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Microreview

Coxiella burnetii: turning hostility into a home

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Summary

***Coxiella burnetii*, the causative agent of the human disease Q fever, is a unique intracellular bacterial pathogen. *Coxiella* replicates to high numbers within a pathogen-derived lysosome-like vacuole, thriving within a low pH, highly proteolytic and oxidative environment. In 2009, researchers developed means to axenically culture *Coxiella* paving the way for the development of tools to genetically manipulate the organism. These advances have revolutionized our capacity to examine the pathogenesis of *Coxiella*. In recent years, targeted and random mutant strains have been used to demonstrate that the Dot/Icm type IV secretion system is essential for intracellular replication of *Coxiella*. Current research is focused towards understanding the unique cohort of over 130 effector proteins that are translocated into the host cell. Mutagenesis screens have been employed to identify effectors that play important roles for the biogenesis of the *Coxiella*-containing vacuole and intracellular replication of *Coxiella*. A surprisingly high number of effector mutants demonstrate significant intracellular growth defects, and future studies on the molecular function of these effectors will provide great insight into the pathogenesis of *Coxiella*. Already, this expanse of new data implicates many eukaryotic processes that are targeted by the arsenal of *Coxiella* effectors including autophagy, apoptosis and vesicular trafficking.**

Introduction

Coxiella burnetii is a unique intracellular bacterium that causes the zoonotic disease Q fever. Human infection, through the inhalation of contaminated aerosols, can cause a broad spectrum of presentations ranging from asymptomatic infection to life-threatening systemic infection. A small proportion of *Coxiella* infections are chronic and predominantly manifest as endocarditis years after exposure. Interestingly, a recent study confirmed that, even with adequate antibiotic treatment, Q fever has a significant long-term health impact with more than one in three patients continuing to suffer from an impaired health status 24 months post-diagnosis (van Loenhout *et al.*, 2015).

Ruminants are the primary reservoir for *Coxiella*. Infection of these animals, predominantly dairy cows, goats and sheep, is generally subclinical, although *Coxiella* can trigger abortion and the placenta and birthing fluid contains a substantial bacterial load that contaminates the local environment (Delsing *et al.*, 2011). Such contamination is a significant threat to both the agricultural industry and public health. This was recently demonstrated in the Netherlands where widespread *Coxiella* infection of goats, in a region of substantial goat farming, led to the largest Q fever outbreak ever recorded (reviewed in van der Hoek *et al.*, 2012). Between 2007 and 2011, over 4000 human cases of Q fever were diagnosed in the Netherlands. In addition to the significant societal impact, this outbreak has cost in excess of €300 million to control (van Asseldonk *et al.*, 2013).

The morbidity and mortality caused by Q fever, in combination with the extremely low infectious dose and environmental stability of *Coxiella*, led to the US Centers for Disease Control and Prevention classifying *Coxiella* as a biological weapon agent (Madariaga *et al.*, 2003). Understanding the interplay between this important pathogen and eukaryotic host cells is an essential step towards developing novel approaches and interventions to disrupt the pathogenic process. Until recently, researchers attempting to investigate the molecular mechanisms of *Coxiella* pathogenesis have been restricted by the obligate intracellular nature of *Coxiella*. However, the advent of axenic culture conditions, using acidified citrate cysteine medium (ACCM), has transformed our capacity to examine this unique pathogen (Omsland *et al.*, 2009). Researchers have developed tools to genetically manipulate *Coxiella* and we are now inundated with important new information about the bacterial factors that contribute

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to *Coxiella* pathogenesis. Here we will provide an overview of these new studies and discuss new insights into the interactions between *Coxiella* and the host cell.

A bacterium that thrives in a lysosome-like vacuole

Phase I *Coxiella*, isolated from sources of infection, are highly pathogenic due to the production of full-length lipopolysaccharide (LPS) (Moos and Hackstadt, 1987). Following *in vitro* passage of phase I organisms, spontaneous mutations lead to phase II *Coxiella* that express truncated LPS and are subsequently strongly attenuated for *in vivo* models of infection (Moos and Hackstadt, 1987; Hoover *et al*., 2002). A well-characterized phase II variant of the Nine Mile strain RSA493 is locked in this avirulent form due to a chromosomal deletion removing the organism's capacity to synthesis O-antigen polysaccharide (Hoover *et al*., 2002). Importantly, it has been demonstrated that the behaviour of this Nine Mile phase II organism in tissue culture models of infection is indistinguishable from phase I *Coxiella*, and thus this strain is commonly used in laboratories as a safer model for researchers to examine the molecular pathogenesis of *Coxiella* (Howe *et al*., 2010).

Coxiella is an intravacuolar pathogen, replicating to high numbers within a membrane-bound compartment of eukaryotic cells termed the *Coxiella*-containing vacuole (CCV). During natural infection, *Coxiella* has a tropism for alveolar phagocytic cells; however, *in vitro* studies have demonstrated that this organism has the capacity to enter and replicate in a wide array of both phagocytic and non-phagocytic cells (Khavkin and Tabibzadeh, 1988; reviewed in Voth and Heinzen, 2007). The CCV undergoes normal endocytic trafficking through early and late endosomes to a lysosome. This hostile environment, with an acidic pH and a myriad of acid hydrolases, normally forms a natural defence against phagocytosed bacteria. However, within this environment *Coxiella* can undergo biphasic development, differentiating from an environmentally stable small cell variant (SCV) into a replicating large cell variant (LCV) (Coleman *et al*., 2004) (Fig. 1A). Our understanding of the dynamics and comparative virulence roles of the SCV and LCV is limited; however, these variants are equally infectious indicating that this differentiation is not required for intracellular replication of *Coxiella* (Coleman *et al*., 2004).

Following this bacterial differentiation, the CCV undergoes dramatic expansion that is followed by slow replication of *Coxiella* until they reach stationary phase, roughly 6 days after infection (Fig. 1B). The expansion of the CCV is a pathogen-directed process that recruits cellular vesicles to fuse with the vacuole membrane (Howe *et al*., 2003). The CCV will fuse with vacuoles containing other microbes or latex beads, and in the event of a single cell being infected with more than one *Coxiella*, homotypic fusion will resolve all *Coxiella* into one CCV (Veras *et al*., 1994). Despite occupying the majority of the host cytosol, replicative CCVs still maintain proteolytic and acidic characteristics.

Reliance on the Dot/Icm type IV secretion system (T4SS)

The first *Coxiella* genome sequence revealed that this organism encodes a T4SS closely related to the Dot/Icm system of *Legionella pneumophila* (Seshadri *et al*., 2003). This secretion system is ancestrally related to conjugation systems and has the ability to translocate bacterial proteins from the bacterial cytosol directly into the host cytoplasm, crossing both the bacterial and vacuolar membranes (Christie and Vogel, 2000). The Dot/Icm system was simultaneously discovered by two research groups who identified clusters of genes responsible for preventing *d*efects in *o*rganelle *t*rafficking (Segal *et al*., 1998) and promoting *i*ntra*c*ellular *m*ultiplication (Vogel *et al*., 1998) of *L. pneumophila*. Indeed in the absence of a functional Dot/Icm system *L. pneumophila* is avirulent, incapable of remodelling the *Legionella*-containing vacuole (LCV) to avoid lysosomal degradation (reviewed in Hubber and Roy, 2010).

