cells **B)** HEK293 cells transfected of a plasmid carrying a CFP encoding gene. **C)** HEK293 cells transfected with mClover construct. **D)** mClover expressing cells with some cells expressing CFP following editing by CRISPR-Cas9 and repair by a provided donor DNA. Cyan arrows indicate CFP expressing cells.

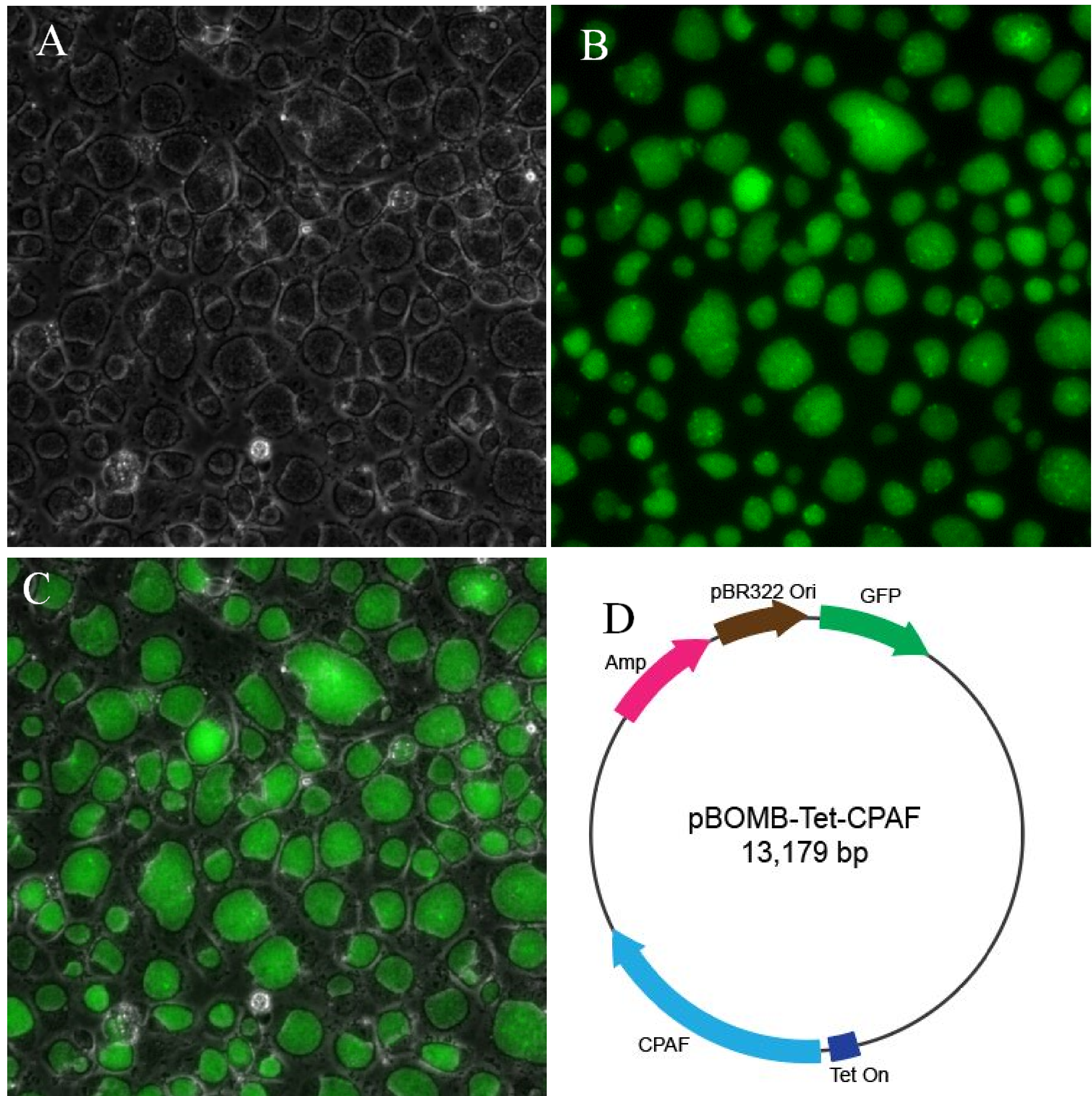

Figure 2.4. Inclusion formation by *C. trachomatis* transformed with a plasmid expressing Green Fluorescent Protein (GFP). **A)** Bright field of Co7 cells infected with *C. trachomatis* with inclusions seen 36HPI. **B)** A green channel of the same field **C)** A merge of the bright field and the green channel.

We hypothesized that the plasmid size either precludes efficient transformation or that leaky expression of Cas9 results in toxicity (Costello et al., 2019; Ryan & Cate, 2014). The inability to transform *C. trachomatis* with wildtype SpyCas9 carrying plasmid was recently reported in a paper by Scot Ouellette (Ouellette, 2018). Based on these data we switched from SpyCas9 to the catalytically dead mutant of *Staphylococcus aureus* Cas9 (dSaCas9). We attempted to transform the EBs with two different plasmid DNA concentration, but neither resulted in successful transformants. While we could not isolate transformants carrying these Cas9-based plasmids, we had confidence in our transformation protocol since we were able to isolate *C. trachomatis* clone that were transformed with a control plasmid (**Fig. 2.4**). Wildtype inclusions was noticeable after the third passage and we considered passage 5 as a good point to determine if the transformation experiments were successful or not.

C. trachomatis Transformation with Campylobacter jejuni Cas9 Gene

We suspected that our inability to transform Chlamydia might be specifically associated with the size of *Spy*Cas9 gene toxicity of the protein. We then hypothesized that *C. trachomatis* could tolerate protein from different Cas9 gene orthologues. Cas9 from *Campylobacter jejuni* (*Cj*Cas9) is 1152bp smaller than SpyCas9 (Kim et al., 2017). We cloned the *Cj*Cas9 into the pBomb4R vector and transformed *C. trachomatis*. After expressing mCherry (**Fig. 2.5**), the presence of the plasmid carrying the *Cj*Cas9 gene was confirmed by a PCR.

