Structural basis for the recognition and degradation of host TRIM proteins by *Salmonella* effector SopA

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The hallmark of *Salmonella* Typhimurium infection is an acute intestinal inflammatory response, which is mediated through the action of secreted bacterial effector proteins. The pro-inflammatory *Salmonella* effector SopA is a HECT-like E3 ligase, which was previously proposed to activate host RING ligases TRIM56 and TRIM65. Here we elucidate an inhibitory mechanism of TRIM56 and TRIM65 targeting by SopA. We present the crystal structure of SopA in complex with the RING domain of human TRIM56, revealing the atomic details of their interaction and the basis for SopA selectivity towards TRIM56 and TRIM65. Structure-guided biochemical analysis shows that SopA inhibits TRIM56 E3 ligase activity by occluding the E2-interacting surface of TRIM56. We further demonstrate that SopA ubiquitinates TRIM56 and TRIM65, resulting in their proteasomal degradation during infection. Our results provide the basis for how a bacterial HECT ligase blocks host RING ligases and exemplifies the multivalent power of bacterial effectors during infection.

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Salmonella enterica serovar Typhimurium is a Gram-negative pathogenic bacterium, which represents a major cause of food- and water-borne disease. Non-typhoidal Salmonella strains, including S. Typhimurium, cause severe gastroenteritis in immunocompetent individuals, whereas systemic infection can arise in immunosuppressed hosts. S. Typhimurium invasion and the concomitant induction of intestinal inflammation are initiated by secreted bacterial effector proteins, which are translocated into host cells via a channel-forming multi-protein complex known as the type-3 secretion system (T3SS)

Bacterial infection is sensed by host pattern recognition receptor-mediated detection of pathogen-associated molecular patterns, such as lipopolysaccharide or bacteria-deriviced nucleic acids, and induces a pro-inflammatory state to combat infection. The propagation of this innate immune response requires posttranslational modification of assembled receptor signalling complexes with ubiquitin (Ub), which in eukaryotes is catalysed by three distinct classes of E3 Ub ligases known as homologous to E6–AP carboxy terminus (HECT), really interesting new gene (RING) and RING-between-RING.

To counteract Ub-dependent induction of host inflammatory signalling and microbialicidal programmes, a wide range of bacteria have acquired strategies to subvert the host Ub proteasome system. Despite lacking the canonical Ub proteasome system, prokaryotic pathogens encode various families of virulence promoting E3 ligases. After their T3SS-mediated translocation, these ligase effectors hijack the host ubiquitination machinery and use their intrinsic catalytic activity to modify specific cellular targets. The Salmonella T3SS effector protein SopA is a HECT-like E3 ligase that promotes Salmonella infection-induced inflammation. Lacking noticeable primary sequence homology to eukaryotic HECT enzymes, the crystal structure of SopA showed the characteristic bi-lobal architecture of HECT ligases. SopA-catalysed ubiquitination proceeds via a thioester-linked SopA ~ Ub intermediate and requires an active site cysteine in its C terminus. Subsequently solved structures of SopA in complex with E2 Ub-conjugating enzymes revealed an E2-binding site located within the N-lobe of SopA and further indicate a high structural flexibility of its C-lobe. Structural characterization of SopA also uncovered the presence of an amino-terminal β-helix domain, whose function so far remains unknown.

Recent work implicates SopA in the regulation of two tripartite-motif containing (TRIM) E3 ligases TRIM56 and TRIM65 (ref. 15). TRIM proteins constitute a large family of ~70 RING-type E3 ligases, which plays a pivotal role in the host innate immune response against various pathogens. TRIM56 and TRIM65 in particular have been demonstrated to stimulate type I interferon expression in conjunction with nucleic acid-sensing receptors such as STING, RIG-I and MDA5. TRIMs are characterized by the presence of three common structural features, consisting of an N-terminal catalytic RING domain, one or two B-boxes and a coiled-coil. Multiple studies demonstrated that the coiled-coil of various TRIMs mediate anti-parallel dimer formation. Furthermore, catalytic activity of TRIM proteins is enhanced by coiled-coil-dependent dimerization and, more recently, was shown to require RING domain dimerization for the proper activation of the E2 ~ Ub conjugate.

Here, using quantitative proteomics, structural and biochemical analysis we elucidate how Salmonella HECT-like ligase SopA specifically targets and inhibits human TRIM56 and TRIM65. We present the structure of the SopA–TRIM56 RING complex providing the molecular basis for SopA target specificity. Analysis of this complex further reveals that SopA occludes the E2 binding site of TRIM56 RING and inhibits TRIM ligase activity. Finally, we demonstrate that SopA directly mediates ubiquitination and proteosomal degradation of TRIM56 and TRIM65.

