An analysis of sub-cellular localization of host sorting nexin 4 and 7 in *Coxiella burnetii* infected Hela cells

Authors: Sydni Smith, Saugata Mahapatra PhD., Edward Shaw PhD.*
Department of Microbiology and Molecular Genetics, Oklahoma State University

Abstract

Coxiella burnetii is a zoonotic obligate intracellular bacterial pathogen that is the causative agent of acute and chronic disease in humans (Kazar 2005). Bacterial replication is dependent on *C. burnetii* protein synthesis to create and maintain enlarged parasitophorous vacuoles (PV) within its eukaryotic host cell (Tang and Ho 2007). Human infection occurs with the inhalation of aerosols containing the small cell variant (SCV) form of *C. burnetii* (Tang and Ho 2007). Once these metabolically inactive SCVs are passively engulfed by mononuclear phagocytes in the lung, they differentiate into metabolically active and replicative large cell variants (LCVs) (Coleman et al. 2004). *C. burnetii* LCVs then undergo a series of maturation steps through the host cell endocytic pathway to develop into a PV with lysosomal characteristics(Coleman et al. 2004). LCV replication is accompanied by a remarkable expansion of the PV(Coleman et al. 2004). The formation of this spacious PV (SPV) indicates that *C. burnetii* manipulates host cell pathways to survive and replicate(Coleman et al. 2004).

Research shows that proteins play a key role in determining *C. burnetii*'s interactions with its eukaryotic host cell(Tang and Ho 2007). The host proteins involved in the maturation of the *C. burnetii* PV remain largely unidentified. To identify host proteins that function during *C. burnetii* PV trafficking and maturation, we have initiated sub-cellular localization studies on sorting nexins (SNXs). SNXs are key regulators of the endosomal sorting system and have diverse roles in membrane remodeling, protein and lipid trafficking, and organelle motility(Cullen 2008, van Weering et al. 2012). Two different GFP-tagged sorting nexin proteins, SNX4 and SNX7, were chosen and their sub-cellular localization in *C. burnetii* infected Hela cells observed using indirect immunofluorescent antibody (IFA) and Laser Scanning confocal microscopy (LSCM). Confocal analysis displays co-localization of *C. burnetii* with SNX4, a protein that may play a crucial role in parasitophorous vacuole trafficking and maturation within an infected eukaryotic Hela cell.

Keywords: Coxiella burnetii, sorting nexin, protein, co-localization

Introduction

Coxiella burnetii is a zoonotic obligate intracellular bacterium that is the causative agent of Q fever in humans (Kazar 2005). *C. burnetii* has been classified as a "Class III" biohazard, and a U.S. Centers for Disease Control and Prevention "Category B" biological warfare agent due to its low infectious dose and ability to survive in the extracellular SCV form for long periods of time(Beare et al. 2009). Due to its numerous modes of infection, it is broadly transmissive and a risk to both human and animal health(Noden et al. 2014).

Obligate intracellular bacteria such as *C. burnetii* have evolved to manipulate their host cells using a range of proteins(Tang and Ho 2007). Exploitation of host cell signaling pathways allows for manipulation of host cells, suggesting that *C. burnetii* relies on these pathways for intracellular replication(Czyz et al. 2014). *C. burnetii* is metabolically complex and shows the ability to produce bacterial proteins that mimic the activity of host cell proteins and exploit the metabolic processes of the host cell(Beare et al. 2009). *C. burnetii* protein synthesis may modify host

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cell gene expression to allow for replication within the host cell while simultaneously evading an immune response(Gallaher). The maturation and protein manipulation mechanisms utilized by *C. burnetii* in its maturation along the endocytic pathway are presently unknown.