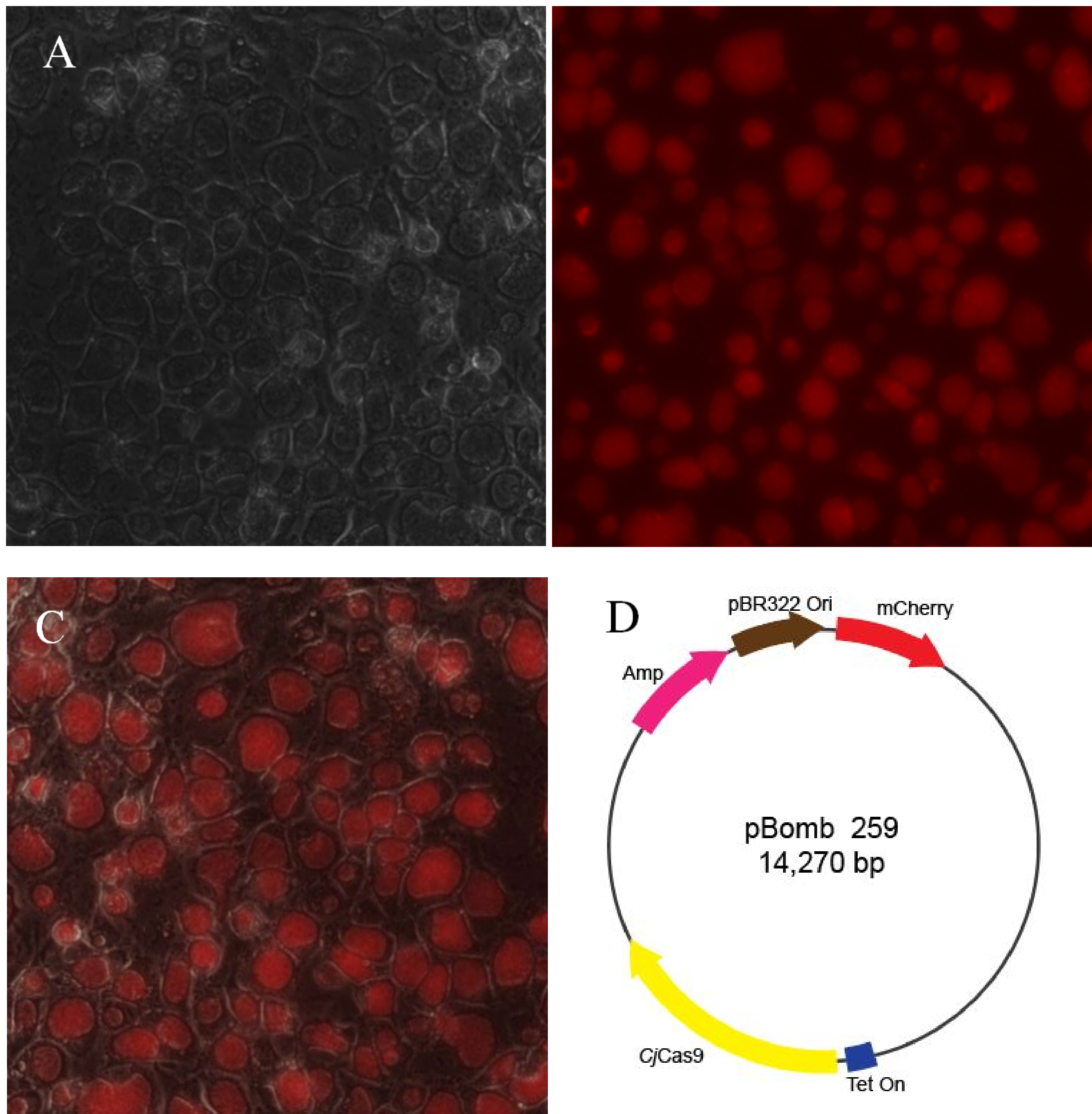

Figure 2.5. mCherry expression of *C. trachomatis* transformed with pBomb 259 plasmid carrying a β-Lactamase, mCherry and *Cj*Cas9 genes. **A)** Bright field of Co7 cells infected with *C. trachomatis* with inclusions seen 36HPI. **B)** A red channel of the same field **C)** A merge of the bright field and the red channel. **D)** Plasmid map of the pBomb 259 vector carrying the *Cj*Cas9

Discussion and Conclusions

Here we attempted to use the *Streptococcus pyogenes* Cas9 (SpyCas9) to perform gene editing in *C. trachomatis,* but we were unable to transform *Chlamydia* with plasmid carrying SpyCas9. Similar results have recently been published (Ouellette, 2018). Based on these data we suspected this could be due to Cas9 toxicity to *Chlamydia*. Genes from the *Chlamydia* indigenous plasmid needs to be included in the plasmid constructs if they are to be stably maintained in *Chlamydia* (Gong, Yang, Lei, Shen, & Zhong, 2013). This results in big constructs of up to 16.5Kb and we suspected this size could also decrease the efficiency of *Chlamydia* transformations. We hypothesized that smaller Cas9 orthologs may be easier to transform and may not results in high levels of toxicity. To test this hypothesis, we replaced the *Spy*Cas9 (4104bp) with a smaller Cas9 gene of 2952bp from *Campylobacter jejuni* Cas9 (*Cj*Cas9) (Kim et al., 2017). We were able to transform *C. trachomatis* with the plasmid (**Fig. 2.5D**). We have cloned the *Cj*Cas9 under a tetracycline inducible promoter. Inducible control will increase the versality of this system since it will allow for expression of the Cas9 at a specific stage during the *Chlamydia* lifecycle (Wickstrum, Sammons, Restivo, & Hefty, 2013).

Low *Chlamydia* transformation efficiencies are still a problem (Ouellette, 2018). The current CaCl2 transformation protocol requires about 2ug of DNA (Mueller et al., 2017). In our hands, it took about one to three trials to get a successful transformation and one transformation takes approximately two weeks to complete. A higher efficiency transformation method is of critical importance to the field. Smaller constructs perhaps

which do not require genes from the *Chlamydia* indigenous plasmid help with improving transformations efficiencies.

In the CjCas9 plasmid transformed *Chlamydia*, we always observed persistent aberrant bodies (growth arrested *Chlamydia* cells), even after continuous passaging in antibiotic selection medium (Elwell et al., 2016). This behavior has also been reported previously (Ouellette, 2018). We hypothesized that, since *Chlamydia* reside in an inclusion, acquisition of the plasmid by some of the *Chlamydia* cell provide antibiotic resistance to the rest of the residents in that inclusion. As a result, some of the cells lose the plasmid and these are observed as aberrant bodies in the next passage. We speculated that this might be due to the mechanism of β-lactamase resistance. The amount of β-lactamase produced by some of the cells inside the inclusion is enough to hydrolyze the penicillin and provide resistance to the entire inclusion. Use of different antibiotic resistance gene for selection might provide a solution to this for example resistance against antibiotics that interfere with protein or DNA synthesis.

The ability transform *Chlamydia* has opened door to use of many genetic manipulation tools (Y. Wang et al., 2011). Since the first successful transformation of *Chlamydia* we have seen the addition of tools that have made targeted mutations in the *Chlamydia* genome possible. Targetron is a group II intron-based system that has been used to knockout genes in *Chlamydia*. Targetron allows for inactivation of genes by insertion of antibiotic resistance cassettes. This system can be programmed to target specific sequence and the antibiotic resistance gene allows selection of knockout strain (Johnson & Fisher, 2013). Fluorescent Reported Allelic Exchange Mutagenesis

(FRAEM) exploits *Chlamydia* natural ability in exchanging DNA to delete genes. Through homologous recombination genes can be replaced by antibiotic resistance cassettes (Mueller et al., 2016). Prior to these targeted mutations, genetic manipulation of *Chlamydia* relied on a tedious approach that combined chemical mutagenesis and forward or reverse genetics (Bastidas & Valdivia, 2016).

We have transformed *C. trachomatis* with a *Cj*Cas9 plasmid, which is an important first step towards developing CRISPR-Cas9 for genome engineering tool in *Chlamydia*. The next step will be to test the feasibility of using this system by providing an sgRNA the mClover encoding gene. The sgRNA will delivered on a plasmid carrying the mClover encoding gene and a donor template. The successful demonstration of the *Cj*Cas9, we will have a system that will allow targeting of any gene in the bacterium by simply providing a specific sgRNA.