Results

Identification of TRIM56 and TRIM65 as SopA interactors. Aiming to understand the molecular basis of SopA function during Salmonella pathogenesis, we set out to identify SopA host interactions using affinity purification coupled mass spectrometry (MS). To this end, we generated a stable HeLa Flp-In T-Rex cell line inducibly expressing GFP-SopA upon the addition of doxycycline (Supplementary Fig. 1a). These cells were differentially labelled using stable isotope labelling by amino acids in cell culture (SILAC) and then either left untreated or treated with doxycycline to induce GFP-SopA expression. Next, SopA-containing complexes were immunoprecipitated using anti-green fluorescent protein (GFP) beads, subjected to tryptic in-gel digest and extracted peptides were analysed by liquid chromatography-coupled MS/MS on an Orbitrap mass spectrometer (Fig. 1a and Supplementary Fig. 1b). We reproducibly identified two TRIM E3 ligases, TRIM56 and TRIM65, as the most significant SopA-enriched hits (Fig. 1b, Supplementary Fig. 1c and Supplementary Data 1). Subsequent immunoblotting experiments confirmed these MS data and revealed that endogenous TRIM56 and TRIM65 indeed specifically co-precipitated with transiently expressed GFP-SopA, but not with its enterohemorrhagic Escherichia coli homologue NleL (Fig. 1c). To additionally identify SopA interactors in the course of infection, we isolated SopA from cells infected with S. Typhimurium strains expressing tagged SopA–HA or empty vector controls. Consistent with results from heterologous expression experiments, we recovered TRIM56 and TRIM65 as major interacting proteins of bacterially secreted SopA in Salmonella-infected cells (Fig. 1d,e and Supplementary Data 2).

Given the reported ability of TRIM proteins to form heterooligomers, we tested whether TRIM56 and TRIM65 interact with each other. We did not detect complex formation between these two TRIM proteins after transient expression of tagged TRIM versions (Supplementary Fig. 1e,f). To further characterize the interaction mode between TRIM56/TRIM65 and SopA, we expressed different truncation constructs of both TRIM proteins individually and tested them for the ability to co-immunoprecipitate SopA (Fig. 2a and Supplementary Fig. 1d). Whereas ablation of both coiled-coil and substrate-binding domains had no effect, deletion of the RING domain in case of both TRIM56 and TRIM65 completely abolished SopA interaction (Fig. 2c,d). Analogously, we expressed SopA truncations to determine the requirements for TRIM56/65 interaction (Fig. 2b). Interestingly, the capacity of SopA to associate with both TRIM56/65 mapped to the N-terminal β-helix domain (Fig. 2e). Consistently, pull-down experiments using recombinant TRIM56 and SopA proteins revealed that SopA directly interacts with the RING of TRIM56 or TRIM65 via its β-helix domain (Fig. 2f and Supplementary Fig. 1g).

Crystal structure of the SopA–TRIM56 complex. To understand the molecular basis of the interaction between SopA and TRIMs, we set out to determine the crystal structure of the SopA–TRIM56 complex. Initial attempts to co-purify bacterially expressed SopA (163–782) and TRIM56 RING domain (1–94) did not yield stoichiometric complex in size-exclusion chromatography (Supplementary Fig. 2a), due to the low affinity binding between these molecules with a dissociation constant of ~9 μM (Fig. 2g). To circumvent this problem, we fused the minimal binding regions of TRIM56 (1–94) and SopA (163–425) using a
flexible linker (Supplementary Fig. 2b). Such end-to-end fusion of two different proteins with short flexible linkers is a commonly employed method in crystallization of low-affinity protein complexes. This fusion construct was purified and crystallized in the space group P 31 1 2 and the optimized crystals diffracted to 2.9 Å resolution (Supplementary Fig. 2c). The structure was determined by molecular replacement and refined until convergence (Fig. 3a and Table 1).

The asymmetric unit contained one copy of the SopA–TRIM56 dimer. The structure reveals that the first Zn\(^{2+}\)-binding loop in the TRIM56 RING domain is packed in a cleft at the interface of the \(\beta\)-helix and the N-lobe domains of SopA (Fig. 3b). The structure of SopA in complex with TRIM56 overlays well with the SopA apo structure with a mean root mean squared deviation (r.m.s.d.) of 0.9 Å over all the C-\(\alpha\) atoms. Both the linker residues between TRIM56 and SopA, as well as the 17 N-terminal residues of TRIM56 were disordered and could not be observed in the electron density. Comparison of the TRIM56 RING domain structure with all the structures in PDB using DALI revealed that it is most similar to the RING domain of RNF146 with a mean r.m.s.d. of 2.5 Å over all the C-\(\alpha\) atoms. Moreover, the RING domains of TRAF6, RING1B, TRIM32 and RNF4 also closely resemble the TRIM56 RING domain with mean r.m.s.d. of 2.6, 1.8, 2.1 and 1.9 Å over >90% of C-\(\alpha\) atoms, respectively. The interface of SopA and TRIM56 contains a mix of hydrophobic/hydrophilic interactions with a buried surface area of 750 Å\(^2\) (Fig. 3b). In TRIM56, residues Leu25 and Glu26 contribute majorly towards interaction with SopA. Although Glu26 makes polar contacts with Arg296, His297 and Lys298 of SopA, Leu25 inserts into a hydrophobic pocket of SopA involving Phe345 and Pro334.
Figure 2 | TRIM56 and TRIM65 RING domains directly interact with the β-helix region of SopA. (a,b) Domain organization of TRIM56 (a) and SopA (b), and corresponding truncation constructs. B1 and B2, B-box type zinc fingers; CC, coiled-coil domain; HECT-N, N-lobe; HECT-C, C-lobe. (c,d) TRIM56/TRIM65 RING domain is required for SopA interaction. Lysates from HEK293T cells co-expressing GFP-SopA C753A and indicated FLAG-TRIM56 (c) or FLAG-TRIM65 (d) constructs were subjected to anti-FLAG IP, followed by SDS–PAGE and immunoblotting. (e) Intact SopA β-helix is required for TRIM56/TRIM65 interaction. Lysates from HEK293T cells expressing indicated GFP-SopA constructs were subjected to anti-GFP IP, followed by SDS–PAGE and immunoblotting. (f) SopA–TRIM56/65 interaction is direct. Recombinant MBP-TRIM56 was incubated with GST or different GST-SopA proteins and subjected to glutathione sepharose pull-down followed by SDS–PAGE and immunoblotting. (g) Isothermal titration calorimetry (ITC) measurement of SopA–TRIM56 RING interaction. 1,000 μM TRIM56 (1–94) was injected gradually into 20 μM SopA (163–782) present in the sample cell. Raw ITC data were plotted and analysed using Origin 7 software. The table shows values obtained for various parameters of the SopA–TRIM56 interaction. ΔH, heat change; $K_d$, dissociation constant; N, occupancy; $\Delta S$, entropy change.
The central α-helix present in TRIM56 also makes several hydrophobic contacts and packs tightly against SopA (Fig. 3b).