The Dot/Icm systems of *Legionella* and *Coxiella* are functionally analogous as specific *dot/icm* mutants of *L. pneumophila* can be complemented by the respective *Coxiella* homologues (Zamboni *et al*., 2003; Zusman *et al*., 2003). In 2011, the Dot/Icm T4SS was demonstrated to be essential for intracellular replication of *Coxiella* (Beare *et al*., 2011; Carey *et al*., 2011). A transposon mutant, disrupting *icmL*, rendered *Coxiella* incapable of Dot/Icm-dependent protein translocation and biogenesis of the replicative CCV (Carey *et al*., 2011). This finding was confirmed by Beare *et al*. (2011) who demonstrated that a transposon insertion in *icmD* also resulted in *Coxiella* unable to replicate inside host cells (Beare *et al*., 2011). A subsequent seminal paper, which described the first targeted gene deletion of *Coxiella*, demonstrated the same findings for deletion of both *dotA* and *dotB* (Beare *et al*., 2012). Both research groups observed that Dot/Icm-deficient *Coxiella* remain viable inside the lysosome of host cells, demonstrating that a functional T4SS is not required for resistance to lysosomal killing (Beare *et al*., 2011; Carey *et al*., 2011; Newton and Roy, 2011).

Despite both *Coxiella* and *Legionella* depending on a functional Dot/Icm system for intracellular replication, these systems are active at different times. This reflects the requirement for different effector functions, at different stages of infection, to facilitate the divergent intracellular niches of these pathogens. The T4SS of *L. pneumophila*

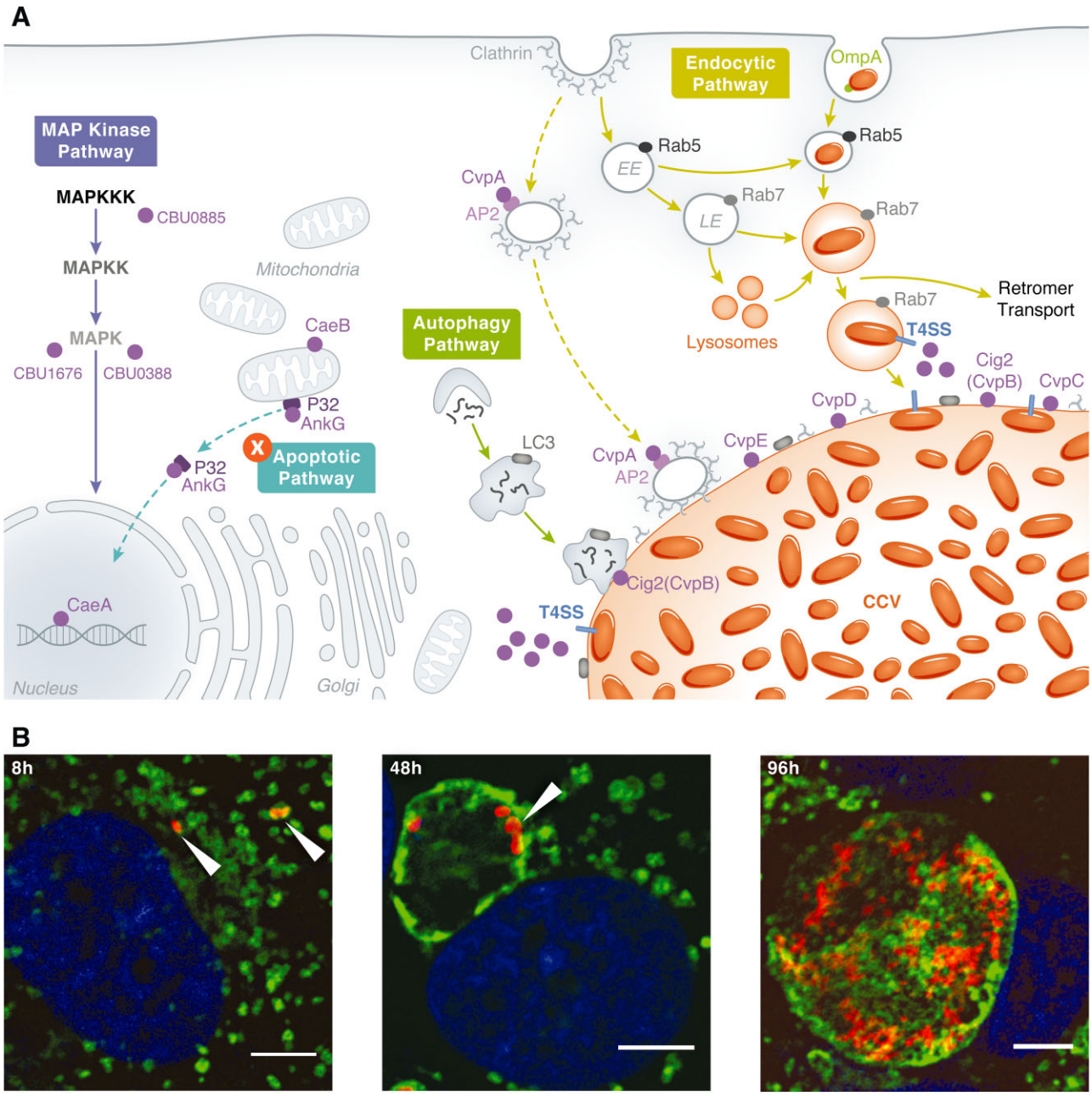

Fig. 1. *Coxiella* replicates within a unique and highly fusogenic, lysosome-derived vacuole remodelled by the actions of Dot/Icm effectors. Schematic representation of the biogenesis of the CCV highlights our current knowledge of mammalian pathways that are possibly influenced by Dot/Icm effectors (purple) (A). *Coxiella* enters the mammalian host cell through phagocytosis, likely facilitated by the action of the invasin OmpA. The early CCV traffics through the endocytic pathway via progressive interaction with EE and LE. Upon developing into an acidified vacuole with phagolysosomal characteristics, the Dot/Icm secretion system is activated and the LCV *Coxiella* replicate. Over 130 effectors are translocated through the Dot/Icm system into the host cytosol where they target specific eukaryotic processes leading to the remodelling of the CCV into a highly fusogenic vacuole that supports bacterial replication. It is known that the CCV fuses with clathrin-coated vesicles, autophagosomes and endolysosomal vesicles and effectors that may be involved in these processes are highlighted. Furthermore, specific effectors interact with signalling pathways and ectopic expression studies have shown that some localize to mitochondria and the nucleus to mediate a strong anti-apoptotic phenotype as seen during infection. Immunofluorescence microscopy of HeLa cells infected with *Coxiella* demonstrates that *Coxiella*, stained in red with an anti-*Coxiella* antibody, mediates expansion of the CCV prior to replication (B). Eight hours post-infection *Coxiella* are contained within a vacuole (arrow) decorated with lysosomal markers such as lysosomal-associated membrane protein 1 (green). Following activation of the Dot/Icm system, the CCV (arrow) rapidly expands through both homotypic and heterotypic fusion (48 h post-infection). Over a period of several days, demonstrated here at 96 h post-infection, the bacteria replicate until the host can no longer support the CCV and replicating LCVs begin to transition into small cell variants. Host nuclei are stained with DAPI (4',6-Diamidino-2-Phenylindole, blue) and scale bars represent 5 μm.