Acknowledgments

I thank Dr. Wiedenheft for his guidance, inputs and helpful discussion on this work. I thank Dr. Scott Grieshaber for his technical support, for providing the Cos7 cells, and the pBomb4R vector. I thank Dr. Emma Loveday for her guidance, training and establishing protocols for isolation and purification of Chlamydia and transformation of Chlamydia. I thank Royce Wilkinson for helping with a construction of different pBomb vectors for Cas9 delivery into Chlamydia and testing of the mClover to CFP conversion in HEK293 cells. Research in the Wiedenheft lab is supported by the National Institutes of Health

(P20GM103500, P30GM110732-03, R01GM110270, R21AI130670-02, and R01GM108888), the M. J. Murdock Charitable Trust, a young investigator award from Amgen, Montana University System Research Initiative, Gordon and Betty Moore Foundation, and the Montana State University Agricultural Experimental Station.

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Table 2.1. Expression and cloning vectors

Plasmid	Genotype and relevant characteristics	Source or reference
pRAW-219	pRAW-219 with an mClover	This study
pRAW-229	#820, pRAW-229 – IhtA shuttle vector for cloning of sgRNAs and donor template	This study
756- pBOMB	pBomb4-Tet-with mCherry replaced by GFP, with CPAF	(Grieshaber & Grieshaber, 2014)
pRAW-247	pBomb4-Tet-mCherry with *Spy*Cas9, 1080 bp CFP donor and mClover sgRNA 185	This study
pRAW-248	pBomb4-Tet-mCherry with *Spy*Cas9, 826 bp CFP donor and mClover sgRNA 185	This study
pRAW-249	pBomb4-Tet-mCherry with *Spy*Cas9, 1080bp CFP donor and mClover sgRNA 194	This study
pRAW-250	pBomb4-Tet-mCherry with *Spy*Cas9, 826bp CFP donor and mClover sgRNA 194	This study
pRAW-251	pBomb4-Tet-mCherry with *Spy*Cas9, 1080 bp CFP donor and mClover sgRNA 203	This study
pRAW-252	pBomb4-Tet-mCherry with *Spy*Cas9, 826 bp CFP donor and mClover sgRNA 203	This study
pRAW-253	pBomb4-Tet-mCherry with *Spy*Cas9n(D10A), 1080bp CFP donor and mClover sgRNA 185	This study
pRAW-254	pBomb4-Tet-mCherry with *Spy*Cas9n(D10A), 826 bp CFP donor and mClover sgRNA 185	This study
pRAW-255	pBomb4-Tet-mCherry with *Spy*Cas9n(D10A), 1080bp CFP donor and mClover sgRNA 194	This study
pRAW-256	pBomb4-Tet-mCherry with *Spy*Cas9n(D10A), 826bp CFP donor and mClover sgRNA 194	This study
pRAW-257	pBomb4-Tet-mCherry with *Spy*Cas9n(D10A), 1080bp CFP donor and mClover sgRNA 203	This study
pRAW-258	pBomb4-Tet-mCherry with *Spy*Cas9n(D10A), 826bp CFP donor and mClover sgRNA 203	This study
pEJK-259	pBomb4-Tet-mCherry with *Cj*Cas9	This study

CHAPTER THREE

IDENTIFICATION AND VALIDATION OF HOST FACTORS NECESSARY FOR REPLICATION OF *C. TRACHOMATIS*

Abstract

Chlamydia trachomatis is an obligate intracellular bacterium that is the most commonly reported sexually transmitted infection worldwide. Despite the impact of this parasite on human health, there is a critical gap in our understanding of specific host factors and molecular pathways that are necessary to support *C. trachomatis* replication. To identify human host factors that are necessary to support *C. trachomatis*, we generated a human genome wide knockout library in HEK293T and challenged the library with *C. trachomatis*. Cells that survived were enriched for deletions in 103 genes. Three of these genes (i.e., AP1B1, AP3S2, and AP1G2) are part of the Adaptor Protein complex, which plays an important role in intracellular membrane trafficking. We hypothesize that *C. trachomatis* utilizes the AP-complex to hijack resources from the host thus maintaining its lifecycle inside the host cell. To test this hypothesis, we are using CRISPR-Cas9 to generate clonal KO cell lines for each of the three AP genes. We are currently testing each KO lines for a *C. trachomatis* phenotype.

Introduction

Chlamydia infections are responsible for a significant health burden worldwide. This genus consists of 11 species which are all pathogenic infecting humans and animals (Bachmann et al., 2014). *C. trachomatis* is the main human pathogen from this genus and is the leading cause of bacterial sexually transmitted diseases and preventable blindness (Derre et al., 2007). Using antibody specificity to the major outer membrane protein, *C. trachomatis* has been divided into several serovars (Derre et al., 2007). The serovars also display tissue-specific tropisms with serovars A-C being associated with the ocular infection trachoma, serovar D-K causing the non-invasive sexually transmitted infections, and L1 to L3 being associated with invasive lymphogranuloma (Bastidas & Valdivia, 2016; Derre et al., 2007; Elwell et al., 2016).

Chlamydia have a unique cycle that involves two alternating forms, the extracellular infectious elementary body (EB) and a replicative intracellular reticulate body (RB). As an obligate intracellular parasite, *Chlamydia trachomatis* have evolved to depend on the host many of its metabolic needs. *C. trachomatis* relies on effector proteins secreted through the Type 3 Secretory System (T3SS) to manipulate and takeover host machineries and resources for its needs. Due to the inherent difficulty and/or lack of a robust tool to directly manipulate and study *Chlamydia* (Kokes et al., 2015), several genome-wide screens have been conducted to identify and study host factors involved in *Chlamydia* infections. Using gene-trap, a loss of function screen was conducted on human haploid cells from which they demonstrated the requirement of heparan sulfate for an efficient *C. trachomatis* infection (Rosmarin et al., 2012). A genome wide knockdown

with RNA interference in Drosophila cells identified a novel role for the mitochondrial Tom complex in *C. caviae* replication (Derre et al., 2007). Recently, a FACS-based genome-wide CRISPR-Ca9 screen reveals a requirement of Coatomer Complex I (COPI) in *Chlamydia trachomatis* Invasion (J. S. Park et al., 2019)

RNAi screens are prone to considerable variability in knockdown levels and there has been a poor overlap of gene knockdown screens. Gene-trap screens only functions in haploid cell and therefore are most likely to miss essential genes that might otherwise be identified in diploid and polyploid cells. CRISPR-Cas9 knockout libraries have been used to understand host-parasite interactions in several different cell types (Evers et al., 2016; Perreira, Meraner, & Brass, 2016). Additionally, while gene-trap and CRISPR-based screens both works by producing genome-wide knockouts comparative studies have shown that CRISPR-Cas9 screens can identify host factors not revealed by the gene-trap screens (Marceau et al., 2016; Timms et al., 2016; T. Wang et al., 2015)

To identify human host factors that are necessary to support *C. trachomatis*, we performed a CRISPR-Cas9 genome-wide knockout screen in HEK293. We have identified three genes from the Adaptor Protein Complex, and we have used CRISPR-Cas9 to generate clonal KO cell lines for each of the three AP genes. We are currently using Fluorescent Activated Cell Sorting (FACS), *Chlamydia* plaque assays and kill curves to test each KO line for a *C. trachomatis* phenotype.

