Manipulating endosomal systems: the molecular mechanisms of transport decisions and Salmonella-induced cancer

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Chapter 4

The interplay between endocytosis and cell manipulation by pathogens

As defined by the World Health Organization (WHO), infectious diseases are caused by pathogenic microorganisms including bacteria, viruses, parasites, or fungi that spread from one person to another, or in the case of zoonotic diseases are transmitted from animals to humans. The effects of these diseases range anywhere from mild to severe and can even result in death. While the human host rarely benefits from infection, pathogens often need the host as a niche for survival and proliferation. This is by definition the case for viruses, which can be considered lifeless carriers of genomic material, unable to replicate by themselves. Therefore, they need to infect a host in order to use their replicative machinery as part of their lifecycle. By contrast, bacteria constitute living organisms on their own merits, with the majority of their species capable of autonomous replication. Still, some bacteria have evolved to make use of the host's protective environment for their proliferation. To achieve this, bacteria must strike a delicate balance whereby host pathways remain sufficiently functional to support the pathogen's lifecycle needs for a successful round of infection. In this light, pathogens often make use of an entry system endogenous to the host: the endocytic pathway. To ensure their optimal uptake and prolonged intracellular survival, as well as circumvent recognition by the defense systems of the host, both bacteria and viruses have developed comprehensive mechanisms to manipulate the host (*Mercer et al., 2010; Cossart and Helenius, 2014; Asrat et al., 2014*).

Endosomes as a Trojan Horse for bacterial and viral infection

In the first step of entry pathogens establish stable contacts with the host cell, typically by interacting with specific cell surface molecules. Viruses bind a broad range of glycoproteins found at the surface of host cells, and some of these have also been described as having additional roles during endocytosis and associated signaling (*Cossart and Helenius, 2014*). Binding to host receptors induces clustering at the plasma membrane and generates micro-domains that serve as platforms for initiation of endocytosis and viral uptake (*Kahmi et al., 2013*).

Viruses have also learnt to adapt to the mechanisms of endosomal uptake. Following the initial interaction with the host membrane, viruses can enter the target cell through a variety of mechanisms, including clathrin-mediated endocytosis, micropinocytosis, caveolin-induced endocytosis and phagocytosis (*Mercer et al., 2010; Grove and Marsh, 2011*). Subsequently, viruses must expose their genomes to relevant molecular machineries present in the cytosol or nucleus of the host to multiply, and must thus escape the endosomal system. To properly time this escape, many viruses have evolved the capacity for acid-activated release, meaning that progressively lower luminal pH acquired as endosomes mature induces conformational changes in viral surface proteins that induces their fusion with the endosomal membrane and delivery of the genetic content in the cytosol (*Vázquez-Calvo et al., 2012; Kubo et al., 2012*).

Bacteria also use host proteins to facilitate their first contact. After binding to adhesins on the host membrane, two mechanisms used by different bacteria can instigate endocytosis. The first is called the zipper mechanism. Here, attachment to the host is followed by so-called zippering of the host membrane around the bacterium, culminating in endosomal uptake. In the second mechanism, which is described as the

trigger mechanism, interaction is followed by direct delivery of bacterial effector molecules inside the host cell. These molecules manipulate endosomal uptake and internal trafficking of the bacterium by modulation of the host endosomal system (*Pizarro-Cerdá and Cossart, 2006*).

While viruses use the endocytic system passively, many bacteria have found ways to actively manipulate the endosomal system in order to facilitate their uptake and intracellular trafficking (*summarized in Figure 1*). Through the release of specific effector molecules, bacteria manipulate host signaling and prime the endosomal system for invasion (*Mattoo et al., 2007; Ham et al., 2011*). Once internalized, bacteria use the endosomal pathway to travel towards their favored niche. While some bacteria, such as *Salmonella* and *Brucella*, prefer to occupy mature late endosomal vacuoles, others, like *Chlamydia*, reside in a compartment associated with the Golgi complex, where it is well positioned to intercept sphingomyelin- and cholesterol-containing exocytic vesicles (*Hackstadt et al., 1996; Gorvel and Méresse, 2001; von Bargen et al., 2012*). Although most other bacteria carefully avoid the proteolytic lysosomal compartment, some species, such as *Coxiella*, have evolved to survive and replicate in lysosomes and are apparently resistant to the resident proteolytic enzymes (*Voth and Heinzen, 2007*). These differences in preferred intracellular niches have led to extensive diversity in modes of host manipulation facilitated by different effector repertoires to influence bacterial uptake, motility and interactions with the endosomal system of the host.