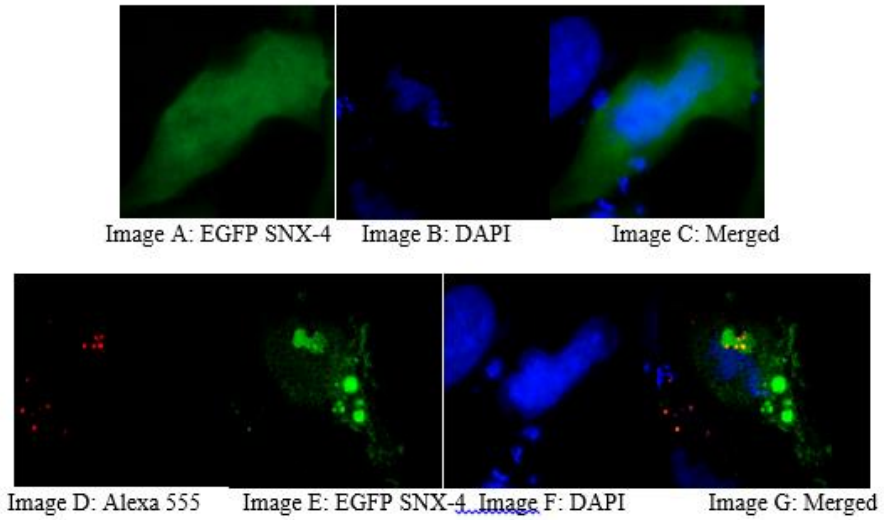

Figure 1: EGFP-SNX4 localizes with *C. burnetii* parasitophorous vacuoles

Sorting nexin (SNXs) proteins are fundamental in the regulation of endosomal sorting (van Weering et al. 2012). Endosomal sorting is crucial in maintaining the internal homeostasis of a cell (van Weering et al. 2012). The endocytic pathway receives cellular material from the cell membrane, Golgi complex, and cytoplasm molecules through the endocytosis process (Bishop 2003). Much of the endocytic pathway relies on the early endosome, which is responsible for the allocation of lipids and proteins throughout the cell (Beck et al. 2012). In this study, we have examined two different GFP-tagged sorting nexin proteins, SNX4 and SNX7, for their sub-cellular localization in *C. burnetii* infected Hela cells. Recent studies have shown that SNX4 localizes with early endosomes, suggesting that SNX4 may play a role in regulating the endosome sorting system (van Weering et al. 2012). Other studies show that SNX4 functions in transferrin receptor sorting, transferrin being the main protein transporting iron in the blood(Cullen 2008) , and displays localization throughout the cytoplasm of the cell (Traer et al. 2007). Unlike SNX4, not much is known about the function of SNX7 (Cullen 2008). However, it is known to bind SNX4 *in vitro* (Cullen 2008).

Materials and Methods

SNX4 and 7, intrinsic to Hela host cells, were selected for their association with vesicle trafficking. Each of these proteins were expressed with a fluorescent tag within infected Hela cells to observe the effect *C. burnetii* may have on the localization of these proteins within the host cell. Uninfected Hela host cells were observed as a control, and employed in the comparison of SNX protein localization within infected Hela cells.

Diluted samples of 1×10^5 Hela cells were seeded onto a sterile cover slip in 24-well tissue culture plates. Cells were transiently transfected with EGFP-SNX4, and EGFP-SNX7 recombinant plasmids. After a twenty-four period of incubation, one cell line was infected with *C. burnetii* for another 24 hours, during which *C. burnetii* reached the early stages of infection. A separate line of SNX-transfected Hela cells were left uninfected to serve as a control and comparison group. All cells were chemically fixed with 4% PFA and MeOH at 24 hours post infection (hpi), and were then stained with a primary anti-*C. burnetii* guinea pig IgG. Goat anti-guinea

pig Alexa 555 was used as a secondary antibody. Host and bacterial DNA were stained using 4',6-diamidino-2-phenylindole (DAPI). Uninfected cells utilized only DAPI staining and the EGFP marker. Indirect immunofluorescent antibody (IFA) and Laser Scanning confocal microscopy (LSCM) was utilized to analyze the co-localization of the SNX4 and -7 proteins within *C. burnetii* infected Hela cells. IFA microscopy was conducted using a Nikon Eclipse TE 2000-S microscope with a Nikon DS FI1 camera and NIS-ELEMENTS F 3.00 software. Laser Scanning confocal microscopy (LSCM) was performed by employing Leica DM E14 upright microscope. IMAGEJ version 1.42n (Wayne Rasband, NIH) was also used for image processing.