Materials and Methods

Cell Culture and bacterial Propagation

HEK293 cells were obtained from ATCC (https://www.atcc.org/). HEK293 cells with stable *S. pyogenes* Cas9 expression were established by lentiviral transduction and Blasticidin selection using pLentiCas9-blast (addgene plasmid# 52962). Blasticidin was used at 5 ug/mL. *Chlamydia trachomatis* serovar L2 (LGV 434/Bu) GFP (CtL2-GFP) was kindly provided by Dr. Scott Grieshaber and was propagated in HeLa cells. EBs were purified using a 30% Renografin density gradient (Caldwell et al., 1981). HEK293, HEK293-Cas9 and HeLa cells were grown in DMEM media (Fisher) supplemented with 10% Fetal+ (Atlas Bio) and 10U/mL of penicillin to maintain the *Chlamydia* plasmid that expresses GFP at 37°C and 5% CO2. 30% renografin purified CtL2-GFP was titered on HeLa cells. Inclusions were calculated at 48 hpi using fluorescent images taken on a Nikon TiEclipse inverted fluorescence microscope.

CRISPR/Cas9 Loss of Function Screen

A total of 120 million HEK293 cells that constitutively express Cas9 were transduced with the lentiGuide-Puro from GeCKO v2 library at an MOI of 0.2 overnight in polybrene (8 ug/ml) media. Cells were selected with puromycin (2 ug/ml) and the CRISPR genetic screens were started 14 days after transduction. Approximately 30 million mutagenized cells for each half of the library (A and B), representing 500x representation of every potential sgRNA present within the library, were infected with

CtL2-GFP using a MOI of 10. At 48 hpi, infected cells were treated with rifampicin and the following day, the cells were collected and spun down 2X at 1200 rpm to remove external bacteria from the population. The cells were then reseeded and allowed to expand for ~1 month with frequent media changes. Following expansion of cells, genomic DNA was extracted from a minimum of 10 million cells using phenol-chloroform and precipitated using isoamylalchohol. The remaining cells were infected at an MOI of 10 and split into split two groups. Both groups were treated rifampicin 48 hpi and one group was the cells were spun down as described above and expanded for sequencing. The other group were sorted using FACS, for GFP-positive (+) and GFP-negative (-) cells. The GFP + and - populations were collected, re-plated in rifampicin media and allowed to expand prior to sequencing. All three populations of cells that were re-challenged were expanded until a minimum of 10 million cells. Together we collected four population over the course of each experiment: cells infected once (1X), cells infected twice (2X), and cells infected 2X, and then sorted for GFP expression and these was done in three replicates.

To prepare samples for next-generation sequencing (NGS), 130 ug of genomic DNA (gDNA) from library A and 120 ug of DNA from library B were PCR amplified using Q5 High-Fidelity DNA Polymerase (Newbiolabs) with 10 ug of DNA in 100 ul reactions. All PCR products obtained from each sample were run on an agarose gel and gel-extracted. The DNA concentration of each sample was measured, and all samples were combined at equal amount of DNA.

Sequence Analysis

Each sequence read in the FASTQ files was trimmed at the 5'-end to start with a 20 nt constant part of the sgRNA. Trimmed FASTQ files were then analyzed using MAGeCK software (Li et al., 2014). Counts were then aggregated gene-wise and sorted. Due to strong selective pressure, the majority of sgRNA reads in the infected populations were 0. Hits were determined by filtering for genes that had a robust rank aggregation (RRA) score < 0.01 and also considered high in treatment over controls. This initial list of genes from each screen performed was then compared to the gene list generated in the additional screens to determine which genes were consistently highly ranked.

Pathway and Interaction Network Analysis

Enrichr was employed to perform gene list-based gene set enrichment analysis on the four CE populations. The top 10 enriched Reactome pathway sets ranked by combined score were visualized using ggplot2 package in R. The algorithm to calculate the combined score has been described (Chen et al., 2013). The protein-protein interaction network was generated using the GeneMANIA (http://www.genemania.org/) app in Cytoscape (http://www.cytoscape.org/) (Montojo et al., 2010; Warde-Farley et al., 2010)

Rifampicin Survival Curves

Prior to challenging the KO library with *C. trachomatis*, we identified the time point in which the majority (> 99%) of wild type cells were infected and not recoverable

following antibiotic treatment. This same time point could then be utilized to identify loss of function (LOF) mutants that impact the bacterial lifecycle, delaying bacterial induced lysis and allowing for recovery of the cell following antibiotic treatment. WT HEK293 cells were infected with *C. trachomatis* L2 at an MOI 10 and rifampicin (10 ug/mL) media was added at 24, 36, 48, 72, and 96 hpi (**Fig. 3.1B**). The cells were collected and spun down to remove extracellular bacteria 24 hours after the addition of the initial rifampicin media and allowed to recover for 6 days prior to counting. The percent of surviving cells was determined and there was a considerable loss of recoverable cells by 48 hpi (< 1%).

Plasmids Construction

We used pX330 vector carrying a *Streptococcus pyogenes* Cas9 (SpyCas9) gene to create 6 plasmids carrying 3 different single guide RNAs (sgRNA) targeting the long isoform of the and 3 sgRNA targeting the short isoform of AP1G2 gene. Briefly, we ordered two oligos for each guide which when annealed would produce the desired DNA sequence for the guide and over-hangs that we used to ligate these fragments into pX330 vector using BbsI sites.

Plasmid Transfections

The plasmid carrying Cas9 and the different sgRNAs were transfected into HEK293T cells using a PEI based transfection methods and this was done in triplicates. Briefly, A 9cm plate of HEK293T cells of ~60% confluency was prepared, the cells were

re-fed with DMED medium with 2% serum for 2h prior to transfection. The transfection solution was prepared in a 15cm poly propylene tube by following the recipe below:

- 520ul DMEM with no serum or antibiotics
- 5ug of DNA (sterile, min. 0.5ug/ml conc.; OD ration 260/280 greater than 1.7)
- 30ul PEI (1mg/ml in sterile ddH_2O, made from 10mg/ml PEI solution*)

The mixture was vortexed quickly twice (50% vortex) then incubated for 10 min at room temperature, afterwards, the ~550ul transfection solution was added to the plate (drop-wise), while gently swirling it followed by an incubation of 3 to 8 hours at 37^0C, and 5% CO_2. The transfection solution was replaced by complete medium (with antibiotics and 10% FBS) and cells were observed for 24 hour and split into two 9 cm dishes. Puromycin selection was initiated 24 hours after splitting and plates were observed daily, and medium changed every 2 to 3 days to get rid of debris. Stable population of cells formed 7-14 days after selection was initiated.

Single Guide RNA Efficiency Determination and Selection

After selection with puromycin, the cells were expanded and split, half was frozen for down-stream and the remaining half was used for DNA extraction to determine the efficiencies of the selected guide. The DNA was used to perform PCR using a set of primers that flank the target sites for each sgRNA and the PCR product was cleaned and sent for Sanger sequencing. The sequencing results were used to determine the efficiency of each guide in editing the gene in a manner that would result in gene knocking out the

gene (indels frameshift mutations). We employed a web-based tool called Tide assay, a web-based tool with an algorithm to analyses the frequency type if the indels mutations and hence determine which guides produces the highest percentage of cells with knockouts from these mutations. Once the sgRNA with the highest rate of knockouts was identified the corresponding cells from this group was thawed and left to recover and subsequently there were seeded into three 96-well plates at a concentration of three to four cells/well, and wells containing groups of cells arising from singles cells (clones) were identified and expanded. These were again split into two groups where half were frozen, and the other half was extracted for DNA, followed by another PCR and sanger sequencing and tide analysis. Clones with AP1G2 homologous knockouts were identified, the knockout of the gene was confirmed by Sanger sequencing and Western blot.