Structure-guided point mutations in the first Zn$^{2+}$-binding loop of TRIM56 RING confirmed the requirement of Leu25 and Glu26 for SopA–TRIM complex formation. The corresponding TRIM56 single and double mutants were unable to co-precipitate SopA upon transient expression, while retaining functional RING E3 activity (Fig. 3c and Supplementary Fig. 2d). Thr338 of SopA is in close proximity to the central α-helix in TRIM56 (Fig. 3b) and we predicted that mutating it to Leucine would create steric clashes and impair the interaction. Indeed, a T338L point mutation in SopA completely abolished interaction with endogenous TRIM56 (Fig. 3d). Interestingly, SopA T338L mutation also prevented binding of SopA to TRIM65, indicating that SopA recognizes both TRIM56 and TRIM65 in a similar manner.

**Figure 3 | Structure of SopA α-helix in complex with TRIM56 RING domain.** (a) Crystal structure of SopA–TRIM56 complex shown in cartoon representation. SopA is shown in green and TRIM56 in cyan, two orientations rotated by x:180° are shown. (b) Enlarged view of the interface of SopA and TRIM56 showing the important residues involved in the interaction. Right conformation depicted in a was rotated by y: 140°, x: 145° and z: 35° and zoomed in; residues 18–48 in TRIM56 RING and 295–299, as well as 330–345 in SopA are shown. Hydrogen bonds with corresponding distances are depicted in deep red. (c) TRIM56 RING mutations abolish SopA interaction. Lysates from cells co-expressing GFP-SopA C753A and indicated FLAG-TRIM56 RING domain mutants were subjected to anti-FLAG IP, followed by SDS–PAGE and immunoblotting. (d) SopA T338L mutation abolishes SopA–TRIM56 and SopA–TRIM65 interaction. Lysates from cells expressing indicated GFP-SopA α-helix point mutants were subjected to anti-GFP IP, followed by SDS–PAGE and immunoblotting.

**Structural basis of SopA specificity for TRIM56 and TRIM65.** Sequence comparison of SopA and its *E. coli* homologue NleL revealed that NleL lacks all the residues involved in SopA–TRIM56 binding (Supplementary Fig. 2e). This readily explained why NleL does not target TRIM proteins (Fig. 1c). On the other hand, multiple sequence alignment of several TRIM proteins and other closely related RING domain-containing proteins revealed that residues Leu25 and Glu26, which are essential for TRIM56–SopA interaction, show a conservation of 75% and 50%, respectively (Fig. 4a). Despite this conservation, no other detected TRIM protein was found significantly enriched in our SopA interactome studies (Supplementary Data 1,2), indicating that the primary sequence of TRIM proteins may not determine SopA specificity. Other factors influencing SopA selectivity may include subcellular targeting and expression levels of various TRIMs.
Table 1 | Data collection and refinement statistics.

|                      | SopA-Trim56 complex |
|----------------------|---------------------|
| Wavelength (Å)       | 0.99987             |
| Resolution range (Å) | 43.48–2.849 (2.95–2.85) |
| Space group          | P 3 1 2             |
| Unit cell            |                     |
| a, b, c (Å)          | 71.01, 71.01, 122.94 |
| α, β, γ (°)          | 90, 90, 120         |
| Unique reflections   | 8,375 (833)         |
| Multiplicity         | 20.0 (19.0)         |
| Completeness (%)     | 98.37 (84.05)       |
| Mean ω (°)           | 31.15 (3.64)        |
| Wilson B-factor (Å²) | 77.87               |
| R-merge              | 0.074 (0.834)       |
| CCI/2                | 1 (0.985)           |
| R-work               | 0.2234 (0.3022)     |
| R-free               | 0.2780 (0.3269)     |
| Number of atoms      | 2395                |
| Macromolecules       | 2392                |
| Ligands              | 2                   |
| Water                | 1                   |
| Protein residues     | 325                 |
| Root mean square (bonds; Å) | 0.004 |
| Root mean square (angles; °) | 0.97 |
| Ramachandran favoured (%) | 92 |
| Ramachandran outliers (%) | 0.94 |
| Clashscore           | 6.51                |
| Average B-factor (Å²) | 86.30              |
| Macromolecules       | 86.30               |
| Ligands              | 88.50               |
| Solvent              | 46.80               |

Statistics for the highest-resolution shell are shown in parentheses.