is assembled prior to host cell uptake, allowing rapid delivery of effectors upon initial contact (Nagai *et al*., 2005). This immediate activity allows *L. pneumophila* to translocate effectors that subvert the endocytic maturation of the LCV and promote fusion with secretory vesicles (reviewed in Prashar and Terebiznik, 2014). In contrast, the *Coxiella* Dot/Icm system does not begin translocating effectors until several hours post-infection (Carey *et al*., 2011; Newton *et al*., 2013). Using a BlaM reporter assay, in which an effector is transcriptionally fused to the β-lactamase reporter and translocation is detected by β-lactamase activity within the host cytosol, translocation by *Coxiella* cannot be detected unless the bacteria reach the acidic confines of the lysosome (Newton *et al*., 2013). *Coxiella* effector translocation does not occur when siRNA is used to silence the genes encoding Rab5 and Rab7, the Rab GTPases that direct endocytic maturation (Newton *et al*., 2013). This finding validates earlier reports regarding the importance of endocytic Rab GTPases in CCV formation that showed sequential acquisition of Rab5 and Rab7 onto the CCV, representing trafficking of the vacuole from early to late endosomes (Romano *et al*., 2007) (Fig. 1A). It is now clear that intracellular replication of *Coxiella* is dependent on the host endocytic process to deliver the pathogen to a lysosome that *Coxiella* then remodels, via the Dot/Icm system, to facilitate replication.

Effectors of the Dot/Icm system

To date, approximately 130 *Coxiella* Dot/Icm effectors have been identified. Given that the *L. pneumophila* effector repertoire accounts for approximately 10% of the genome, it is possible that there are 200 or more *Coxiella* effectors. However, there is significant functional redundancy within the *L. pneumophila* effector cohort and many effectors are present as families of paralogs (Luo and Isberg, 2004). The vast majority of known *Coxiella* effectors are unique, and therefore the known effectors may already represent close to the full cohort.

Identification of effectors

Initial studies to identify *Coxiella* Dot/Icm effectors used *L. pneumophila* as a surrogate host due to the ease with which this organism can be genetically manipulated and the functional analogy between the T4SSs (Pan *et al*., 2008; Voth *et al*., 2009; Chen *et al*., 2010). However, new genetic techniques have facilitated the development of methods to measure translocation by *Coxiella* using reporter systems such as β-lactamase and adenylate cyclase–effector fusions (Chen *et al*., 2010; Carey *et al*., 2011; Voth *et al*., 2011).

Both experimental and bioinformatic approaches have been employed to identify *Coxiella* Dot/Icm effectors. A common feature of *L. pneumophila* effectors is the presence of eukaryotic-like motifs (Hubber and Roy, 2010) and the genome sequence of *Coxiella* revealed genes encoding a range of eukaryotic motifs including ankyrin repeats and homology to eukaryotic kinases (Seshadri *et al*., 2003; Beare *et al*., 2009). Many of these proteins have subsequently been shown to be effectors (Pan *et al*., 2008; Voth *et al*., 2009; Chen *et al*., 2010; Weber *et al*., 2013; Larson *et al*., 2015). Computational modelling and machine learning techniques, based on characteristics of known Dot/Icm effectors, also identified novel effectors (Lifshitz *et al*., 2013; 2014). Bioinformatic predictions were combined with experimental verification to identify effectors that are regulated by the PmrAB two-component system (Zusman *et al*., 2007; Beare *et al*., 2014). This regulatory network is required for intracellular replication of *Coxiella* as it also controls the expression of components of the T4SS apparatus (Beare *et al*., 2014; Newton *et al*., 2014). Another study identified effectors based on their ability to interact with the inner membrane T4SS component DotF (Chen *et al*., 2010). Finally, we undertook a random genetic screen to identify fragments of *Coxiella* DNA that were capable of directing reporter translocation within *L. pneumophila* (Carey *et al*., 2011).

Heterogeneity in effector repertoire

Interestingly, following comparative genome analysis, a significant proportion of *Coxiella* effectors were classified as pseudogenes in some strains; genes interrupted by IS elements, small indels or nonsense mutations (Beare *et al*., 2009). This is very clearly demonstrated by the ankyrin repeat domain-containing effectors (Anks). The Nine Mile reference strain encodes only four intact Anks in comparison with 11 within the Dugway isolate (Voth *et al*., 2009). Similarly, there is a cohort of 12 plasmid-encoded effectors that vary among different isolates depending on the plasmid that they carry (Maturana *et al*., 2013). The high level of pseudogenization likely reflects genome reduction, and the loss of certain effectors may have contributed to the pathoadaptation of *Coxiella*. Presumably, those effectors that are highly conserved among *Coxiella* isolates represent effectors that make important contributions to the intracellular survival of the organism.

Some genes classified as pseudogenes may produce N-terminally truncated effectors still capable of translocation via the C-terminal translocation signal. However, these proteins may lack important functional domains. One such example is the Nine Mile effector CBU0080. The pseudogene *cbu0080* is transcribed by *Coxiella* and is capable of translocation, when expressed by

L. pneumophila; however, it lacks an N-terminal 19.4 kDa region present in the full-length K-strain homologue, CbuK1976. This region, absent in CBU0080, encodes a predicted nuclear localization signal and a helix-loop-helix motif that are presumably important for effector function (Carey *et al*., 2011).

Clues to effector functions

A range of phenotypic assays have been used to begin to attribute function to individual effectors. Microscopy analysis of ectopically expressed effectors in mammalian cells has revealed that some effectors specifically target a subcellular compartment such as the nucleus, mitochondria, microtubules, Golgi apparatus, autophagosomes and endocytic vesicles. Close to 100 effectors have been screened for the capacity to disrupt trafficking within the mammalian secretory pathway by examining the impact effector expression has on trafficking of the secreted alkaline phosphatase (SEAP) reporter (Carey *et al*., 2011; Weber *et al*., 2013). Ectopic expression of CBU0635, CBU1556 (CvpC), CBU1825 and CBUA0019 significantly diminishes the secretion of SEAP, suggesting these effectors may function to manipulate host vesicular trafficking (Carey *et al*., 2011; Weber *et al*., 2013).

Anti-apoptotic effectors. The host cell must maintain viability in order to support *Coxiella* replication. Several studies have ascertained that *Coxiella* actively inhibits host cell death machinery by preventing the activation of both the intrinsic, mediated by mitochondria, and extrinsic, death receptor-mediated, apoptotic pathways. In particular, inhibition of cytochrome *c* release from mitochondria and sustained activation of the pro-survival kinases Akt and Erk1/2 has been observed during *Coxiella* infection (Luhrmann and Roy, 2007; Voth *et al*., 2007; Voth and Heinzen, 2009).