Western blots

For visualization of the AP1G2 protein we used a 10% SDS. After pouring the gel, a few drops isopropanol were added to get rid of bubbles and flattens the surface. Then the stacking gel was poured on top of the resolving gel. Sample were measured for concentration and diluted whenever necessary with RIPA buffer and 25ug of protein was loaded per lane mixed with 3ul of 5x loading dye and these samples + loading dye were incubated at 98^{0}C for 5 min prior to loading. The samples and controls were sun at 100V through the stacking gel, then at 150V through the resolving gel. The proteins were transferred on to a nitrocellulose membrane (Bio-Rad) for 1 hour at 100V. Then the

membrane was blocked in 5% non-fat milk in TBST solution for 1 hour at room temperature, followed by overnight incubation with primary antibody at 4^0C. The primary antibodies were diluted in blocking buffer at 1:1,000 and 1:100,000 dilutions for APIG2 and Actin β primary antibodies respectively. On the next day, the membrane was washed 3 times (for 5 min each time) with TBST, then incubated with anti-rabbit secondary conjugated to HRP for 1 hour at room temperature. The Secondary antibody was diluted at 1:10,000 in blocking buffer. After that the membrane was 3 washed with TBST (5 min. each) and was detected by chemiluminescence using 750 ul peroxide and luminol (Biorad). After 1 min incubation of the substrate on the membrane a film was developed on Konika Minolta model SRX-101A, for 3 min.

Chlamydia trachomatis Plaque Assays

Plaque assays were done following (Caldwell et al., 1981), Stock solutions were prepared as follow:

- 1.1% agarose in distilled water,
- Phenol red-free DMEM
- 100 mg/ml of cycloheximide in phosphate-buffered saline (PBS) without Mg and Ca ions
- 3% neutral red in PBS
- Filter all the solutions through a 0.2mm pore size filter and store them at 4°C. Autoclave the agarose solution and keep at room temperature.
- Plate preparation

i. Obtain PLL or PDL hydrobromide (Sigma P6282 or P6407). Make 0.1 mg/ml solution (working concentration) with sterile water. Store lysine solution frozen at –20.

ii. Add 0.5-1 ml per well. Incubate for 1-2 hours at 37ºC.

iii. Aspirate and wash 2x with sterile water/PBS.

iv. Let plates dry in TC hood 30-60 minutes before use. Store extra plates at 4º C.

A 293T cells monolayers was prepared in six-well culture plates, enough plates were seeded to perform 3 technical replicates of 3 biological replicates (3clones in triplicates) for the knockout clones and 3 technical replicates for the wildtype 293T (control). So, for one knockout seed six 12-well plates. Then *C. trachomatis* were serially diluted (7 serial dilutions) in HBSS and these dilutions were used to infect the HEK 293T cells by incubating at 37^{0}C for 2 hours. Then the infection mixture was removed and an overlay medium (DMEM) mixed 1:1 with the 1.1% agarose containing 10% FBS and 1mg/mL cycloheximide was overlaid on the cells (2mL/well) and after solidification at room temperature 2ml/well of DMEM containing 10% FCS was added on top of the agarose medium and incubate at 37°C in an atmosphere of 5% CO2. 7 days post inoculation, a final agarose medium, (1/100 of neutral red stock added to primary agarose medium) was overlaid on to the first agarose medium 2 ml/well. Plaques were counted 10 to14 days post inoculation.

Results

Genome-wide CRISPR Knockout Screen for Host Factors Associated with Chlamydia Infection

We performed a genome-scale loss of function genetic screen using the Genome-Scale CRISPR Knockout (GeCKO) library to identify host genes that are important for the completion of the *C. trachomatis* lifecycle (Sanjana et al., 2014; Shalem et al., 2014). Briefly, clonal Cas9 expressing HEK293 cells were transduced with the pooled GeCKO lentivirus library (V2), which contains 123,411 single guide RNAs (sgRNAs) and a puromycin resistance gene. Cells were selected for 14 days in puromycin media to generate a redundant knockout library in HEK293 cells. To identify host gene knockouts that confer reduced susceptibility to *C. trachomatis* infection we inoculated the KO library with the *C. trachomatis* L2 strain expressing green fluorescent protein (CtL2-GFP) at a multiplicity of infection (MOI) of 10 and treated the inoculated cells at 48 hours post infection (hpi) with the antibiotic rifampicin (Agaisse and Derre, 2013; Vromman et al., 2014). We hypothesized that, despite survival advantage provided by some of the knockouts these resistance mutants cells die as collateral damage from the mass death produced *C. trachomatis.* We used the Rifampicin treatment to increase the survival of these resistance mutants HEK293 cells. Surviving HEK293 cells were expanded to 150 cells/sgRNA based on the number of sgRNAs (123,411) in the initial library prior to re-challenge. We performed two consecutive rounds of inoculation and expansion of surviving cells to enrich for sgRNAs associated with host genes that are critical for successful *C. trachomatis* infection **(Fig. 3.1A)**. Following the second round

of inoculation, cells were sorted into two populations at 48 hpi using fluorescence activated cell sorting (FACS): GFP-positive (GFP-pos) cells that permitted entry and replication of *C. trachomatis* and GFP-negative (GFP-neg) non-infected cells. The overall screening protocol was repeated three individual times. To identify gene KOs that are enriched in this surviving population of cells, we extracted total genomic DNA from cells collected from each round of infection (1X and 2X) and from the FACS sorted populations. The 20 nt guide of the sgRNAs were PCR amplified, sequenced and analyzed using Model-based Analysis of Genome-wide CRISPR/Cas9 Knockouts (MAGeCK) (Li et al., 2014). To evaluate positively selected genes found in our screen, we focused on the sorted cells as they represent specific subpopulations that survived the two rounds of infection. Genes with a robust rank aggregation (RRA) score < 0.01 from each replicate screen for each population (GFP-pos or GFP-neg) were then compared to each other to identify significant hits that were present in at least two of the three screens for further analysis. This analysis reveals a total of 103 genes shared between at least 2 of the 3 replicate screens (**Fig. 3.2**), among them three AP complex genes namely AP1B1, AP1G2 and AP3S2 (**Fig. 3.2E**). We hypothesized that *C. trachomatis* utilizes the AP-complex to hijack resources from the host thus maintaining its lifecycle inside the host cell, therefore we focused our investigation on these three genes.

Development of AP1G2 knockout HEK 293 Cell line

We initially focused on the *AP1G2* gene, which encodes for γ2 (adaptin) of the clathrin associated Adaptor Complex I (S. Y. Park & Guo, 2014). AP1G2 has previously

been implicated in other infections by obligate intracellular parasite such as Hepatitis B and HIV1 (Hartmann-Stuhler & Prange, 2001; Jurgens et al., 2013; Tavares et al., 2017). Moreover, the AP1 complex is involved in vesicle trafficking between the trans-Golgi network, recycling endosome and lateral transport from the cell, which suggests that *Chlamydia* might be hijacking membrane components that are being recycled and direct them to the inclusion for its own needs such as inclusion and *Chlamydia* inner and outer membranes formation and enlargement (S. Y. Park & Guo, 2014). We hypothesized that *C. trachomatis* hijacks transport vesicles using gamma2 adaptin of the AP1 complex and redirects them to the inclusion for various purposes membrane synthesis and metabolic needs.