Salmonella's hitchhiking guide through the endocytic system

Salmonella has long served as a classical—and thus thoroughly studied—example of a bacterium that modulates its host during its infectious cycle (*Haraga et al., 2008*). *Salmonella* is a gram negative, rod shaped bacterium whose genus consists of multiple species and subspecies, resulting in over 2500 distinct members in the *Salmonella* genus, called serotypes. Depending on the serotype, *Salmonella* can cause a diverse range of disease phenotypes ranging from food poisoning to typhoid fever by *S. typhi*. While food poisoning is caused by *Salmonella* bacteria that mainly reside in the digestive track, such as *S. typhimurium* and *S. enteritidis*, typhoid fever results from bacteria entering the patients' bloodstream, which can give rise to fever, diarrhea and, in severe cases, sceptic shock (*Coburn et al., 2007*).

In order to enter host cells and successfully modulate its uptake and survival, *Salmonella* releases effector molecules into the host cytoplasm. It does so by forming a needle-like structure called the injectosome, which is part of the *Type III secretion system* (T3SS). To start the assembly of the T3SS, *Salmonella* releases 'translocon' proteins through a hollow export channel. These molecules then regulate the build-up of the injectosome on the extracellular side of the bacterial membrane, as well as help to form a pore in the host membrane. Once assembled, an ATPase subunit provides energy for active recruitment and preparation of protein transport through the needle into the host cell (*Mattëi et al., 2011; Portaliou et al., 2016*).