One of the first *Coxiella* effectors to be identified, AnkG, was shown to block apoptosis on a physiologically relevant scale (Pan *et al*., 2008; Luhrmann *et al*., 2010). Expression of AnkG by *L. pneumophila* allowed this pathogen to replicate in normally restrictive mammalian host cells. Further investigation revealed that AnkG interacts with the host mitochondrial protein p32 and that this interaction is important for delivering AnkG to the nucleus where it exerts an anti-apoptotic phenotype (Luhrmann *et al*., 2010; Eckart *et al*., 2014).

Screening of previously identified *Coxiella* effectors (Carey *et al*., 2011) for the capacity to block intrinsic apoptosis revealed two more anti-apoptotic effectors, CBU1524 and CBU1532, renamed CaeA and CaeB (C*. burnetii a*nti-apoptotic *e*ffector) (Klingenbeck *et al*., 2013). Ectopically expressed CaeB, a mitochondrial effector, was able to inhibit both staurosporine and UV-induced apoptosis, while CaeA, a nuclear localized effector, was only able to inhibit UV-induced apoptosis, suggesting that these two proteins interfere with different aspects of apoptotic signalling (Klingenbeck *et al*., 2013). The discovery of three independent anti-apoptotic effectors indicates some functional redundancy; however, AnkG, CaeA and CaeB possess distinct mechanisms to block apoptosis and therefore may all contribute to the strong anti-apoptotic properties of *Coxiella*-infected cells. These studies only screened a small proportion of known *Coxiella* effectors and it is plausible that several more effectors contribute to maintaining host viability.

The data demonstrating the anti-apoptotic function of AnkG, CaeA and CaeB were not in the context of *Coxiella* infection. It still remains to be determined whether the absence of these effectors, individually or in combination, impacts on host cell survival during infection and the subsequent ability of *Coxiella* to replicate. The developing capacity to genetically manipulate *Coxiella* will provide the means to address these important questions.

Effectors that perturb yeast growth. Studies in both *L. pneumophila* and *Chlamydia* have demonstrated that expression of specific effectors in *Saccharomyces cerevisiae* can interfere with the rate of yeast replication due to their capacity to perturb eukaryotic pathways (Campodonico *et al*., 2005; Sisko *et al*., 2006; de Felipe *et al*., 2008). Two screens have expressed many effector proteins in yeast and identified only 10 effectors that exert some toxicity (Carey *et al*., 2011; Weber *et al*., 2013). However, no further functional characterization has been performed to determine how this toxicity is mediated.

A recent study identified CBU1818 (Cem11) and CBU0388 as effectors that are toxic to yeast (Lifshitz *et al*., 2014). This research examined the interplay between *Coxiella* effectors and the yeast MAP kinase pathways. Expression of effectors was performed in the presence of caffeine to activate the cell wall integrity MAP kinase pathway, revealing that CBU1676 (Cem9) and a homologue, CBU0885, inhibit yeast growth selectively in the presence of caffeine. These data indicate that these effectors likely modulate eukaryotic MAP kinase signalling. Further, the caffeine-induced toxicity of Cem9 was suppressed by the expression of CBU0388, suggesting that these effectors mediate opposing functions within this pathway (Lifshitz *et al*., 2014). As with the anti-apoptotic effectors, the importance of effectors that interfere with yeast growth has not yet been examined in the context of *Coxiella* infection.

The virulence contribution of individual effectors

The ability to introduce a replicative plasmid to introduce a transposon into the chromosome and to create targeted

gene deletions of *Coxiella* has transformed our capacity to understand the bacterial factors that contribute to *Coxiella* virulence. Several recent reports, characterizing *Coxiella* mutants, have demonstrated that many individual effectors have a measurable impact on intracellular replication (Larson *et al*., 2013; 2015; Weber *et al*., 2013; Martinez *et al*., 2014; Newton *et al*., 2014).

Transposon mutagenesis screens. In a study that identified novel *Coxiella* effectors, Weber *et al*. (2013) began to characterize a cohort of transposon mutants that disrupt genes encoding Dot/Icm effectors. Of 20 effector mutants, six were unable to replicate intracellularly and four showed significantly reduced replication. Multiple independent transposon mutants disrupting five of the six essential effectors (CBU0041, CBU0425, CBU0937, CBU2052 and CBU2059) reproduced the replication defect and the effectors were renamed CirA-E, respectively, for C*oxiella* effector for *i*ntracellular *r*eplication (Weber *et al*., 2013).

Martinez *et al*. (2014) conducted a large-scale transposon mutagenesis screen, testing over 1000 *Coxiella* mutants for internalization, intracellular replication and anti-apoptotic capacity using a sophisticated automated imaging approach. In addition to identifying OmpA as an outer membrane invasin, this screen demonstrated a reduced replication rate for mutants in 17 Dot/Icm effectors, including CirC, and indicated a putative role for CBU1639 in blocking host cell death (Martinez *et al*., 2014). Multiple independent mutants corroborated the findings for some effectors although it should be noted that for both the Weber *et al*. (2013) and Martinez *et al*. (2014) no further characterization of these effector mutants has been reported and no complementation data were presented.

We have also conducted a transposon mutagenesis screen, examining over 3000 independent *Coxiella* mutants for their ability to form replicative vacuoles in HeLa cells (Newton *et al*., 2014). We identified the Dot/Icm effectors Cig57, CoxCC8 and CBU1754 as being potentially important for intracellular growth of *Coxiella*. The *cig57::Tn* phenotype was verified through complementation (Newton *et al*., 2014). Our findings, of significantly fewer effectors impacting intracellular growth, potentially reflect the subjective visual analysis that was performed. Only the most severe growth defects were likely detected using this method.

Cig2 and vacuole biogenesis. We also identified the effector Cig2 as being important for CCV biogenesis. Multiple *Coxiella cig2::Tn* mutant strains demonstrated a striking multi-vacuole phenotype that could be complemented by introducing a plasmid-encoded copy of *cig2* (Newton *et al*., 2014). Interestingly, this phenotype was reminiscent of the multi-vacuole phenotype observed when expression of the host autophagy mediator Syntaxin-17 is silenced with siRNA (McDonough *et al*., 2013). Autophagy is a process the host cell uses to remove misfolded proteins, damaged organelles and intracellular pathogens via delivery to the lysosome for degradation (reviewed in Levine and Kroemer, 2008). The importance of autophagy for homotypic fusion of CCVs was confirmed with several different approaches to block autophagy, all resulting in the same multi-CCV phenotype (Newton *et al*., 2014). Several reports indicated that LC3 associates with CCVs in a Dot/Icm-dependent manner (Gutierrez *et al*., 2005; Romano *et al*., 2007; Winchell *et al*., 2014) and we demonstrated that, while autophagy is not up-regulated by *Coxiella* infection, LC3 is abundantly present on and in replicating CCVs. Importantly, the fusion of CCVs with autophagosomes is dependent on the effector Cig2 as CCVs formed by the *cig2* mutant did not show LC3 accumulation. Understanding any direct link between *Coxiella* subversion of autophagosomes and Cig2 will require elucidation of the biochemical function of Cig2.