To test the role of AP1G2 in *C. trachomatis* infection, we generated an AP1G2 knockout line in HEK293T cells using CRISPR-Cas9. AP1G2 gene has several genetic isoforms which are either long or short isoforms, therefore we picked a total of six sgRNAs (three per isoform), three targeting the long isoforms and three for the short isoforms. The sgRNAs were cloned into pX330 carrying a *Streptococcus pyogenes* Cas9 (*Spy*Cas9) gene and were transfected into HEK293T cells. This was followed by puromycin selection and expansion of transfected cells. The cells were split into two groups, group one was used to determine knockout efficiency of each guide and the other was stored at -80 for further analysis. We PCR amplified the target sequence of each guide and deep sequenced it to determine the efficiency of each guide using a web-based Inference of CRISPR Edits (ICE) algorithm (https://ice.synthego.com/#/) (Osborn et al., 2018). We used three sgRNAs to target the long isoform of AP1G2 (T4, T5 and T6).

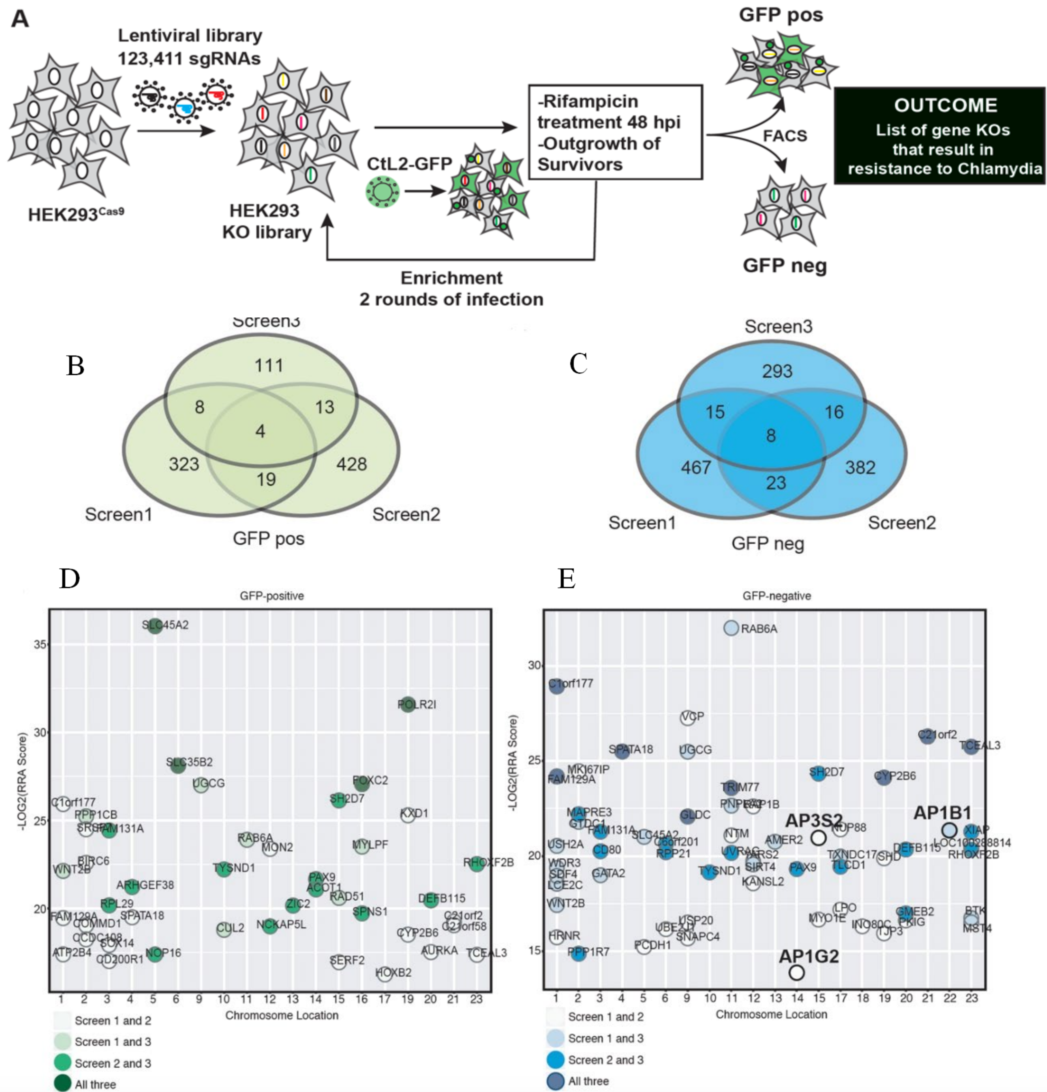

Figure 3.1. Screening strategy and identification of key host gene deletions that enhanced resistance to *C. trachomatis* infection. **A)** Overview of GeCKO screen in human HEK293 cells. Cas9 expressing HEK293 cells were transduced with lentivirus containing the GeCKO sgRNA library and selected for 14 days in Puromycin to generate the KO library. The KO library was infected with *C. trachomatis* at an MOI 10 and cells were treated with antibiotics at 48 hpi. Surviving cells were expanded and subjected to a second round of infection and treatment with antibiotics. Surviving cells were FAC sorted for GFP positive and GFP negative cells at 48 hpi, treated with antibiotics and expanded. To assess sgRNA distributions in the surviving cells, genomic DNA was isolated and the sgRNA barcodes were PCR amplified and sequenced. (**B and C**) Venn diagrams of overlapping genes with an RRA score < 0.01 and high in treatment compared to controls identified in the GFP-pos and GFP-neg populations. (**D and E**) The cumulative -Log2 RRA score is plotted for the most significant host genes present in 2 out of 3 screens in the GFP-pos and GFP-neg KO library. The The chromosome for each gene is shown on the x-axis. Genes are colored based on the overlap between the different screens.

T4 showed the highest knockout efficiency (~70%), compared to T5 (62%) and T6 (49.5%) (**Fig. 3.2A**). We re-plated and dilutionally cloned cells edited with T4. We PCR amplified DNA from the clones and sequenced the sgRNA target which was followed by analysis of the sequencing data using ICE. The analysis revealed four clones with ~100% deletion of AP1G2 (**Fig. 3.2B**). However, western blot showed that there was still expression of the target protein (**Fig. 3.2C**). We suspect that this detection could be from the AP1G1 isoform. AP1G1 and AP1G2 are isoforms with about 60% amino acid sequence similarity. The polyclonal antibody used for AP1G2 detection might be cross-reacting with the AP1G1.