Here we investigated whether the crystal structure of the SopA–TRIM56 complex explains the specificity of SopA. We superimposed the TRIM56 RING domain with RINGs of various TRIM proteins (only TRIM32 and TRIM39 are shown as representatives), as well as RNF4 and TRAF6 (Fig. 4b–e). Although, the first Zn⁺⁺⁺⁺⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻˓...

**SopA mediates degradative ubiquitination of TRIM56.** To identify SopA substrates, we compared the diGly-modified proteome of cells infected with wild-type Salmonella (SL1344 WT) or a sopA deletion strain (AsopA) (Fig. 6a and Supplementary Fig. 3a). Two replicate SILAC diGly proteomics experiments resulted in the quantification of ~9,000 diGly sites in ~4,500 proteins (Supplementary Fig. 3b). Importantly, we identified multiple ubiquitination sites in TRIM56 (K87, K270 and K377) and TRIM65 (K206) as SopA-regulated events with the highest SILAC ratios of all quantified peptides (Fig. 6b, Supplementary Fig. 3c and Supplementary Data 3). To elaborate these findings, we purified ubiquitinated proteins from cells, which were infected with SL1344 WT, a non-invasive mutant defective in effector secretion (ASPII) or with various sopA deletion (AsopA) and complemented strains (AsopA + sopA, AsopA + sopA C753A) in the presence or absence of the proteasome inhibitor MG132 using tandem Ub-binding entities (TUBEI; Fig. 6c). Indeed, we observed the appearance of ubiquitinated high molecular weight species of endogenous TRIM56 upon Salmonella infection (Fig. 6c). Infection of cells with complemented sopA deletion strains, expressing higher amounts of SopA relative to SL1344 WT Salmonella (Supplementary Fig. 3g), resulted in increased modification of TRIM56. Interestingly, ubiquitination of TRIM56 increased upon proteasome inhibition and was completely dependent on the presence of catalytically competent SopA. Moreover, infection of cells with Salmonella expressing the binding-deficient SopA T338L mutant did not result in TRIM56 modification (Fig. 6d). In agreement with the results obtained from infected cells, we were able to reconstitute SopA-mediated ubiquitination of TRIM56 in vitro using recombinant proteins. Importantly, to monitor SopA catalytic activity and to exclude TRIM-mediated chain formation, we used the E2 enzyme UbcH7 in these experiments (Fig. 5b). Both the isolated RING domain and full-length TRIM56 were robustly ubiquitinated by SopA in vitro (Fig. 6e and Supplementary Fig. 3d), whereas TRIM56 RING mutants unable to interact with SopA were not modified (Supplementary Fig. 3e). As conjugation of substrates with different types of polyubiquitin chains can have distinct functional outcomes, we decided to examine the Ub linkage preference of SopA as well as TRIM56. For this purpose, we performed in vitro ubiquitination reactions using Ub mutants harbouring single lysine residues. Although TRIM56 catalytic activity showed a bias towards K11- and K63-linked polyubiquitin (Supplementary Fig. 3f), SopA preferentially modified TRIM56 with K48- and K11-linked Ub chains implicated in proteasomal targeting (Fig. 6f).

**Infection induces proteasomal turnover of TRIM56/TRIM65.** Given the MG132-sensitive nature of SopA-mediated TRIM56 ubiquitination and its preference for the synthesis of mostly degradative K48- and K11-chain types, we monitored the abundance of TRIM56 and TRIM65 proteins after infection of cells with S. Typhimurium WT, sopA-deficient or sopA-complemented...
**Figure 4 | Structural features of TRIMs define specificity of SopA towards TRIM56.** (a) Multiple sequence alignment of the first Zn^{2+}-binding loop region from various TRIMs and the closely related RING domain-containing proteins reveal Glu25 and Leu26 (residues in TRIM56 that are involved in binding to SopA) are fairly conserved (coloured in red). Zn^{2+}-coordinating cysteines are indicated using an asterisk. (b, c, d, e) TRIM56 RING domain was aligned with various closely related RINGs showing the clashes (circled) of SopA with the central β-helix of various RING domains (b, TRIM32; c, TRAF6; d, RNF4; e, TRIM39). PDB codes of the structures used in the alignment are indicated in brackets: (2JMD), (4AP4). (f) SopA does not bind TRIM32, TRIM39 and RNF4. Lysates from cells co-expressing GFP-SopA C753A and indicated FLAG-RING E3 ligase constructs were subjected to anti-FLAG IP, followed by SDS-PAGE and immunoblotting.
strains. These experiments revealed that protein levels of both TRIMs decreased in a SopA ligase activity-dependent manner (Fig. 7a). To address whether SopA-mediated reduction of TRIM56 and TRIM65 arises due to diminished protein translation or due to destabilization of TRIM56 and TRIM65 proteins, we performed cycloheximide chase experiments. Infection of cycloheximide-treated cells induced robust SopA-driven degradation of TRIM56 and TRIM65 (Fig. 7b). We were able to recapitulate these findings in doxycycline-inducible SopA-expressing cells, in which SopA induction triggered TRIM56 and TRIM65 degradation. Additional treatment of these cells with MG132 restored TRIM protein levels and resulted in the appearance of high molecular weight ubiquitinated species (Fig. 7c). Interestingly, a previous study suggested SopA targeting of TRIM56 and TRIM65 to be non-degradative. Here we provide multiple lines of evidence, which strongly support the notion that SopA-mediated ubiquitination inhibits and triggers the proteasomal degradation of TRIM56 and TRIM65 during Salmonella infection (Fig. 8).

