Biochemical and Biophysical Research Communications 447 (2014) 172–177

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Identification of TRAF6 as a ubiquitin ligase engaged in the ubiquitination of SopB, a virulence effector protein secreted by *Salmonella typhimurium*

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ARTICLE INFO

Article history:
Received 21 March 2014
Available online 2 April 2014

Keywords:
TRAF6
SopB
Ubiquitin ligase
Effector
Ubiquitination
Salmonella typhimurium

ABSTRACT

The phosphoinositide phosphatase SopB is one of the effectors injected by *Salmonella typhimurium* (*S. typhimurium*) that diversifies its function through a ubiquitin-dependent differential localization. However, it is unclear which E3 ubiquitin ligase is responsible for ubiquitination of SopB. Based on the E1-E2-E3 trio of enzymes responsible for the ubiquitin activation and translocation to substrate proteins, we constructed an *in vitro* assay of SopB ubiquitination. Using this assay, we purified an E3 ubiquitin ligase, TRAF6, from the Henle-407 S100 extraction that may be responsible for the ubiquitination of SopB. To investigate the functional correlation of TRAF6, we showed that recombinant TRAF6 specifically ubiquitinates SopB in a dose-dependent manner *in vitro*. Upon infection, the ubiquitination of SopB was absolutely blocked by TRAF6 deletion, as shown in *Traf6*$^{-/-}$ mouse embryonic fibroblasts (MEFs) compared with *Traf6*$^{+/+}$ MEFs. However, the ectopic expression of TRAF6 in *Traf6*$^{-/-}$ MEFs rescued the two species of ubiquitin-conjugated SopB, which strengthens the role of TRAF6 in SopB ubiquitination. The analysis of E2 revealed that UbcH5c and not other E2 conjugating enzymes are required for TRAF6-mediated SopB ubiquitination both *in vitro* and *in vivo*. In summary, these results suggest the relevance of UbcH5c/TRAF6 in SopB during *S. typhimurium* infection and thereby imply that *S. typhimurium* has evolved a mechanism of utilizing the host's E3 ubiquitin ligase to modify and modulate the function of its effector protein in order to ensure pathogen and host cell survival.

1. Introduction

Salmonella typhimurium is a Gram-negative facultative intracellular bacterial pathogen that causes gastrointestinal diseases in humans and a typhoid-like systemic infection in mice [1]. These bacteria have the ability to invade host cells and grow intracellularly by injecting effector proteins directly into the cytosol of the host cells [2]. Most *S. typhimurium* effector proteins participate in the modulation of a variety of cellular processes to promote bacterial entry and survival [3–5]. At least >30 effector proteins are delivered by *S. typhimurium* at different times during the infection process [6].

SopB is translocated by the *Salmonella* pathogenicity island [SPI]-1-encoded type III secretion system (TTSS). Upon translocation from the bacteria, SopB localizes to the plasma membrane of the infected cells [7–9], from where it mediates actin cytoskeleton reorganization [10,11], bacterial entry [12], and Akt activation [13–15]. Shortly after its translocation, SopB is ubiquitinated. This modification acts as a signal for its removal from the host-cell plasma membrane and its subsequent delivery to internal vesicular compartments and/or for its "trapping" on the nascent bacterial phagosome [8,9]. The observation that SopB diversifies its function by localizing to different cellular compartments in a ubiquitin-dependent manner prompted us to identify the E3 ubiquitin ligases that contribute to the ubiquitination of SopB. Early in 2002, the transfected SigD/SopB fusions were found to be ubiquitinated inside

Abbreviations: S. typhimurium, *Salmonella typhimurium*; MEFs, mouse embryonic fibroblasts; SPI, *Salmonella* pathogenicity island-1; TTSS, type III secretion system; TNF, tumor necrosis factor; TRAF, TNF receptor-associated factor; cDNA, complementary DNA; IPTG, isopropyl β-D-thiogalactopyranoside; RING, really interesting new gene; WCE, whole-cell extract.