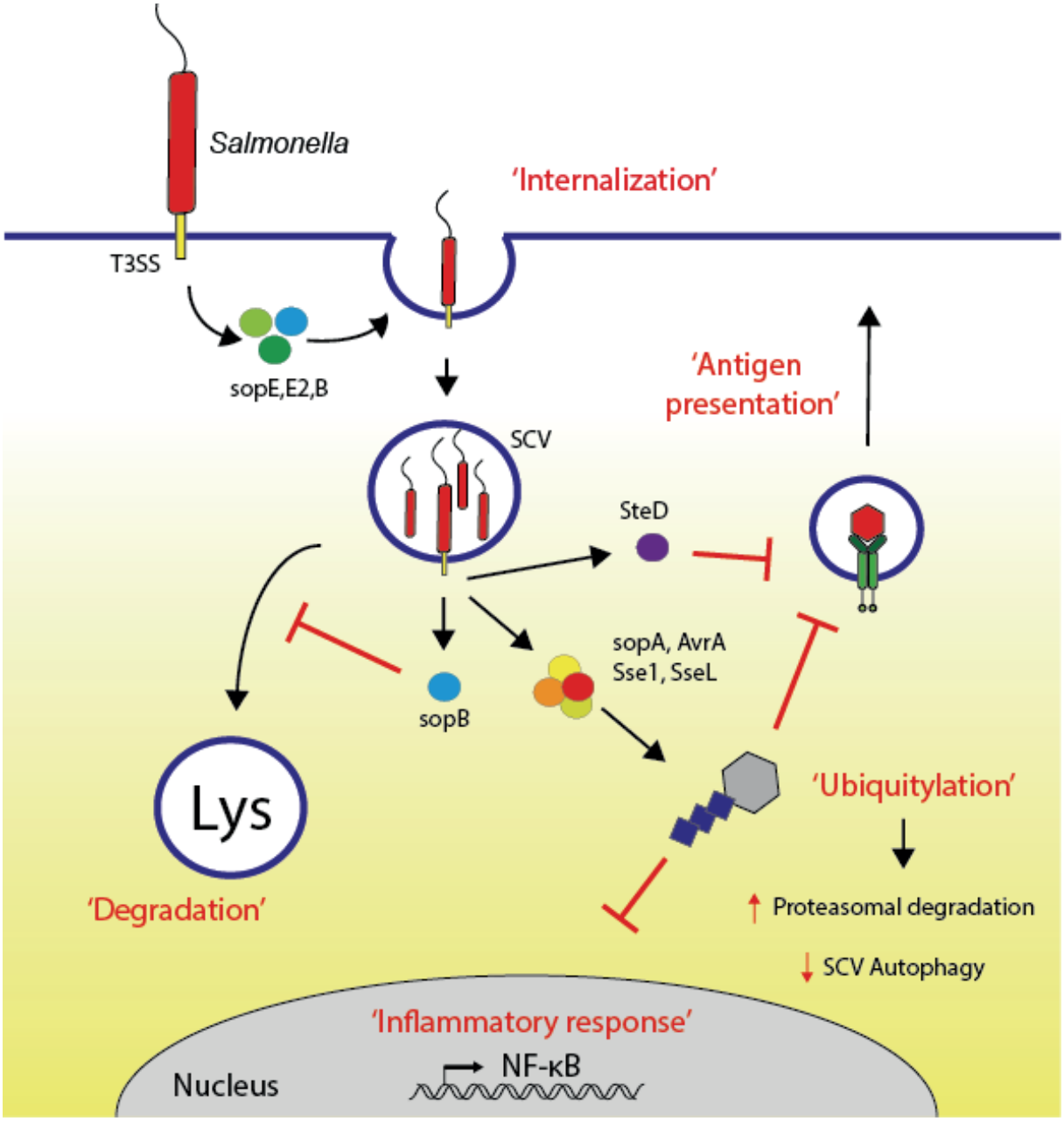

Figure1: Host cell manipulation by Salmonella
Upon contact, Salmonella releases various effector molecules inside the host cell through the Type III secretion system (T3SS). In order to facilitate its uptake, release of SopE, -E2 and -B induces membrane ruffling, thereby stimulating internalization of Salmonella in the endocytic track ('Internalization'). Upon maturation of the Salmonella containing vacuole (SCV), effector molecules are released that preserves the late endosomal niche favored by Salmonella. Through SopB release, excessive endosomal maturation of the SCV is prevented. This results in a failure to deliver Salmonella to the lysosomal compartment and prevents the bacterium from being degraded ('Degradation'). Additionally, Salmonella releases effector molecules, including SopA, AvrA, Sse1 and SseL, to highjack the host ubiquitin system ('ubiquitylation'). By controlling various ubiquitylation and de-ubiquitylation events, Salmonella is able to target host proteins for proteasomal degradation, prevent SCV autophagy and influence host transcription activity in response to infection ('Inflammatory response'). Additionally, through the release of SteD, the antigen presenting Major Histocompatibility Complex II (MHCII) is ubiquitylated and targeted for degradation, further hampering immune recognition upon infection ('Antigen presentation').

Salmonella is known to be capable of releasing around 30 different effectors into the host, with widely different functions (*Jennings et al., 2017*). For instance, some of these effectors pave the way to bacterial entry by remodeling the host membrane. Through the release of SopE and its close relative SopE2, which functions as a *Guanine Exchange Factors* (GEF) activating host GTPases Cdc42, Rac1 and RhoG, *Salmonella* stimulates membrane ruffling. Stimulation of Rho GTPases also activates the host's MAPK signaling cascade, resulting in altered host gene expression and production of pro-inflammatory cytokines (*Hobbie et al., 1997*). Another *Salmonella* effector is SopB, a *Phosphoinositide* (PI) phosphatase that modulates actin polymerization through the actin nucleator Arp2/3 and mediates phagocytosis by way of Rho and its kinase, as well as the actin motor Myosin II (*Patel and Galan, 2006*). Through these combined effects on host membrane integrity, *Salmonella* creates a situation that favors its uptake into the host's endocytic system. Once inside cells, release of additional effectors enables *Salmonella* to steer its travels through the vesicular compartments of the host. Here again SopB alters the PI content of the newly formed *Salmonella containing vacuole* (SCV) membrane, thereby promoting recruitment of the early endosomal GTPase Rab5. Along with Rab5 comes its effector *Phosphoinositide 3-kinase* (PI3K) VPS34, which mediates accumulation of PI3-*phosphate* (PI3P) on the SCV membrane and serves to inhibit recruitment of the recycling GTPase Rab35 (*Bakowski et al., 2010; Asrat et al., 2014*). In this way, SopB activity prevents bacterial recycling from the early endosomal compartment back to the plasma membrane and instead targets the SCV for further maturation towards its preferred niche—the acidic *Late Endosomal* (LE) compartment marked by the GTPase Rab7.

Because unrestrained phagosome maturation will invariably result in entry into the lysosome and subsequent degradation of the invading bacterium, *Salmonella* acts to prevent excessive SCV maturation in order to keep its niche intact. This is once again achieved through injection into the host cytosol of the effector SopB, resulting in local activation of the host protein kinase Akt that inhibits further maturation in two ways. Firstly, phosphorylation of Akt exerts local effects on actin dynamics via host GTPases Rac1 and Rho. Secondly, Akt phosphorylates host protein AS160, a *GTPase activating protein* (GAP) that inactivates host Rab14. Full maturation towards the lysosomal compartment requires recycling GTPases, such as Rab14, to be removed from the LE. However, upon phosphorylation, AS160 is prevented from binding to the SCV membrane, keeping Rab14 in an activated state that then inhibits maturation of the SCV towards the lysosomal compartment (*Kuijl et al., 2007*).