Cvp effectors. Recently, Larson *et al*. (2013) observed the involvement of clathrin-mediated vesicular trafficking in CCV biogenesis. Clathrin-mediated endocytosis is a complex membrane budding process that occurs at the plasma membrane for the internalization of membrane proteins, lipids and certain nutrients. Construction of a clathrin-coated vesicle involves an assortment of accessory and adaptor proteins that can recognize sorting motifs within the cytoplasmic domains of transmembrane proteins (Kirchhausen, 1999; Braulke and Bonifacino, 2009). Clathrin was shown to be present on the CCV membrane and siRNA treatment to diminish clathrin and adaptor proteins significantly inhibited *Coxiella* replication. Bioinformatic analysis identified a protein encoding multiple endocytic sorting motifs, CBU0665. This protein, now renamed CvpA (C*oxiella v*acuolar *p*rotein A), was shown to be a Dot/Icm effector and a gene deletion mutant demonstrated that CvpA is required for bacterial replication (Larson *et al*., 2013). The mutant phenotype was complemented in a manner dependent on the CvpA endocytic sorting motifs. CvpA was shown to interact with the clathrin–adaptor complex AP2 and traffic through the endocytic recycling network (Larson *et al*., 2013). Taken together, these findings suggest that the interaction between CvpA and host cell clathrin transport machinery aid CCV biogenesis and intracellular replication, presumably by introducing important factors into the CCV.

In a subsequent study, the role of additional effectors that localize to the CCV membrane was examined (Larson *et al*., 2015). Deletion mutants of four CCV-localized effectors CvpB (Cig2, important for homotypic fusion of CCVs; Newton *et al*., 2014), CvpC (CBU1556,

previously shown to perturb the host secretory pathway; Weber *et al*., 2013), CvpD (CBU1818, shown to mediate yeast toxicity; Lifshitz *et al*., 2014) and the novel effector CvpE (CBU1863) were characterized. All of these mutants demonstrated a measurable intracellular replication defect, developing smaller CCVs with fewer bacteria. Phenotype complementation was demonstrated for CvpD and CvpE by Tn7 *cis*-complementation (Lifshitz *et al*., 2014).

Currently, with *Coxiella* mutagenesis still a very new technique, some results from mutagenesis studies have been conflicting. Different researchers have reported divergent phenotypes for mutations within the same effectors. This may reflect different ways of measuring intracellular replication and that some of the high-throughput screen data are yet to be validated using quantitative growth curves. The inability to functionally complement the growth defect attributed to some effector mutants is of concern and raises the possibility of undetected secondary mutations (Weber *et al*., 2013; Larson *et al*., 2015). These inconsistencies will likely be resolved as we continue to develop the *Coxiella* genetic toolbox.

Manipulation and dependence on host cell function

Elucidating effector functions provides an insightful way to understand the host cell processes that contribute to the intracellular success of *Coxiella* and how the pathogen actively modulates these processes. The recent deluge of effector information has highlighted important roles for autophagy, trafficking of clathrin-coated vesicles, apoptosis and signalling transduction during *Coxiella* infection. This information must be collated with studies that have examined host cell behaviour during infection to allow us to understand the global impact of *Coxiella* infection on a mammalian cell.

Vesicular trafficking

To date, the most comprehensive analysis of host pathways important for *Coxiella* infection was undertaken by McDonough *et al*. (2013). This study utilized a genome-wide siRNA screen to identify host factors involved in both CCV establishment and *Coxiella* replication in mammalian host cells. Using a high-throughput fluorescence-based screen, automated microscopy was employed to determine the size of vacuoles within siRNA-treated host cells. This technique allowed both an increase or a decrease in CCV size to be observed during gene silencing (McDonough *et al*., 2013).

As expected many proteins involved in either acidification of endocytic compartments, such as the host vacuolar ATPase (V-ATPase), or pH maintenance of lysosomal compartments, including CLN3 and CLCN5 (Golabek *et al*., 2000; Novarino *et al*., 2010), impacted vacuolar biogenesis. Additionally, the importance of several endocytic Rab proteins, including Rab5 and Rab7, to early establishment of CCV biogenesis was corroborated (McDonough *et al*., 2013).

The screen revealed the first evidence for the importance of the host retromer complex in *Coxiella* replication within host cells. Silencing of the retromer subunits VPS35 and VPS29 as well as the cargo adapters, sorting nexins SNX2, SNX3, SNX5, SNX6, resulted in smaller CCVs (McDonough *et al*., 2013). The retromer complex is involved in the retrograde transport of proteins from endosomes to the trans-Golgi network (Seaman *et al*., 1998). A receptor involved in the transport of acid hydrolases to lysosomes, the cation-independent mannose-6-phosphate receptor, is one example of proteins sorted by the retromer complex (Kornfeld, 1992). Further investigation demonstrated that Dot/Icm-dependent effector translocation was diminished in the absence of host retromer, indicating that retrograde trafficking contributes to developing the lysosomal environment that triggers Dot/Icm activation (McDonough *et al*., 2013).

Another family of proteins that are involved in mammalian vesicular trafficking, SNAREs (soluble N-ethylmaleimide-sensitive factor attachment protein receptor), are responsible for the docking and fusion of numerous vesicle-mediated transport events (Hong, 2005). Not surprisingly Campoy *et al*. (2013) observed localization of the endocytic SNAREs Vamp3, Vamp8, Vamp7 and Vti1b on the CCV. Vamp3 is involved in the recycling of plasma membrane receptors (Galli *et al*., 1998; Polgar *et al*., 2002), Vamp7 participates in the heterotypic fusion of late endosomes and lysosomes alongside Vti1b, and Vamp8 mediates homotypic fusion of late endosomes (Pryor *et al*., 2004). Either siRNA silencing of the endogenous Vamp7 or overexpression of a Vamp7 truncated mutant results in smaller CCVs (Campoy *et al*., 2013). Interestingly, treatment of infected cells with chloramphenicol, halting bacterial translation, correlated with diminished Vamp7 recruitment to the CCV, suggesting that the presence of Vamp7 is, at least in part, bacterially driven possibly through effector action (Campoy *et al*., 2013). Ectopic expression has identified effectors that localize to various organelles within the endomembrane network, including the CCV, and those effectors that disrupted SEAP trafficking may also be candidates for modulating host vesicular trafficking.

