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# Unveiling ubiquitinome rearrangements induced by *Salmonella* infection

Tihana Bionda and Christian Behrends

Institute of Biochemistry II, Goethe University School of Medicine, Frankfurt am Main, Germany

**ABSTRACT**

Ubiquitination plays a critical role in the activation of host immune responses to infection and serves as a signal for pathogen delivery to phagophores along the xenophagy pathway. We recently performed systematic ubiquitination site profiling of epithelial cells infected with *Salmonella* Typhimurium. Our findings specifically highlight components of the NFKB, membrane trafficking pathways and RHO GTPase systems as ubiquitination hubs during infection. In addition, a broad spectrum of bacterial effectors and several outer membrane proteins are ubiquitinated in infected cells. This comprehensive resource of ubiquitinome dynamics during *Salmonella* infection enables further understanding of the complex host-pathogen interplay and may reveal novel targets for the inhibition of *Salmonella* invasion and inflammation.

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Bacterial pathogens have evolved strategies to circumvent host cell defense mechanisms and promote their intercellular proliferation. Being an important part of the cellular immune response, the ubiquitin (Ub) system is a prominent target hijacked by bacterial effectors that are secreted to the host cytosol. Ubiquitination serves as a signal triggering NFKB activation, which by inducing inflammation dampens bacterial proliferation. Ub also marks bacteria for autophagosome-mediated degradation. While the majority of intracellular *Salmonellae* reside in the *Salmonella*-containing vacuole (SCV), a fraction of bacteria escapes to the cytosol where they are tagged with a dense polyubiquitin coat. Ub-binding macroautophagy/autophagy cargo receptors CALCOCO2/NPD52, SQSTM1 and OPTN mediate delivery of ubiquitinated bacteria to phagophores via interactions with LC3 and GABARAP proteins. Despite the key role of Ub during bacterial infection, the extent of host ubiquitinome remodeling induced by pathogen insults remained unexplored. Another unanswered question concerns the identity and origin of the ubiquitination targets constituting the Ub coat.

In our recent study we performed systematic ubiquitination site profiling of *Salmonella*-infected epithelial cells using quantitative diGly proteomics. Coupling stable isotope labeling by amino acids in cell culture (SILAC) with immunoaffinity-based diGly enrichment enabled us to quantitatively dissect the dynamics of the host ubiquitinome in response to *Salmonella* infection. To robustly capture ubiquitinome dynamics, we performed all experiments in 2 epithelial cell lines (HCT116 and HeLa), in multiple biological replicates and at early (0.5 and 2 h) as well as later (6 h) time points post infection (pi). Functional annotation analysis of regulated diGly sites revealed that dynamic ubiquitinome changes correlate with the progression of *Salmonella* interaction with specific host pathways. Components of the actin cytoskeleton, the NFKB and autophagy pathways, and the Ub and RHO GTPase systems constitute the most prominent ubiquitination hubs at 0.5 and 2 h pi. In line with transient as well as sustained activation of the NFKB pathway by bacterial lipopolysaccharides, ubiquitination of certain NFKB-related components (RBCK1, SHARPIN, OTULIN) increases 0.5 h pi and is followed by a steep decline 2 h pi, whereas another subset of NFKB constituents (IKBKG, SHARPIN, RNF31, TRAF6, TRAF2) remains highly ubiquitinated throughout the early stage of infection. A similar trend is observed for the RHO GTPase network, including sites in RHO GTPases, GTPase activating proteins and guanine nucleotide dissociation inhibitors (CDC42, RHOG, ARHGAP1, ARHGAP29, ARHGDIA), whose increased ubiquitination correlates with *Salmonella*-induced cytoskeleton rearrangements during and upon bacterial invasion. Notably, several components of the Ub system (CUL4A, CUL4B, TRIM25, TRIM32, TRIM56, UBR2, UBA6, USP38, USP45, USP24, USP13) and factors with established roles in autophagy, such as LGALS8, TMEM59 and SQSTM1, are upregulated 2 h pi. Ubiquitination of these early-regulated diGly sites is largely dependent on SPI-1-encoded *Salmonella* effectors. A significantly different profile of the regulated ubiquitinome is found at later stages of infection where proteins regulating vesicle-mediated transport, membrane organization and lysosomes are modified by ubiquitination. Secretory carrier membrane proteins (SCAMPs) and SNARE proteins are the most prominent ubiquitination targets at 6 h pi, reflecting the dependence of vacuolar bacteria on host vesicular trafficking pathways for maintenance of SCVs, development of *Salmonella*-induced filaments and intra-vacuolar replication. Ubiquitination of SCAMP3, which participates in

**CONTACT** Christian Behrends behrends@biochem2.de
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maintenance of SCVs juxtaposed to the Golgi, and the SNAREs VAMP2 and VAMP7, is highly increased at multiple lysine residues and is dependent on SPI-2-encoded *Salmonella* effectors. Functional analysis showed that *Salmonella*-induced ubiquitination specifically promotes CDC42 and linear Ub chain assembly complex/LUBAC activity, both being required for NFKB activation and induction of inflammation.

In contrast to these specific host rearrangements, the bacterial ubiquitinome exhibits extensive ubiquitination of various effectors, some carrying both degradative and non-degradative signals that are likely important for their half-life, activity and/or localization. Furthermore, a longstanding question in the xenophagy field concerns the origin of proteins constituting the Ub coat around bacteria, where both host and bacterial proteins may be ubiquitination targets. In contrast to the known xenophagy components SQSTM1 and LGALS8 that localize to cytosolic *Salmonella*, none of the other tested host candidates with infection-induced ubiquitination contribute to the Ub coat. Even though ubiquitinated variants of SQSTM1 and LGALS8 can still participate in coat formation, we provide the first evidence that bacterial outer membrane proteins (OMPs) are bona fide ubiquitination substrates. The majority of the ubiquitination sites detected in OMPs is exposed to the host cytosol and might thus contribute to the Ub coat. As only a small fraction of bacteria escapes SCVs and is rapidly cleared by autophagy, these ubiquitination targets might be intrinsically difficult to detect by conventional biochemical app-roaches. Assuming that ubiqui-tination sites in multiple bacterial OMPs are redundant as docking sites for autophagic cargo receptors, depletion of one candidate OMP and/or mutation of single ubiquitination sites are not likely to result in a detectable diminution of the Ub halo surrounding *Salmonella*. This is reminiscent of events following mitochondria depolarization, where multiple mitochondrial proteins are redundantly ubiquitinated to promote rapid mitophagy, and might represent an evolutionarily conserved theme between mitochondria and their proteobacterial ancestors.

Our systematic and quantitative study unveils a high plasticity of the host ubiquitinome, corroborating established knowledge and allowing the deduction of cellular processes and signaling pathways affected by *Salmonella*. Given the increased occurrence of antibiotic resistance in bacterial pathogens, which prompts the need for alternative antibacterial strategies, this comprehensive dataset might serve as a resource to reveal targets for the inhibition of *Salmonella* invasion and inflammation.

## Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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