**Discussion**

By using an unbiased multi-layered proteomics approach in Salmonella-infected cells, we recovered two host TRIM RING ligases, TRIM56 and TRIM65, as interactors and substrates of the Salmonella HECT-like ligase SopA. Using co-immunoprecipitation of various constructs of SopA and TRIMs, we showed that the N-terminal RING domains of TRIM proteins are both...
Figure 6 | TRIM56 and TRIM65 are substrates of degradative SopA ubiquitination. (a) Workflow for SopA-dependent ubiquitinome analysis using SILAC diGly proteomics of Salmonella SL1344 WT and ΔsopA-infected HCT116 cells 30 min post infection. (b) SopA ubiquitinates TRIM56 and TRIM65. Scatter plot of replicate SopA ubiquitinome experiments. (c,d) SopA-mediated ubiquitination of TRIM56 on infection is MG132 sensitive and depends on SopA catalytic activity (c) and on SopA–TRIM56 binding (d). Lysates from HeLa cells infected with Salmonella SL1344 WT or indicated mutant strains in the absence or presence of 20 μM proteasome inhibitor MG132 were subjected to TUBE pulldown followed by SDS-PAGE and immunoblotting. (e) SopA ubiquitinates TRIM56 RING domain in vitro. Purified SopA WT and catalytic mutant (C753A) were incubated with E1, UbcH7, ubiquitin, ATP and TRIM56 RING domain. TRIM56 ubiquitination is seen with WT SopA but not in the presence of catalytic-dead SopA. (f) Testing SopA ubiquitin chain specificity. WT SopA was incubated with TRIM56(1–207) and WT ubiquitin or various ubiquitin mutants that contain only a single surface lysine. E1, UbcH7, ubiquitin and ATP were added to all the reactions. TRIM56 alone is not active under these conditions (lane 1).
Data 3), thus supporting the juxtaposition of these domains in activity-dependent manner. Lysates from HCT116 cells infected with previously determined SopA structure carrying the C-lobe 9 appears to determine the specificity for SopA binding. (cycloheximide and infected with the indicated PAGE and immunoblotting. (Figure 7 | Salmonella infection induces proteasomal degradation of TRIM56 and TRIM65. (a) TRIM56 and TRIM65 protein levels decrease in SopA activity-dependent manner. Lysates from HCT116 cells infected with Salmonella SL1344 WT, ΔSpi1 or indicated sopA mutant strains were subjected to SDS-PAGE and immunoblotting. (b) SopA-mediated degradation of TRIM56 and TRIM65 on infection. Lysates from HCT116 cells treated with 100 μg ml⁻¹ cycloheximide and infected with the indicated sopA mutant Salmonella strains for indicated time points were subjected to SDS-PAGE and immunoblotting. (c) SopA-mediated degradation of TRIM56 and TRIM65 on heterologous expression. Lysates from inducible HeLa Flp-In T-REx GFP-SopA cells left untreated or treated with indicated combinations of 1 μg ml⁻¹ doxycycline and 20 μM MG132 were subjected to SDS-PAGE and immunoblotting.

Figure 8 | Model for dual SopA-mediated targeting of TRIM56 and TRIM65. Binding of SopA to the RING domains of TRIM56 and TRIM65 impedes TRIM E3 Ub ligase activity by occluding the RING-E2 interaction surface (left panel). The SopA HECT ligase activity deposits degradative ubiquitin chains linked via K48 and K11 on TRIM56 and TRIM65, resulting in their proteasomal degradation during Salmonella infection (right panel).

Our model (Supplementary Fig. 4c). However, Lys87 is disordered in the crystal structure of SopA–TRIM56, indicating that Ub conjugation to the HECT-active site may be necessary to stabilize the substrate lysine. Structural investigation of TRIM56 in complex with Ub-conjugated SopA will provide more insights into the SopA mechanism of action and HECT catalytic mechanism in general.

Interestingly, a recent study demonstrated the requirement of RING dimerization for the E3 ligase activity of TRIM25 and TRIM32 (ref. 26). We did not observe a dimer of TRIM56 RING in our crystal structure, raising the possibility that SopA binding may interfere with TRIM56 RING dimerization. To address this, we aligned one RING domain of the TRIM32 dimer with TRIM56 (residues 163–425) and TRIM56 (residues 1–94) in complex. The structure revealed the interface of SopA and TRIM56 in atomic detail and also provided an explanation for the specificity of SopA towards TRIM56 and TRIM65. The placement of the central α-helix in various RING domains in combination with the defined sequence features of the first Zn²⁺-binding loop appears to determine the specificity for SopA binding. Comparison of our SopA–TRIM56 complex structure with a previously determined SopA structure carrying the C-lobe 9 uncovers that the catalytic cysteine in the C-lobe is positioned in close proximity to the C terminus of the TRIM56 RING (Supplementary Fig. 4c). Strikingly, we found that the ubiquitination site at Lys87 in the RING domain of TRIM56 is one of the most upregulated modification events in cells infected with SopA-containing Salmonella (Fig. 6b and Supplementary Data 3), thus supporting the juxtaposition of these domains in our model (Supplementary Fig. 4c). However, Lys87 is disordered in the crystal structure of SopA–TRIM56, indicating that Ub conjugation to the HECT-active site may be necessary to stabilize the substrate lysine. Structural investigation of TRIM56 in complex with Ub-conjugated SopA will provide more insights into the SopA mechanism of action and HECT catalytic mechanism in general.