Signal transduction

Effectors have been identified that modulate MAP kinase signalling in yeast and Hussain *et al*. (2010) demonstrated a role for calmodulin kinase II, myosin light chain

kinase, protein kinase C and cAMP-dependent protein kinase (PKA) in CCV development (Hussain *et al*., 2010). PKA regulatory subunit I traffics to the CCV membrane in a manner that is believed to be effector driven (MacDonald *et al*., 2014). Further investigation revealed that PKA activation and the phosphorylation of its downstream targets are differentially regulated throughout the course of *Coxiella* infection. In particular, a significant increase in the phosphorylation of the pro-apoptotic protein Bad was observed (MacDonald *et al*., 2012; 2014). During infection, Bad binds the adapter 14-3-3β and the complex is recruited to the CCV where it can be phosphorylated by PKA to consolidate CCV localization. This physical separation of Bad from mitochondrial proteins, such as Bcl-2, aids in preventing host cell apoptosis (MacDonald *et al*., 2014). Identification of specific effector targets, such as PKA, Bad and 14-3-3β, provides another approach to assign effector functions. Screening mutant libraries of *Coxiella* should elucidate the effector(s) responsible for the altered PKA localization and sequestration of Bad and 14-3-3β.

Future directions in understanding *Coxiella* pathogenesis

Axenic culture conditions have opened the door to a new world exploring the molecular pathogenesis of *Coxiella*. The recent reports of transposon mutant libraries and the ability to construct targeted clean deletions of this organism allow us to ask defined questions about the molecular requirements for intracellular replication of *Coxiella* and subsequent pathogenicity. Indeed we already have a growing list of *Coxiella* Dot/Icm effectors that play crucial roles in CCV biogenesis. The next challenge will be to move beyond identifying the effectors that are important for intracellular replication of *Coxiella* to understanding how they function. Clearly, we now know of several eukaryotic processes that are targeted by *Coxiella* effectors, but the challenge will lie in elucidating the effector functions to understand how these processes are manipulated.

The use of tissue culture models of infection may limit our ability to understand the function of some effectors. Given the co-evolution of *Coxiella* and mammalian hosts, it seems likely that some effectors will modulate innate immune signalling to impact on disease burden within the multicellular host. Studies using effector mutants within phase I, virulent, *Coxiella* and an animal model of infection will be required to fully investigate the impact such effectors have on disease progression.

It is clear that *Coxiella* utilizes the Dot/Icm system to remodel the lysosome-derived CCV in order for replication to proceed. The collective action of the Dot/Icm effectors facilitates this replication by manipulating a variety of host processes. An intriguing question that remains is how *Coxiella* combats the low pH, proteolytic and oxidative confines of the lysosome-derived CCV. Dot/Icm-deficient *Coxiella* remain viable within the lysosome, indicating that there are other determinants that provide the unique lysosomal resistance of this bacterium. Transposon mutant screens may shed light on genes responsible for this phenotype; however, growth in ACCM, used to recover transposon mutants, may already select for *Coxiella* that is lysosome resistant. Understanding this phenomenon may require the continued development of novel strategies to manipulate *Coxiella*.

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References

van Asseldonk, M.A., Prins, J., and Bergevoet, R.H. (2013) Economic assessment of Q fever in the Netherlands. *Prev Vet Med* **112:** 27–34.

Beare, P.A., Unsworth, N., Andoh, M., Voth, D.E., Omsland, A., Gilk, S.D., *et al*. (2009) Comparative genomics reveal extensive transposon-mediated genomic plasticity and diversity among potential effector proteins within the genus *Coxiella*. *Infect Immun* **77:** 642–656.

Beare, P.A., Gilk, S.D., Larson, C.L., Hill, J., Stead, C.M., Omsland, A., *et al*. (2011) Dot/Icm type IVB secretion system requirements for *Coxiella burnetii* growth in human macrophages. *MBio* **2:** e00175–11.

Beare, P.A., Larson, C.L., Gilk, S.D., and Heinzen, R.A. (2012) Two systems for targeted gene deletion in *Coxiella burnetii*. *Appl Environ Microbiol* **78:** 4580–4589.

Beare, P.A., Sandoz, K.M., Larson, C.L., Howe, D., Kronmiller, B., and Heinzen, R.A. (2014) Essential role for the response regulator PmrA in *Coxiella burnetii* type 4B secretion and colonization of mammalian host cells. *J Bacteriol* **196:** 1925–1940.

Braulke, T., and Bonifacino, J.S. (2009) Sorting of lysosomal proteins. *Biochim Biophys Acta* **1793:** 605–614.

Campodonico, E.M., Chesnel, L., and Roy, C.R. (2005) A yeast genetic system for the identification and characterization of substrate proteins transferred into host cells by the *Legionella pneumophila* Dot/Icm system. *Mol Microbiol* **56:** 918–933.

Campoy, E.M., Mansilla, M.E., and Colombo, M.I. (2013) Endocytic SNAREs are involved in optimal *Coxiella burnetii* vacuole development. *Cell Microbiol* **15:** 922–941.

Carey, K.L., Newton, H.J., Luhrmann, A., and Roy, C.R. (2011) The *Coxiella burnetii* Dot/Icm system delivers a unique repertoire of type IV effectors into host cells and is required for intracellular replication. *PLoS Pathog* **7:** e1002056.

Chen, C., Banga, S., Mertens, K., Weber, M.M., Gorbaslieva, I., Tan, Y., *et al*. (2010) Large-scale identification and