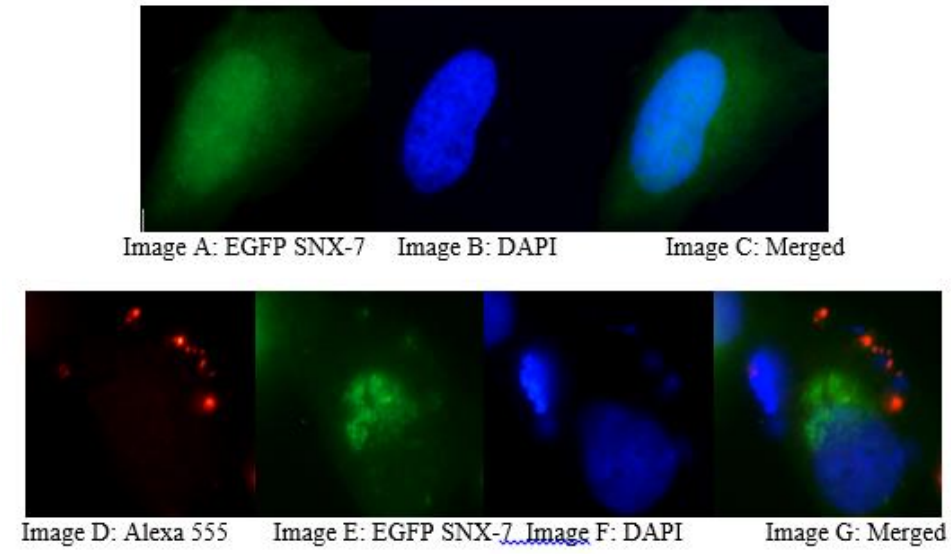

Figure 2: EGFP-SNX7 does not localizes with *C. burnetii* parasitophorus vacuoles

Results

Figure 1: EGFP-SNX4 localizes with *C. burnetii* parasitophorus vacoules: HeLa cells were transiently transfected with GFP-tagged SNX4. At 18h post-transfection, cells were infected with *C. burnetii* for 24h.

A. The SNX4 protein tagged with GFP (green) localizes evenly throughout the cytoplasm of the uninfected control cell
B. The Hela cell nucleus of the uninfected cell, stained with DAPI (blue)
C. Merged image of EGFP-SNX4 and the DAPI stained nucleus shows localization of EGFP-SNX4 protein throughout the host cell cytoplasm
D. *C. burnetii* stained with guinea pig anti-*C. burnetii* primary antibody and Goat anti-guinea pig IgG tagged with Alexa 555 secondary antibody (red) at 24hours post infection in the early stages of infection and beginning to form the basis of a parasitophorous vacuole.
E. The EGFP-SNX4 protein at 24 hours post-infection is considerably less diffused in the cytoplasm and localizes as circular compartments of various sizes throughout the host cell cytosol
F. The Hela cell nucleus of the infected cell, stained with DAPI (blue)
G. Merged image of EGFP-SNX4 and *C. burnetii* shows co-localization of EGFP-SNX4 protein with *C. burnetii* in the peri-nuclear region of the host cell

Figure 2: EGFP-SNX7 does not localize with *C. burnetii* parasitophorus vacoules: HeLa cells were transiently transfected with GFP-tagged SNX7. At 18h post-transfection, cells were infected with *C. burnetii* for 24h.