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Further analysis of the SopA–TRIM56 structure indicated that SopA binding to the RING domain obstructs the E2-binding site of TRIM56. This led us to hypothesize that SopA interferes with TRIM ligase activity. Titration experiments indeed provided support for SopA interaction-mediated inhibition of TRIM56 activity. It should be noted that in our assays SopA (residues 163–425) was added at 1 × 5 × and 10 × molar excess compared with E2 and the effective inhibition was only achieved with the last two conditions. It remains to be seen whether the inhibitory action of SopA on TRIM56 ligase activity is promoted by localized interactions in the context of full size proteins during infection.

This interaction mode of SopA is however an effective way for proteins to ubiquitinate E3 ligases in general without getting ubiquitinated themselves. This is achieved, because binding of SopA and E2 to the TRIM56 RING domain is mutually exclusive. Moreover, acute inhibition of TRIM56 and TRIM65 by binding of SopA functions in conjunction with SopA-mediated degradation ubiquitination of TRIM56/65 to downregulate activity and abundance of TRIM proteins in vivo. Taken together, our results provide evidence for an inhibitory mechanism underlying targeting of TRIM56/65 via SopA.

It is interesting to note that a recent study proposed a different model of action for SopA-mediated TRIM56/65 ubiquitination15. SopA-catalysed ubiquitination of TRIM56 and TRIM65 was suggested to be non-degradative and to promote TRIM56 and TRIM65 activity towards their respective targets RIG-I and MDA5, resulting in an increased transcription of type I interferon. Here we provide multiple lines of evidence in support of SopA-mediating degradation of TRIM65 and TRIM65. We observed that SopA-dependent ubiquitination of TRIM56 is robustly increased after treatment of infected cells with proteasome inhibitor. Moreover, both proteomics and biochemical data revealed that TRIM56 and TRIM65 are degraded in the course of infection and heterologous SopA expression with protein levels being restored by proteasome inhibition. In vitro ubiquitination experiments further indicated that SopA preferentially modifies TRIM65 with K48- and K11-linked polyubiquitin, two chain types known to confer degradative and to promote TRIM56 and TRIM65 ligase activity. It should be noted that in our assays SopA (residues 163–425) was then electrophoretically separated via gel electrophoresis. The incorporation of labelled amino acids to at least 95% was verified.

**Plasmids and bacterial strains.** All generated plasmids and Salmonella strains in this study are listed in Supplementary Table 1. S. Typhimurium strains are all in the virulent background. The SL1344 strain (invAΔ) was a kind gift from Jorge Galan and described previously35. The sopA deletion strain was generated using the lambda Red recombination method39. Therefore, lambda Red recombine expressing temperature-sensitive plasmid pKD46 (kind gift from Dirk Bumann) was introduced into SL1344 at 30 °C. Electrocompetent SL1344 carrying pKD46 was then electrophoretically separated via gel electrophoresis. The incorporation of labelled amino acids to at least 95% was verified.

Follow plasmids were made for purification of various proteins for use in in vitro ubiquitination assays, binding studies and crystallization experiments. Plasmids of mouse Ub1 and human UbC7 in PET28a vector with C-terminal HAB tag were used for expression/purification in E. coli BL21 DE3 and subsequently used in in vitro ubiquitination assays. SopA (163–782), various constructs of TRIM56 (full-length, 1–94, 1–207 and 95–207) were cloned into a modified pET15b vector with an N-terminal His tag cleavable by 3C protease. The fusion construct of SopA (163–425) and TRIM56 (1–94) was generated by inserting a linker sequence coding for GSSG (invAΔ) was a kind gift from Jorge Galan and described previously35. The sopA deletion strain was generated using the lambda Red recombination method39. Therefore, lambda Red recombine expressing temperature-sensitive plasmid pKD46 (kind gift from Dirk Bumann) was introduced into SL1344 at 30 °C. Electrocompetent SL1344 carrying pKD46 was then electrophoretically separated via gel electrophoresis. The incorporation of labelled amino acids to at least 95% was verified.
After incubation with horseradish peroxidase-conjugated secondary antibodies, membranes were developed using chemiluminescence reagents Immunocruz (Santa Cruz) or TMA-6 (Lumigen). The following commercial primary antibodies were used: α-HA (16B12, Covance MMS-101, 1:1,000, α-GFP (B-2, Santa Cruz sc-9996, 1:1,000), α-FLAG (M2, Sigma F3165, 1:5,000), α-Ub (P4D1, Santa Cruz sc-8017, 1:1,000), α-TRIM56 (Abcam ab154662, 1:1,000), α-TRIM65 (Sigma HPA085869, 1:50), α-γ-Tubulin (Sigma T6260, 1:5,000) and α-Vinculin (Sigma V5105, 1:1,000). Unscraped scans of developed membranes are provided in Supplementary Figs 6.7,8 and 9 as part of the Supplementary Information.