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http://dx.doi.org/10.1016/j.bbrc.2014.03.126
0006-291X/

mammalian cells [16]. Moreover, the three known *Salmonella*-secreted E3 ubiquitin ligases, namely SopA, SspH2, and Slrp, were all excluded from the function correlation with the ubiquitination of SopB [8]. Therefore, we speculated that the E3 ubiquitin ligase is derived from mammalian cells. Based on this hypothesis, we constructed an *in vitro* SopB ubiquitination assay. Using our assay, we subsequently purified TRAF6, a member of the tumor necrosis factor (TNF) receptor-associated factor (TRAF) family in Henle-407 cells, and found that it ubiquitinates SopB both *in vitro* and *in vivo*.

2. Materials and methods

2.1. Bacterial strain and cell lines

The wild-type strain of *S. typhimurium* LT2 used in this study was obtained from the China General Microbiological Culture Collection Center (CGMCC 7020). Henle-407 cells were purchased from the American Type Culture Collection (ATCC CCL-6). The MEF cells (*Traf6*$^{+/+}$ and *Traf6*$^{-/-}$) were gifted by Dr. Jun-ichiro Inoue (Tokyo University, Tokyo, Japan). The Phoenix-293 cells were gifted by Dr. Zhen-Yi Ma (Tianjin Medical University, Tianjin, China).

2.2. Plasmids

To construct 3HA-SopB for recombinant expression in *Escherichia coli* and *S. typhimurium*, the DNA for SopB was amplified with the following primers: forward primer, 5′-CGGAATTCTACCCATACGATGTTCCAGATTACGCTTACCCATACGATGTTCCAGATTACGCTTACCCATACGATGTTCCAGATTACGCTATGCAAATACAGAGCTTCTATCAC-3′; and reverse primer, 5′-CGGTCGACTCAAGATGTGATTAATGAATAAAT-3′. In the forward primer, the 3 × HA epitope-encoding sequence, which is shown underlined, was inserted before the initiation codon of SopB. The amplified SopB fragment was then cloned into the pET28a and pBAD24 vectors. The complementary DNA (cDNA) for TRAF6 was amplified from a HeLa cDNA library and cloned into the pET28a vector for recombinant expression in *E. coli* and the pcDNA4-FLAG vector for mammalian expression as previously described [17]. In addition, pET28a/E1, pGEX-6p-2/UbcH6, pGEX-6p-2/UbcH7, pGEX-6p-2/Cdc34, pGEX-6p-2/Ubc3b, pGEX-6p-2/UbcH8, pET28a/Ube2g2, pGEX-6p-2/UbcH5a, pGEX-6p-2/UbcH5b, pGEX-6p-2/UbcH2a, pGEX-6p-2/UbcH2b, pGEX-6p-2/UbcH10, pET21a/UbcH5c were all gifts from Dr. Hong-Tao Li (Xinan University, Chongqing, China). The point mutations, including TRAF6 C70A, UbcH5c S22R, and UbcH5c F62A, were all generated using the QuickChange Site-Directed Mutagenesis Kit (Stratagene). All of the clones were verified by DNA sequencing.

2.3. Cell culture

All of the Henle-407 cells, MEFs, and Phoenix-293 cells were maintained in high-glucose DMEM (Thermo Scientific HyClone) supplemented with 10% fetal bovine serum (Gibco) at 37 °C in an atmosphere of 5% CO_2 without antibiotics.

2.4. Induction and purification of recombinant proteins in E. coli

The expression of all of the proteins, including E1, E2s, TRAF6, and the 3HA-SopB construct in *E. coli* was induced by isopropyl β-D-thiogalactopyranoside (IPTG) through the standard procedure. All of the His fusion proteins encoded by pET28a were purified by Ni–NTA Sepharose beads (Qiagen), and the GST fusion proteins encoded by pGEX-6P-2 were purified by glutathione-Sepharose 4B (GE Healthcare). UbcH5c and its variants cloned into pET21a were purified as described previously [18]. FLAG-tagged ubiquitin and its variant proteins were gifted by Dr. Hong-Tao Li (Xinan University, Chongqing, China).