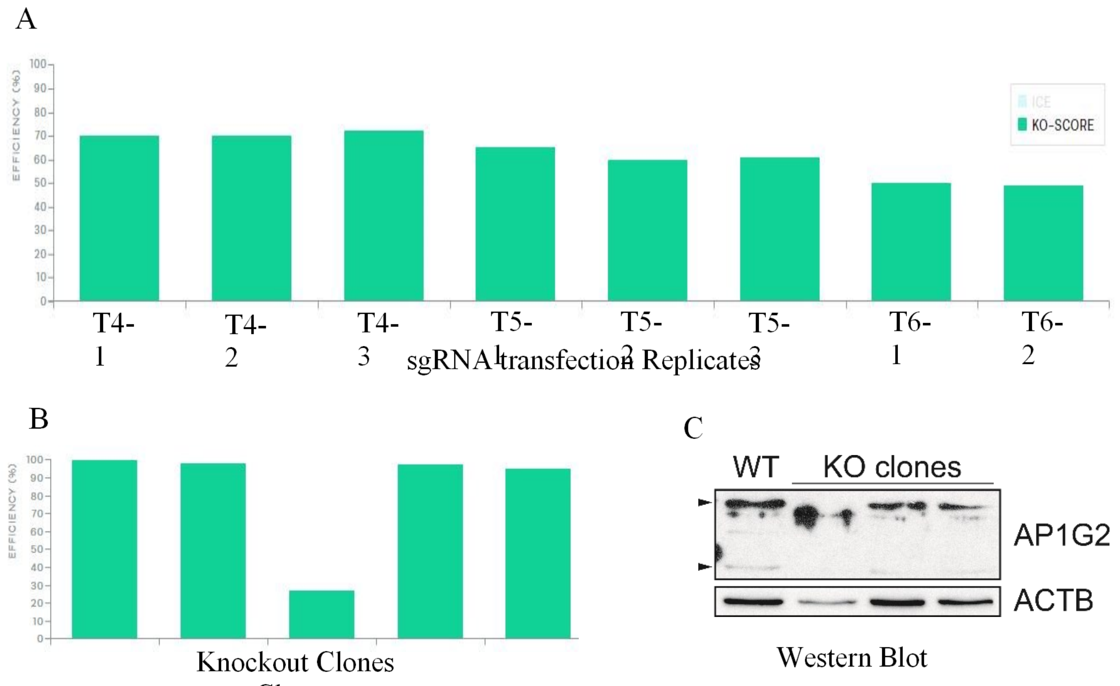

Figure 3.2. Selection of efficient single guide RNAs for deletion of AP1G2. **A)** Determination of knockout efficiency in HEK 293T with three different sgRNA using ICE. Transfections were done in triplicates, PCR amplified and sequenced before analysis of knockout efficiency. T6 has only two replicates since it had poor sequencing results for one replicate. **B)** Clonal expansion and knockout efficiency determination using cells from the T4 sgRNA. **C)** Western blot on the three AP1G2 knockout clones to assess expression of AP1G2. The upper arrow shows the long isoform while the lower arrow shows a faint band of the short isoform of the AP1G2 gene

Fluorescent Activated Cell Sorting

To determine if the AP1G2 knockout has a phenotype, we infected the cells with GFP expressing *C. trachomatis* (Ct-GFP) and determined the percentage of infected cells compared to wildtype HEK 293T. Cells were infected at an MOI of 10 in triplicate, and harvested 24 hours post infection. Infection efficiencies were quantified by counting GFP

positive cells. However, we did not measure any difference in the infection burden between the AP1G2 knockout (35.5%) and wildtype (33.7%) HEK293T cells (**Fig. 3.3**).

However, the GFP based assay only measures differences in GFP expression rather than virulence. To measure resistant to *C. trachomatis* infection we perform plaque assays (Matsumoto, Izutsu, Miyashita, & Ohuchi, 1998). We observed that the HEK 293T cells started to round up on the second day and by third and fourth day the cells looked unviable. Consequentially we kept losing the infection in both the AP1G2 knockout and wildtype cells. We are currently trouble shooting this protocol.

Discussion

AP1G2 was detected in a CRISPR-Cas9 based genome-wide knockout screen. We sought to elucidate the function of these gene in *C. trachomatis* infection because as part of the AP1 it is involved vesicle trafficking between the trans-Golgi network and basolateral secretion (S. Y. Park & Guo, 2014). Also, AP1G2 has previously been implicated in other infections such as HIV-1 and HBV (Hartmann-Stuhler & Prange, 2001; Tavares et al., 2017). The AP1G2 can produce long and short isoforms (NCBI, 2019). Here we have been able to develop a clonal knockout of AP1G2, for the long

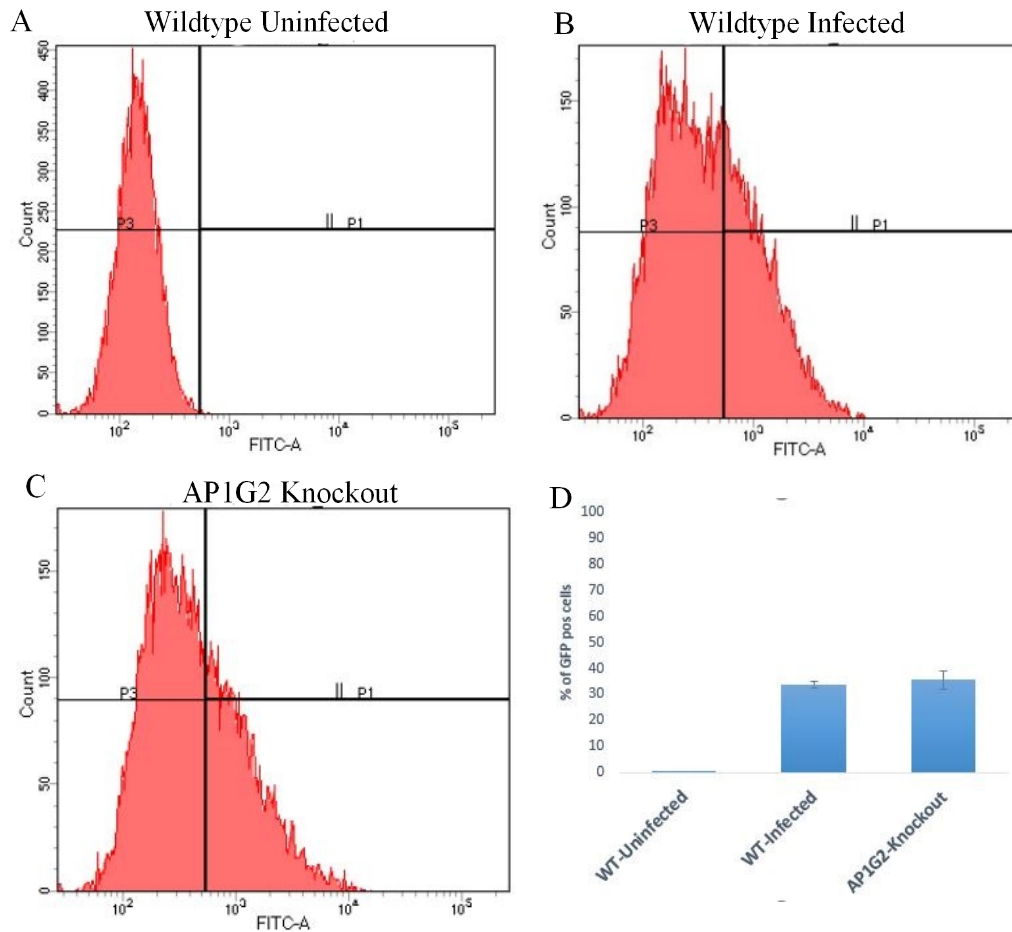

Figure 3.3. FACS count reveals no difference in infection burden between wildtype HEK 293T cells and the AP1G2 knockout. Cells were infected with GFP expressing *C. trachomatis* and were harvested 24 hours post infection for FACS counting. A) Wildtype uninfected HEK293T cells B) Wildtype HEK 293T cells infected with Ct-GFP C) AP1G2 knockout HEK 293T cells infected with Ct-GFP D) Percentages of GFP expressing HEK293T cells among wildtype uninfected, wildtype infected and AP1G2 knockout HEK 293T cell lines.

isoforms. Our sequencing data confirms that we have at least three clones of AP1G2 knockout HEK293T cells. We have used a GFP expressing *C. trachomatis* strain (Ct-GFP) to evaluate infection burden between the AP1G2 knockout and wildtype HEK293T cells. We performed a FACS count to evaluate infection burden between the AP1G2

knockout and wildtype HEK293T cells which revealed no difference in the infection burden. At this point it hard to conclude on whether AP1G2 is required for *C. trachomatis* infection of HEK293 cells for several reasons. First, we suspect that the shorter isoform might still be functional, which may obviate the phenotype. Another knockout that will target the shorter isoform of this gene is required before we can make firm conclusions. Second, during the screen that identified AP1G2, the infected cells were rescued by rifampicin treatment 48 hours post infection and survivors were expanded. It is possible this approach has identified genes that do not increases resistance to *C. trachomatis,* but somehow slow down the *C. trachomatis* infection.