**Salmonella infection.** Cultures of S. Typhimurium strains were inoculated from single colonies in LB with 0.3 M NaCl and grown overnight for 16 h at 37 °C with shaking 180 r.p.m. The next day, bacteria were diluted 1:33 and grown at 37 °C and 180 r.p.m. for 3 h until an optical density of 0.8 was reached. Cell lines were grown to 80–90% confluency in DMEM + 10% FCS with penicillin and streptomycin. At more than 90% confluency, cells were washed and incubated in antibiotics-free medium. Infections were performed for 30 min at 37 °C at a multiplicity of infection of 50 in DMEM + 10% FCS. The infection medium was aspirated and cells were incubated for 1 h in DMEM + 10% FCS containing 100 μg ml⁻¹ gentamicin to kill extracellular bacteria. For the remainder of the experiment, cells were kept in full medium with 16 μg ml⁻¹ gentamicin.

**Protein immunoprecipitation.** For transient co-expression experiments, 8 × 10⁵ HEK293T cells were seeded in six-well plates. Transfection was performed 24 h later with 1 μg of corresponding plasmid DNA using Genejuice (Novagen) according to manufacturer’s instructions. Cells were harvested and lysed 24 h after transfection. For stable inducible protein expression, corresponding cells were treated with 1 μg ml⁻¹ doxycycline for 8 h. Cells were washed once in ice-cold PBS and subsequently incubated for 10 min on ice in immunoprecipitation buffer containing 10 mM Tris-HCl pH 7.5, 100 mM KCl, 2 mM MgCl₂, 0.5% NP-40 and 1 × Protease Inhibitor Cocktail EDTA-free (Roche). Insoluble material was pelleted by centrifugation for 10 min at 15,000 r.p.m., 4 °C and supernatants were applied to equal volume of buffer equivalent to 1:1 dilution of laemmli buffer. For respective IPs, anti-FLAG (Sigma) and anti-GFP (Chromotek) matrices were used and incubated for 2 h at 4 °C with gentle rotation. Protein-bound beads were washed 5 × with IP lysis buffer and eluted by boiling in 2× Laemmli buffer. For SILAC-coupled IP, the same amount of heavy and light labelled cells were harvested and proteins were immunoprecipitated separately. SILAC-IPs from differently labelled conditions were pooled during the last wash step and eluted together.

**Recombinant protein expression and purification.** GST- or MBP-fusion coding plasmids were transformed into E. coli BL21 (DE3). Overnight cultures were diluted 1:40 in LB medium with 200 μM ZnSO₄ and cells were grown to OD₆₀₀ of 0.6 at 37 °C and 140 r.p.m. Cultures were cooled down to 16 °C and protein expression was induced with 0.25 mM isopropyl-β-D-thiogalactoside. After overnight incubation (≥18 h), cells were lysed by sonication in lysis buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride and 0.5% β-mercaptoethanol) and incubated in lysis buffer with 0.5% Triton X-100 for 5 min on ice. Lysates were cleared by centrifugation and the supernatants were incubated with glutathione sepharose 4B beads (GE Healthcare) or amylose resin (NEB) at 4 °C with gentle rotation. Fusion protein-bound beads were washed 3 × with lysis buffer containing 100 mM NaCl. MBP-fusion proteins were eluted using Laemmli buffer and maltose-containing buffer (25 mM Tris-HCl pH 7.5 and 10 mM maltose). C-terminal CD3-His-tagged fusion of TRIM56 (1–94) and SopA (163–425) was expressed in Rosetta DE3. Overnight cultures were diluted 1:40 in LB medium with 200 μM ZnSO₄ and cells were grown to OD₆₀₀ of 0.6 at 37 °C and 140 r.p.m. Cultures were cooled down to 16 °C and protein expression was induced with 0.25 mM isopropyl-β-D-thiogalactoside. After overnight incubation (≥18 h), cells were lysed by sonication in lysis buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride and 0.5% β-mercaptoethanol) and incubated in lysis buffer with 0.5% Triton X-100 for 5 min on ice. Lysates were cleared by centrifugation and the supernatants were incubated with glutathione sepharose 4B beads (GE Healthcare) or amylose resin (NEB) at 4 °C with gentle rotation. Fusion protein-bound beads were washed 3 × with lysis buffer containing 100 mM NaCl. MBP-fusion proteins were eluted using Laemmli buffer and maltose-containing buffer (25 mM Tris-HCl pH 7.5 and 10 mM maltose).

**In vitro ubiquitination.** Unless otherwise mentioned, in vitro ubiquitination reaction mixture contained 20 μM purified Ubc1 (1E), 1 μM E2 (UbcUex3a/b), 1 μM SopA (163–728) WT or C753A mutant and ~8 μg of various TRIM56 constructs. Ub was added to a final concentration of 25 μM and the reaction was initiated by adding 2.5 mM ATP. Ubiquitination buffer contained 50 mM Tris pH 7.5, 50 mM NaCl, 10 mM MgCl₂ and 0.5 mM DTT. Reaction was allowed to proceed for 1 h at 37 °C and stopped by boiling in Laemmli buffer. Samples were analysed by SDS–PAGE followed by western blotting. For the in vitro ubiquitination experiment shown in Supplementary Fig. 3e, bacterial lysates expressing WT and various mutants of TRIM56/RING domain were used instead of purified TRIM56 constructs.