2.5. In vitro ubiquitination assays of SopB

To construct the ubiquitination of SopB *in vitro*, cultured Henle-407 cells were harvested for S100 extraction as previously described [17]. The *in vitro* SopB ubiquitination assays were conducted in a 30-μl reaction buffer (50 mM Tris–HCl, pH 7.4, and 5 mM $MgCl_2$) containing 100 ng of E1, 1 μg of FLAG-ubiquitin, 0.5 μg of 3HA-SopB, and 15 μl of the S100 extraction. The reactions were initiated by the addition of γ-ATP (Promega) to a final concentration of 2 mmol/L and then incubated at 37 °C for 30 min. Due to an N-terminal His-tag in the recombinant 3HA-SopB, 3HA-SopB and ubiquitinated 3HA-SopB could be pulled down with Ni–NTA beads. Both the SopB and ubiquitinated SopB could be detected by anti-HA (sc-7392, Santa Cruz Biotechnology) immunoblotting, but only the ubiquitinated SopB could be detected by anti-FLAG (F3165, Sigma) immunoblotting.

To screen the E2 ubiquitin-conjugating enzymes involved in the cascade of SopB ubiquitination, we executed and pulled down SopB as described above with the exception that we also added 0.3 μg of recombinant E2 enzymes, including UbcH10, UbcH5a, UbcH5c, UbcH2a, UbcH2b, Cdc34, UbcH8, Ube2g2, UbcH7, UbcH6, Ubc3b, and UbcH5b, to the mixture during the reaction process.

For the detection of E3 ubiquitin ligase activity in the column fractions during purification, 10 μl of the column fractions were added to 30-μl reaction mixtures containing 100 ng of E1, 0.3 μg of UbcH5c, 1 μg of FLAG-ubiquitin, and 0.5 μg of 3HA-SopB. For the analysis of the TRAF6-catalyzed ubiquitination of SopB *in vitro*, the assays were conducted using the procedure described for the analysis of the column fractions with the exception that 1 μg of TRAF6 was added to the reaction mixture instead of 10 μl of the column fractions. The detection of other proteins in the reactions, including ubiquitin, UbcH5c, and TRAF6, was determined by immunoblotting the reactions with anti-FLAG (F3165, Sigma), anti-UbcH5c (ab58251, Abcam), and anti-TRAF6 (sc-7221, Santa Cruz Biotechnology), respectively.

2.6. Purification of ubiquitin ligase activity and mass spectrometry

Purification of ubiquitin ligase activity and mass spectrometry analysis were listed in Supplementary materials.

2.7. Transfection, infection, and SopB immunoprecipitation

The transfection of pcDNA4-FLAG-TRAF6 for mammalian expression was performed using VigoFect (Vigorous Biotechnology). For bacterial infection, the *S. typhimurium* LT2 strains were transformed with pBAD24-3HA-SopB. The expression of 3HA-SopB in *S. typhimurium* was performed as previously described [8]. The 3-h-induced *S. typhimurium* was collected and added to serum-starved cells at an MOI of 30 for infection as previously described [13]. For anti-SopB immunoprecipitation, the cells were collected and lysed in buffer A (50 mM HEPES, pH 7.4, 150 mmol/L NaCl, and 1% NP-40) supplemented with 1% protease inhibitor cocktail (Roche). After centrifugation at 12,000 rpm for 20 min, the supernatants were pre-cleared using protein A Sepharose beads at 4 °C for 1 h. Then, 2 μg of the SopB antibodies (a rabbit polyclonal antibody prepared in our lab as described in patent No. 201310029506.0) was added to the pre-cleared lysates, and the mixture was incubated for 4 h. Then, 20 μL of protein A Sepharose beads were added to precipitate the immunocomplex at 4 °C for 1 h. After washing three times with buffer A, the proteins immunoprecipitated with protein A Sepharose beads were separated by SDS–PAGE and immunoblotted with anti-HA

(sc-7392, Santa Cruz Biotechnology) or anti-ubiquitin (sc-8017 Santa Cruz Biotechnology).

2.8. Construction of lentivirus and viral infection

To silence UbcH5c in Henle-407 cells, the shUbcH5c lentiviral particles were prepared with method described previously [19]. After 8 h of infection, the Henle-407 cells were washed and allowed to recover for 24 h prior to arabinose-induced *S. typhimurium* infection. A virtually identical procedure was used to silence Ubc13 with a targeting sequence of 350–368.