A. The SNX7 protein tagged with GFP (green) localizes evenly throughout the cytoplasm of the uninfected control cell
B. The Hela cell nucleus of the uninfected cell, stained with DAPI (blue)
C. Merged image of EGFP-SNX7 and the DAPI stained nucleus shows localization of EGFP-SNX7 protein within the host cell cytosol
D. *C. burnetii* stained with guinea pig anti-*C. burnetii* primary antibody and Goat anti-guinea pig IgG tagged with Alexa 555 secondary antibody (red) at 24 hours post infection in the early stages of infection and beginning to form the basis of a parasitophorous vacuole.
E. The EGFP-SNX7 protein at 24 hours post-infection remains diffused in the cytoplasm and does not colocalize with *C. burnetii* in the host cell cytoplasm
F. The Hela cell nucleus of the infected cell, stained with DAPI (blue)
G. Merged image of EGFP-SNX7 and *C. burnetii* shows no co-localization of EGFP-SNX7 protein with *C. burnetii* within the host cell

Conclusion

After imaging with IFA and LSCM microscopy and comparing the control uninfected cells to the infected cells, co-localization is apparent in SNX4, and little to no co-localization is observed with SNX7, as shown in the Figures 1 and 2. From this experimental data, it is likely that *C. burnetii* may play a part in regulating the production and localization of certain SNX proteins within an infected host cell. In particular, studies have shown that SNX4 is a key contributor in the endosomal sorting of transferrin. As the IFA and LSCM images suggest, *C. burnetii* may co-localize with SNX4 in the early stages of infection, leading to the possibility that *C. burnetii* employs SNX4 to exploit the transferrin trafficking pathway. Further testing and analysis is required to draw more definitive results.

C. burnetii trafficks within the endocytic pathway in order to reach a low pH environment and allows lysosomal fusion to become metabolically active (Cullen 2008). Recent studies have suggested that the protein SNX4 may be a key regulator in the endosome sorting system (van Weering et al. 2012). The host endocytic pathway is critical in the continual maintenance of internal cell homeostasis (Cullen 2008). Constant membrane flux within the endosomal trafficking network is highly dynamic (Cullen 2008). Cargo within endocytic vesicles are rapidly delivered to membrane-bound endocytic organelles called early endosomes (EE) that serve as the focal point for endocytic sorting (Cullen 2008). This sorting process involves synchronized mobilization of cargo to EE microdomains followed by a change in EE morphology (Cullen 2008). Cargo is either delivered to the plasma membrane via recycling pathways, the *trans*-Golgi network via biosynthetic pathway (retrograde transport), or the lysosome via a degradative pathway (Cullen 2008).

Sorting nexins (SNXs) are key regulators of endosomal sorting and have functionally diverse roles in membrane remodeling, cargo trafficking, and organelle motility (Cullen 2008; van Weering et al. 2012). In particular, other studies have shown that SNXs are increasingly associated with pathogen invasion, with SNX4 in particular being a key contributor in the endosomal sorting of transferrin(Cullen 2008). As the confocal images suggest, *C. burnetii* co-localizes with SNX4, leading to the possibility that *C. burnetii* recruits the SNX4 protein so that it may exploit the

transferrin SNX4 is so closely linked with. Transferrin circulates throughout the blood, and is the main protein responsible for the binding and transport of iron(Ponka et al. 1998). Iron is a crucial element in cells as it is a key component in oxygen and electron transport, DNA synthesis, and other types of metabolic processes(Ponka et al. 1998). Due to its potential hazardous deleterious effects, specialized sub-cellular molecules have evolved to control the transport and storage of iron (Ponka et al. 1998). Transferrin is a plasma glycoprotein, and is one of these iron-transporting molecules(Ponka et al. 1998). Transferrin carries iron to cells by binding to the cell's transferrin receptor, where it is internalized by an endocytic vesicle, and upon an endosomal decrease in pH, releases the iron substrate so that the cell may begin utilizing it in its metabolic processes (Ponka et al. 1998). Although significant progress has been made in deciphering the functional roles of SNXs related to *C. burnetii* infection, many questions remain unanswered.

Acknowledgements

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