**MS sample preparation.** For SILAC interactome analysis, IP eluates were subjected to in-gel trypsin digestion. To this end, eluates were run on a 10% Tris-glycine SD–PAGE and lanes were cut into six slices each. Proteins were reduced with 10 mM DTT, alkylated with 55 mM chloroacetamide and digested overnight with 12.5 ng μl⁻¹ trypsin. The next day, peptides were sequentially extracted from gel pieces using 30, 80 and 100% acetonitrile-containing solvents. For proteomics and diglycine proteomics experiments, SILAC labelled cells were washed twice with ice-cold PBS and used under denaturing conditions in 5 ml denaturation buffer (6 M urea, 1% SDS, 50 mM Tris pH 8, 50 mM NaCl, 1 × Protease Inhibitor Cocktail EDTA-free (Roche) and 50 mM DUB inhibitor PR-619). To fragment DNA, lysates were sonicated 2 × 90 s with 1 s pulses. Protein content was measured using BCA assay (Thermo Scientific) and differentially labelled lysates were mixed in a 1:1 ratio. Peptides were precipitated using methanol/chloroform and resuspended in 7 ml thiourea-containing denaturation buffer (6 M urea, 2 M thiourea and 50 mM Tris-HCl pH 8). Samples were reduced using 5 mM DTT and alkylated with 10 mM chloroacetamide. The protein yield from digestion was performed initially with Lys-C (Wako; 1:200 substrate-enzyme ratio) for at least 4 h. Digests were stopped by addition of 25 mM Tris-HCl pH 8 and incubated for at least 16 h with trypsin (Promega; 1:200 substrate-enzyme ratio) at room temperature. Digests were stopped by addition of trifluoroacetic acid (TFA) to 0.4% and diglycine proteomics samples were desalted using IC18-Sep-Paks (Whatman), eluted in 30% acetonitrile and dried by lyophilization.

**Purification of diglycine-modified peptides.** After lyophilization, peptides were resuspended in 1.5 ml IPA buffer (50 mM MOPS pH 7, 10 mM NaHPO₄ and 50 mM NaCl). The pH of the peptide solution was adjusted using 1 M Tris pH 10 and insoluble material was removed (9,000 g/8 min). Supernatants were incubated with 128 μg IPA buffer equilibrated tr-(lys)-Gγ(GG) antibody coupled agarose (Cell Signaling) for 4 h at 4 °C. Beads were washed 3 × with IPA buffer and twice with MilliQ water. Bound diglycine peptides were eluted 2 × with 80 μl 0.15% TFA by centrifugation and were further separated into multiple fractions using H₂O-based strong cation exchange chromatography. Each fraction peptide was desalted using C18 material containing stage tips.

**Peptide identification and data analysis.** Each peptide sample was applied onto an EasyLC nano-HPLC-coupled Orbitrap Elite setup (Thermo Scientific). Peptides were digested in 0.5% acetic acid on a 15 cm-long C18-filled silica capillary column. Samples were separated at a flow of 200 nl min⁻¹ using a 226 min gradient of 5–33% solvent B (80% acetonitrile and 0.5% acetic acid). Peptide MS spectra were acquired in the Orbitrap ranging from m/z 300 to 2,000 with a resolution of 120,000. The mass spectrometer was operated in data-dependent acquisition mode. For each cycle, the most intense peptide ion and its charge state were sequentially isolated and CID MS/MS spectra were acquired in the iontrap. The target values of the mass analysers were 10⁶ (Orbitrap) and 5 × 10⁵ (Iontrap). Peptide ions with unsigned charge state and in case of diglycine proteomics samples below m/z 3 were excluded from fragmentation. Raw data were processed with MaxQuant (1.3.16.0) and fragmented peaks were searched against human and Salmonella SL1344 UNIPROT databases. Up to two missed tryptic cleavages were allowed. Carbamidomethylation of cysteine was set as fixed modification. Variable
modifications included N-terminal acetylation, methionine oxidation and GlyGly modification. For protein quantification, a minimum of two quantified peptides was required. Both peptide and protein false discovery rate were set to 1%. Perseus was used for data sorting.

**Isothermal titration calorimetry.** Purified SopA (163–782) and TRIM56 (1–94) were buffer exchanged into 10 mM HEPES, 100 mM NaCl and 1 mM TCEP. Using VP-ITC Microcal calorimeter (GE Healthcare), 1.4 ml of 20 μM SopA was titrated with 1 mM of TRIM56 at 25 °C with 5 μl injections with a gap of 5 min between each injection. Heat of dilution was also measured by injecting 1 mM TRIM56 into the buffer and the resulting values were subtracted from the SopA–TRIM56 titration curve. Data were analysed using Origin 7 software provided by Microcal.

**Data availability.** The atomic coordinates of the SopA–TRIM56 structure have been deposited in the Protein Data Bank under accession number 5W7L. All the remaining data can be obtained from the corresponding author upon reasonable request. The following reported PDB files were used: 4AP4, 2JMD, 2QZA and 2CT2.

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**Author contributions**

E.F. and LC conceived the study and initiated the project. LC supervised the project. E.F. performed and analysed MS experiments, *Salmonella* infections, immunoprecipitation experiments, biochemical experiments with GST- and MBP-fusion proteins, cycloheximide chase assays, generated stable cell lines and *Salmonella* sopA knockout and

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complemented strains. S.B. performed and analysed crystallography experiments including protein purification of SopA–TRIM56 fusion and HIS-tagged proteins, crystallization, structure determination, isothermal titration calorimetry, nickel pull-down experiments, modelling and in vitro ubiquitination experiments. L.H. and M.H. performed immunoprecipitation and infection experiments. S.K. performed in vitro ubiquitination experiments. E.F., S.B. and I.D. wrote and all authors edited the manuscript.

Additional information

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