translocation of type IV secretion substrates by *Coxiella burnetii*. *Proc Natl Acad Sci USA* **107:** 21755–21760.
Christie, P.J., and Vogel, J.P. (2000) Bacterial type IV secretion: conjugation systems adapted to deliver effector molecules to host cells. *Trends Microbiol* **8:** 354–360.
Coleman, S.A., Fischer, E.R., Howe, D., Mead, D.J., and Heinzen, R.A. (2004) Temporal analysis of *Coxiella burnetii* morphological differentiation. *J Bacteriol* **186:** 7344–7352.
Delsing, C.E., Warris, A., and Bleeker-Rovers, C.P. (2011) Q fever: still more queries than answers. *Adv Exp Med Biol* **719:** 133–143.
Eckart, R.A., Bisle, S., Schulze-Luehrmann, J., Wittmann, I., Jantsch, J., Schmid, B., *et al*. (2014) Antiapoptotic activity of *Coxiella burnetii* effector protein AnkG is controlled by p32-dependent trafficking. *Infect Immun* **82:** 2763–2771.
de Felipe, K.S., Glover, R.T., Charpentier, X., Anderson, O.R., Reyes, M., Pericone, C.D., and Shuman, H.A. (2008) *Legionella* eukaryotic-like type IV substrates interfere with organelle trafficking. *PLoS Pathog* **4:** e1000117.
Galli, T., Zahraoui, A., Vaidyanathan, V.V., Raposo, G., Tian, J.M., Karin, M., *et al*. (1998) A novel tetanus neurotoxin-insensitive vesicle-associated membrane protein in SNARE complexes of the apical plasma membrane of epithelial cells. *Mol Biol Cell* **9:** 1437–1448.
Golabek, A.A., Kida, E., Walus, M., Kaczmarski, W., Michalewski, M., and Wisniewski, K.E. (2000) CLN3 protein regulates lysosomal pH and alters intracellular processing of Alzheimer's amyloid-beta protein precursor and cathepsin D in human cells. *Mol Genet Metab* **70:** 203–213.
Gutierrez, M.G., Vazquez, C.L., Munafo, D.B., Zoppino, F.C., Beron, W., Rabinovitch, M., and Colombo, M.I. (2005) Autophagy induction favours the generation and maturation of the *Coxiella*-replicative vacuoles. *Cell Microbiol* **7:** 981–993.
van der Hoek, W., Morroy, G., Renders, N.H., Wever, P.C., Hermans, M.H., Leenders, A.C., and Schneeberger, P.M. (2012) Epidemic Q fever in humans in the Netherlands. *Adv Exp Med Biol* **984:** 329–364.
Hong, W. (2005) SNAREs and traffic. *Biochim Biophys Acta* **1744:** 493–517.
Hoover, T.A., Culp, D.W., Vodkin, M.H., Williams, J.C., and Thompson, H.A. (2002) Chromosomal DNA deletions explain phenotypic characteristics of two antigenic variants, phase II and RSA 514 (crazy), of the *Coxiella burnetii* nine mile strain. *Infect Immun* **70:** 6726–6733.
Howe, D., Melnicakova, J., Barak, I., and Heinzen, R.A. (2003) Fusogenicity of the *Coxiella burnetii* parasitophorous vacuole. *Ann N Y Acad Sci* **990:** 556–562.
Howe, D., Shannon, J.G., Winfree, S., Dorward, D.W., and Heinzen, R.A. (2010) *Coxiella burnetii* phase I and II variants replicate with similar kinetics in degradative phagolysosome-like compartments of human macrophages. *Infect Immun* **78:** 3465–3474.
Hubber, A., and Roy, C.R. (2010) Modulation of host cell function by *Legionella pneumophila* type IV effectors. *Annu Rev Cell Dev Biol* **26:** 261–283.
Hussain, S.K., Broederdorf, L.J., Sharma, U.M., and Voth, D.E. (2010) Host kinase activity is required for *Coxiella burnetii* parasitophorous vacuole formation. *Front Microbiol* **1:** 137.
Khavkin, T., and Tabibzadeh, S.S. (1988) Histologic, immunofluorescence, and electron microscopic study of infectious process in mouse lung after intranasal challenge with *Coxiella burnetii*. *Infect Immun* **56:** 1792–1799.
Kirchhausen, T. (1999) Adaptors for clathrin-mediated traffic. *Annu Rev Cell Dev Biol* **15:** 705–732.
Klingenbeck, L., Eckart, R.A., Berens, C., and Luhrmann, A. (2013) The *Coxiella burnetii* type IV secretion system substrate CaeB inhibits intrinsic apoptosis at the mitochondrial level. *Cell Microbiol* **15:** 675–687.
Kornfeld, S. (1992) Structure and function of the mannose 6-phosphate/insulinlike growth factor II receptors. *Annu Rev Biochem* **61:** 307–330.
Larson, C.L., Beare, P.A., Howe, D., and Heinzen, R.A. (2013) *Coxiella burnetii* effector protein subverts clathrin-mediated vesicular trafficking for pathogen vacuole biogenesis. *Proc Natl Acad Sci USA* **110:** E4770–E4779.
Larson, C.L., Beare, P.A., Voth, D.E., Howe, D., Cockrell, D.C., Bastidas, R.J., *et al*. (2015) *Coxiella burnetii* effector proteins that localize to the parasitophorous vacuole membrane promote intracellular replication. *Infect Immun* **83:** 661–670.
Levine, B., and Kroemer, G. (2008) Autophagy in the pathogenesis of disease. *Cell* **132:** 27–42.
Lifshitz, Z., Burstein, D., Peeri, M., Zusman, T., Schwartz, K., Shuman, H.A., *et al*. (2013) Computational modeling and experimental validation of the *Legionella* and *Coxiella* virulence-related type-IVB secretion signal. *Proc Natl Acad Sci USA* **110:** E707–E715.
Lifshitz, Z., Burstein, D., Schwartz, K., Shuman, H.A., Pupko, T., and Segal, G. (2014) Identification of novel *Coxiella burnetii* Icm/Dot effectors and genetic analysis of their involvement in modulating a mitogen-activated protein kinase pathway. *Infect Immun* **82:** 3740–3752.
van Loenhout, J.A., Hautvast, J.L., Vercoulen, J.H., Akkermans, R.P., Wijkmans, C.J., van der Velden, K., and Paget, W.J. (2015) Q-fever patients suffer from impaired health status long after the acute phase of the illness: results from a 24-month cohort study. *J Infect* **70:** 237–246.
Luhrmann, A., and Roy, C.R. (2007) *Coxiella burnetii i*nhibits activation of host cell apoptosis through a mechanism that involves preventing cytochrome c release from mitochondria. *Infect Immun* **75:** 5282–5289.
Luhrmann, A., Nogueira, C.V., Carey, K.L., and Roy, C.R. (2010) Inhibition of pathogen-induced apoptosis by a *Coxiella burnetii* type IV effector protein. *Proc Natl Acad Sci USA* **107:** 18997–19001.
Luo, Z.Q., and Isberg, R.R. (2004) Multiple substrates of the *Legionella pneumophila* Dot/Icm system identified by interbacterial protein transfer. *Proc Natl Acad Sci USA* **101:** 841–846.
MacDonald, L.J., Kurten, R.C., and Voth, D.E. (2012) *Coxiella burnetii* alters cyclic AMP-dependent protein kinase signaling during growth in macrophages. *Infect Immun* **80:** 1980–1986.
MacDonald, L.J., Graham, J.G., Kurten, R.C., and Voth, D.E. (2014) *Coxiella burnetii* exploits host cAMP-dependent

protein kinase signalling to promote macrophage survival. *Cell Microbiol* **16:** 146–159.

McDonough, J.A., Newton, H.J., Klum, S., Swiss, R., Agaisse, H., and Roy, C.R. (2013) Host pathways important for *Coxiella burnetii* infection revealed by genome-wide RNA interference screening. *MBio* **4:** e00606-12.

Madariaga, M.G., Rezai, K., Trenholme, G.M., and Weinstein, R.A. (2003) Q fever: a biological weapon in your backyard. *Lancet Infect Dis* **3:** 709–721.

Martinez, E., Cantet, F., Fava, L., Norville, I., and Bonazzi, M. (2014) Identification of OmpA, a *Coxiella burnetii* protein involved in host cell invasion, by multi-phenotypic high-content screening. *PLoS Pathog* **10:** e1004013.

Maturana, P., Graham, J.G., Sharma, U.M., and Voth, D.E. (2013) Refining the plasmid-encoded type IV secretion system substrate repertoire of *Coxiella burnetii*. *J Bacteriol* **195:** 3269–3276.

Moos, A., and Hackstadt, T. (1987) Comparative virulence of intra- and interstrain lipopolysaccharide variants of *Coxiella burnetii* in the guinea pig model. *Infect Immun* **55:** 1144–1150.

Nagai, H., Cambronne, E.D., Kagan, J.C., Amor, J.C., Kahn, R.A., and Roy, C.R. (2005) A C-terminal translocation signal required for Dot/Icm-dependent delivery of the *Legionella* RalF protein to host cells. *Proc Natl Acad Sci USA* **102:** 826–831.