We also tried to use plaque assays to evaluate the effect of the AP1G2 knockout on *C. trachomatis* infection. However, we were unable to maintain the HEK293 cells viability during the course of the assay which takes about 10 to 14 days to complete (Matsumoto et al., 1998). While trouble shooting this assay, we are also working on performing, kill curve assays that will mimic conditions used in the original screen.

Acknowledgments

I thank Dr. Wiedenheft for his guidance, inputs and helpful discussion on this work. I thank Dr. Scott Grieshaber for his technical support specifically during the genome wide knockout screen. I thank Emma Loveday for performing the screen on which this work is built on. I also thank Artem Nemudryi and Anna Nemudraia for their guidance and help on development and testing of knockouts clones for the individual Adaptor Protein (AP) complex genes identified from the screen. And I also thank Myndi Holbrook for helping

with developing and testing of the AP gene knockouts. Research in the Wiedenheft lab is supported by the National Institutes of Health (P20GM103500, P30GM110732-03, R01GM110270, R21AI130670-02, and R01GM108888), the M. J. Murdock Charitable Trust, a young investigator award from Amgen, Montana University System Research Initiative, Gordon and Betty Moore Foundation, and the Montana State University Agricultural Experimental Station.

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Table 3.1. Expression vectors used in the study

Plasmid	Genotype and relevant characteristics	Source or reference
pX330-Puro	pX330 with puromycin resistance gene for expression of SpyCas9, and sgRNA targeting individual AP complex gene in	(Cong et al., 2013)

CHAPTER FOUR

FUTURE DIRECTION

<u>CRISPR-Cas9 Editing in *Chlamydia*</u>

CRISPR-Cas9 has revolutionized the field of biology by providing a facile and reliable method for manipulating genomes (Ding et al., 2016). We have made progress towards developing a CRISPR-Cas9 tool for direct manipulation of the *Chlamydia* genome. Attempts to transform expression vectors containing SpyCas9 failed, however we successfully transform *C. trachomatis* with a plasmid carrying the *cas9* gene *Campylobacter jejuni* (CjCas9) (Kim et al., 2017). Our failure to transform *C. trachomatis* with SpyCas9 is consistent with what was reported by Ouellette *(Ouellette, 2018)*. As a result of this failure to transform *C. trachomatis* with a SpyCas9 plasmid, Ouellette ended up using catalytically dead Cas9 from *Staphylococcus aureus* (dSaCas9) to perform conditional knockdown of the IncA protein in *C. trachomatis (Ouellette, 2018)*. Our success in transforming *C. trachomatis* with CjCas9 may prove to be a crucial step towards developing a tool for engineering the *Chlamydia* genome. Most genes in *Chlamydi*a are expected to be essential. This requires a versatile tool that will enable manipulation of these genes through targeted hypomorphic mutations and gene knockdowns. However, direct verification of CjCas9 expression is an important next step. Western blots can be used to detect the protein but converting mClover to CFP is quantitative functional assay. IncA is a *Chlamydia* inclusion protein required for homotypic fusion of inclusions (Fields, Fischer, & Hackstadt, 2002; Johnson & Fisher,

2013). IncA knockout can provide an alternative proof of principle since it has an easy phenotypic readout of production of multiple inclusions in the IncA knockout strain compared to fusogenic inclusions in the wildtype *C. trachomatis*. Targeting IncA will comparatively be easier to achieve as the target is already in the *C. trachomatis* genome. Upon observation of non-fusogenic inclusions, the editing of the IncA can be confirmed by DNA sequencing.

In addition to CjCas9, I would also like to develop alternative Cas9 systems for editing in *Chlamydia*. *Cj*Cas9 recognizes an extensive PAM sequence (i.e., NNNNRYAC) where R and Y stands for purines and pyrimidines, respectively (Kim et al., 2017). This PAM sequence occurs approximately 32,500 times compared to the *Spy*Cas9 NGG PAM that occurs approximately 107,380 times in the *Chlamydia* genome and thus restricts the versatility of *Cj*Cas9. To address the critical need of more tools for *Chlamydia* research, I propose further development of Cas9 orthologs from different organism such *Neisseria meningitidis* and *Fancisella novicida* (Hirano et al., 2016; Lee, Cradick, & Bao, 2016). these will provide different PAMs and increase the number of target sequences on the *Chlamydia* genome (Ding et al., 2016).

Determining the role of the AP-complex in *C. trachomatis* Infection

The Adaptor Protein Complex 1 (AP1) is involved in clathrin associated vesicle trafficking between the Trans-Golgi Network (TGN), recycling endosome and the basolateral sorting in epithelial cells (S. Y. Park & Guo, 2014). The AP1 complex has two isoform gamma subunits, AP1G1 and AP1G2. AP1G2 is highly similar to AP1G1

with 60% amino acid identity, never the less, it is hypothesized to participate in transport steps different from those of AP1G1 (Doring, Gotthardt, Stieler, & Prange, 2010; Takatsu, Sakurai, Shin, Murakami, & Nakayama, 1998). AP1G2 has been implicated in other infections such as Human Immunodeficiency Virus-1 and Hepatitis B virus (Hartmann-Stuhler & Prange, 2001; Jia et al., 2014). Our CRISPR-Cas9-based loss of function genome wide screen for *C. trachomatis* infection detected AP1G2 as a host factor necessary for *C. trachomatis*. We have created an AP1G2 knockout cell line and challenged it against *C. trachomatis* infection and evaluated the effect of the knockout using FACS count. The FACS count revealed no difference in infection burden between AP1G2 knockout and wildtype cell lines. But due to the different isoforms of the AP1G2 produced through alternative splicing (NCBI, 2019), we believe we cannot make any conclusion on the requirement of AP1G2 during *C. trachomatis* infection until we have knocked out both the long and short isoforms. The next step would be development of a second knockout that target the shorter isoform (isoform 2) and cell lines with knockouts on individual and both long and short isoforms can be challenged against *C. trachomatis* infection.

C. trachomatis uses different T3SS effectors at different stages of its lifecycle and these have been shown to hijack and recruit different host factors during throughout the *Chlamydia* lifecycle (Elwell et al., 2016). The protective effect that may be produced by the AP1G2 knockout could be coming during the later stage of the *Chlamydia* lifecycle. If these knock-outs truly provide protective effect, they could present good drug targets. Kill curve may provide a good assay to assess the effect of the AP1G2 knockout in

HEK293T cells as it may detect the protective effect regardless of the time point of the *Chlamydia* lifecycle.

Acknowledgments

I thank Dr. Wiedenheft for his guidance, inputs and helpful discussion. Research in the Wiedenheft lab is supported by the National Institutes of Health (P20GM103500, P30GM110732-03, R01GM110270, R21AI130670-02, and R01GM108888), the M. J. Murdock Charitable Trust, a young investigator award from Amgen, Montana University System Research Initiative, Gordon and Betty Moore Foundation, and the Montana State University Agricultural Experimental Station.

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