But there is more. The effector molecule *Salmonella-induced Filaments A* (SifA) stimulates SCV membrane remodeling and formation of *Salmonella-induced tubules* (SITs) (*Boucrot et al., 2005*). Following injection into the host cytosol, SifA inserts into the SCV membrane, where it recruits the host effector *SifA and Kinesin-Interacting Protein* (SKIP; also termed PLEKHM2). The latter serves as an adaptor for the microtubule-based motor protein Kinesin-1 (*Leone and Méresse, 2011*), which generates outward force on the phagosomal membrane, resulting in formation of tubules emanating from the bacteria containing phagosome. Although the functional

significance of these structures remains largely unclear, SITs are known to contain proteins from both late endosomal and secretory origins, suggesting that *Salmonella* may utilize both of these vesicular pathways to create an ideal niche for proliferation and survival. While some studies have indicated that SKIP mediates SCV transport towards the plasma membrane and may even be involved in extracellular release of bacteria during reinfection, other studies have suggested a role for SKIP in nutrient diversion towards the bacterial vacuole (*Kaniuk et al., 2011; Liss et al, 2017*).

Salmonella's manipulation of the host Ubiquitin system

Residing in the late endosomal compartment provides a relatively safe niche since *Salmonella* is protected from many host regulatory mechanisms that degrade intracellular pathogens. However, rupturing of the SCV can result in bacterial release into the cytoplasm (*Knodler, 2015*). While this endosomal escape initially results in rampant bacterial replication due to nutrient rich conditions, the cell will be triggered to respond and clear itself from the bacterium. It has been reported that such clearance is mediated by ubiquitylation-mediated degradation of bacteria via the autophagy pathway (*Malik-Kale et al., 2012; Herhaus and Dikic, 2017*). Once exposed to the cytosol, Salmonella can be recognized by host E3 ubiquitin ligases that link poly-ubiquitin chains to the bacterium, thus instigating recruitment of ubiquitin-binding autophagic adaptor proteins, such as p62. The latter simultaneously binds to LC3 present on the nascent phagophore membrane and thereby facilitates the formation of a double membrane phagosome around the bacterium. These structures can then fuse to the lysosome to deliver *Salmonella* for degradation (*Gomes and Dikic, 2014*). Although the bacterium itself lacks a functional ubiquitin system, it does produce ubiquitin-specific enzymes capable of interfering with the ubiquitin system of the host (*Perret et al., 2011; Ashida et al., 2014*). One of these is the effector SopA (*Zhang et al., 2006*). Release of SopA results in modification of host proteins ranging in functions from cytoskeletal remodeling to signaling molecules and enzymes of the host ubiquitin system. Specifically, *Salmonella*-induced ubiquitylation events mediate cytoskeletal and host membrane rearrangements, and manipulation of the NF-κB signaling cascade meddles with the inflammatory response employed by cells to warn the rest of the host's organism of the infection (*Ashida et al., 2014*). Furthermore, amongst the E3 ligases ubiquitylated by SopA are TRIM56 and TRIM65, both of which are known to regulate the innate immune response against various pathogens. Ubiquitylation of these host proteins targets them for proteasomal degradation, attenuating host immune responses against the *Salmonella* infection (*Fiskin et al., 2017*).

Effector molecules can also exert inhibitory effects on the ubiquitin system of the host by de-ubiquitylating host proteins normally activated by conjugation with ubiquitin in response to *Salmonella* SCV escape. As a result, release of effector molecules prevents proteasomal degradation of cytoplasmic *Salmonellae*. For example, *Salmonella* effectors AvrA and Sse1 have been shown to reduce IκBα ubiquitylation levels in the host, resulting in poor NF-κB signaling. Furthermore, the Deubiquitinating enzyme (DUB) effector SseL has been described to take a more direct approach to protection of the bacterium through removal of ubiquitin chains from the SCV and

consequent impairment of autophagosome formation around the cytosolic bacterium (*Ashida et al., 2014*).
Last but not least, activation of the adaptive immune response following infection can also be manipulated by *Salmonella*. When antigen presenting cells, such as dendritic cells, become infected by *Salmonella*, the bacterial effector SteD reduces the abundance of the antigen presenting *Major Histocompatibility Complex II* (MHCII) on the surface of infected cells. SteD binds both MHCII and its E3 ligase MARCH8 in order to promote ubiquitylation of MHCII. The resulting depletion of cell surface MHCII suppresses T cell activation and thus protects *Salmonella's* intracellular niche from adaptive immune responses (*Bayer-Santos et al., 2016*).

Concluding remarks
An interaction between a pathogen and its host cell constitutes a microcosm of a persistent battle between organisms. While one (the host) is trying to evade or clear pathogenic infections, the other (the pathogen) has developed elaborate ways to avoid recognition, evade clearance and guard its intracellular niche. The many ways pathogens, as exemplified by *Salmonella*, manipulate the host have given rise to a diverse landscape of maladies associated with infection. While the most obvious ones are related to the immune response of the host desiring to clear itself from infection, interference with its basic cellular functions can have far reaching long-term effects on the host organism. These can be manifested in a variety of disease phenotypes, rather unexpectedly including cancer. The role that infection by *Salmonella* plays in cellular transformation is explored in the next chapter.

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