Newton, H.J., and Roy, C.R. (2011) The *Coxiella burnetii* Dot/Icm system creates a comfortable home through lysosomal renovation. *MBio* **2:** e00226–11.

Newton, H.J., McDonough, J.A., and Roy, C.R. (2013) Effector protein translocation by the *Coxiella burnetii* Dot/Icm type IV secretion system requires endocytic maturation of the pathogen-occupied vacuole. *PLoS ONE* **8:** e54566.

Newton, H.J., Kohler, L.J., McDonough, J., Temoche-Diaz, M., Crabhill, E., Hartland, E.L., and Roy, C.R. (2014) A screen of *Coxiella burnetii* mutants reveals important roles for Dot/Icm effectors and host autophagy in vacuole biogenesis. *PLoS Pathog* **10:** e1004286.

Novarino, G., Weinert, S., Rickheit, G., and Jentsch, T.J. (2010) Endosomal chloride-proton exchange rather than chloride conductance is crucial for renal endocytosis. *Science (New York, N.Y.)* **328:** 1398–1401.

Omsland, A., Cockrell, D.C., Howe, D., Fischer, E.R., Virtaneva, K., Sturdevant, D.E., *et al.* (2009) Host cell-free growth of the Q fever bacterium *Coxiella burnetii*. *Proc Natl Acad Sci USA* **106:** 4430–4434.

Pan, X., Luhrmann, A., Satoh, A., Laskowski-Arce, M.A., and Roy, C.R. (2008) Ankyrin repeat proteins comprise a diverse family of bacterial type IV effectors. *Science (New York, N.Y.)* **320:** 1651–1654.

Polgar, J., Chung, S.H., and Reed, G.L. (2002) Vesicle-associated membrane protein 3 (VAMP-3) and VAMP-8 are present in human platelets and are required for granule secretion. *Blood* **100:** 1081–1083.

Prashar, A., and Terebiznik, M.R. (2014) *Legionella pneumophila*: homeward bound away from the phagosome. *Curr Opin Microbiol* **23C:** 86–93.

Pryor, P.R., Mullock, B.M., Bright, N.A., Lindsay, M.R., Gray, S.R., Richardson, S.C., *et al.* (2004) Combinatorial SNARE complexes with VAMP7 or VAMP8 define different late endocytic fusion events. *EMBO Rep* **5:** 590–595.

Romano, P.S., Gutierrez, M.G., Beron, W., Rabinovitch, M., and Colombo, M.I. (2007) The autophagic pathway is actively modulated by phase II *Coxiella burnetii* to efficiently replicate in the host cell. *Cell Microbiol* **9:** 891–909.

Seaman, M.N., McCaffery, J.M., and Emr, S.D. (1998) A membrane coat complex essential for endosome-to-Golgi retrograde transport in yeast. *J Cell Biol* **142:** 665–681.

Segal, G., Purcell, M., and Shuman, H.A. (1998) Host cell killing and bacterial conjugation require overlapping sets of genes within a 22-kb region of the *Legionella pneumophila* genome. *Proc Natl Acad Sci USA* **95:** 1669–1674.

Seshadri, R., Paulsen, I.T., Eisen, J.A., Read, T.D., Nelson, K.E., Nelson, W.C., *et al.* (2003) Complete genome sequence of the Q-fever pathogen *Coxiella burnetii*. *Proc Natl Acad Sci USA* **100:** 5455–5460.

Sisko, J.L., Spaeth, K., Kumar, Y., and Valdivia, R.H. (2006) Multifunctional analysis of *Chlamydia*-specific genes in a yeast expression system. *Mol Microbiol* **60:** 51–66.

Veras, P.S., de Chastellier, C., Moreau, M.F., Villiers, V., Thibon, M., Mattei, D., and Rabinovitch, M. (1994) Fusion between large phagocytic vesicles: targeting of yeast and other particulates to phagolysosomes that shelter the bacterium *Coxiella burnetii* or the protozoan *Leishmania amazonensis* in Chinese hamster ovary cells. *J Cell Sci* **107** (Part 11)**:** 3065–3076.

Vogel, J.P., Andrews, H.L., Wong, S.K., and Isberg, R.R. (1998) Conjugative transfer by the virulence system of *Legionella pneumophila*. *Science (New York, N.Y.)* **279:** 873–876.

Voth, D.E., and Heinzen, R.A. (2007) Lounging in a lysosome: the intracellular lifestyle of *Coxiella burnetii*. *Cell Microbiol* **9:** 829–840.

Voth, D.E., and Heinzen, R.A. (2009) Sustained activation of Akt and Erk1/2 is required for *Coxiella burnetii* antiapoptotic activity. *Infect Immun* **77:** 205–213.

Voth, D.E., Howe, D., and Heinzen, R.A. (2007) *Coxiella burnetii* inhibits apoptosis in human THP-1 cells and monkey primary alveolar macrophages. *Infect Immun* **75:** 4263–4271.

Voth, D.E., Howe, D., Beare, P.A., Vogel, J.P., Unsworth, N., Samuel, J.E., and Heinzen, R.A. (2009) The *Coxiella burnetii* ankyrin repeat domain-containing protein family is heterogeneous, with C-terminal truncations that influence Dot/Icm-mediated secretion. *J Bacteriol* **191:** 4232–4242.

Voth, D.E., Beare, P.A., Howe, D., Sharma, U.M., Samoilis, G., Cockrell, D.C., *et al.* (2011) The *Coxiella burnetii* cryptic plasmid is enriched in genes encoding type IV secretion system substrates. *J Bacteriol* **193:** 1493–1503.

Weber, M.M., Chen, C., Rowin, K., Mertens, K., Galvan, G., Zhi, H., *et al.* (2013) Identification of *C. burnetii* type IV secretion substrates required for intracellular replication and *Coxiella*-containing vacuole formation. *J Bacteriol* **195:** 3914–3924.

Winchell, C.G., Graham, J.G., Kurten, R.C., and Voth, D.E. (2014) *Coxiella burnetii* type IV secretion-dependent

recruitment of macrophage autophagosomes. *Infect Immun* **82:** 2229–2238.

Zamboni, D.S., McGrath, S., Rabinovitch, M., and Roy, C.R. (2003) *Coxiella burnetii* express type IV secretion system proteins that function similarly to components of the *Legionella pneumophila* Dot/Icm system. *Mol Microbiol* **49:** 965–976.

Zusman, T., Yerushalmi, G., and Segal, G. (2003) Functional similarities between the *icm/dot* pathogenesis systems of *Coxiella burnetii* and *Legionella pneumophila*. *Infect Immun* **71:** 3714–3723.

Zusman, T., Aloni, G., Halperin, E., Kotzer, H., Degtyar, E., Feldman, M., and Segal, G. (2007) The response regulator PmrA is a major regulator of the *icm/dot* type IV secretion system in *Legionella pneumophila* and *Coxiella burnetii*. *Mol Microbiol* **63:** 1508–1523.