3. Results and discussion

3.1. In vitro screening for E3 ubiquitin ligase

Previous studies revealed that SopB is ubiquitinated regardless of whether it is transfected or translocated into mammalian cells [8,9,16]. It has been established that the E3 ubiquitin ligase is responsible for the translocation of ubiquitin to substrate proteins. However, to the best of our knowledge, it is unclear which E3 ubiquitin ligase is engaged in the ubiquitination of SopB.

Firstly, we designed an assay for detecting the activity of candidate E3 ubiquitin ligases. In this assay, 3HA-SopB and FLAG-tagged ubiquitin were used as the two substrates. As shown in Fig. 1A, compared with the only band of 3HA-SopB obtained through both non-S100 and non-ATP initiated reactions, a new band with a slower migration was observed through anti-HA immunoblotting as a result of S100 catalysis. Furthermore, this slower band could be immunoblotted by anti-FLAG, which suggests that the new band should be ubiquitin-conjugated 3HA-SopB. These results indicate that it is feasible to detect the activity of E3 ubiquitin ligase upon the ubiquitination of SopB *in vitro* and thus supply a method for the purification of the candidate E3 ubiquitin ligase.

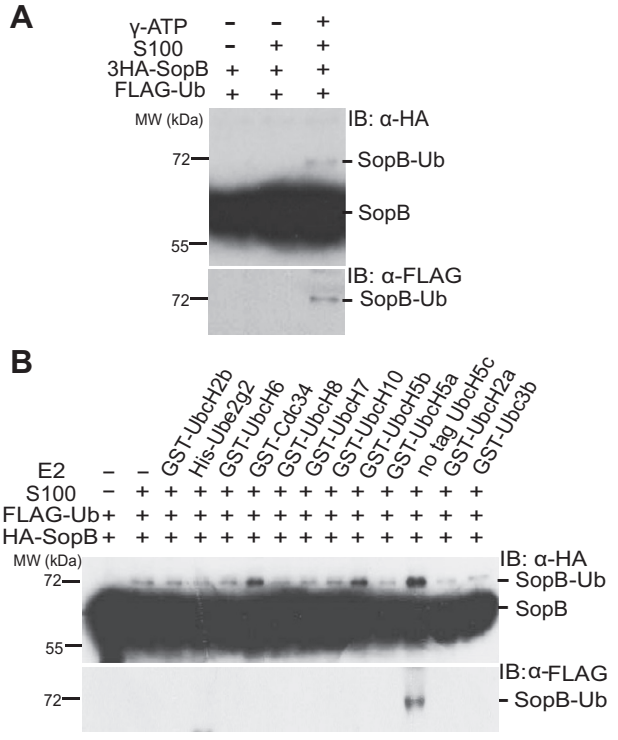

Fig. 1. Construction of *in vitro* assay of SopB ubiquitination. (A) SopB ubiquitination catalyzed by Henle-407 S100 extraction. (B) Profiles of E2 ligases that conjugated the ubiquitination of SopB catalyzed by Henle-407 S100 extraction.

It is worth mentioning that only a marginal degree of ubiquitinated SopB was detected in response to S100 catalysis. This result is qualitative rather than quantitative similar to the results obtained with transfected and translocated SopB in mammalian cells [8,9,16]. This disparity may be explained by the dissolution of nearly all of the proteins into the buffers during cell lysis, which resulted in the dilution of the candidate E3 ubiquitin ligases in contrast to their enriched subcellular localization in intact cells.

Due to the requirement of a specific E2 for ubiquitin ligase enzymes, dozens of recombinant E2 enzymes, including UbcH10, UbcH5a, UbcH5c, UbcH2a, UbcH2b, Cdc34, UbcH8, Ube2g2, UbcH7, UbcH6, Ubc3b, and UbcH5b, were tested to determine whether one or many of them mediate the E3-catalyzed SopB ubiquitination. As shown in Fig. 1B, only the addition of UbcH5c revealed a robust stimulation of 3HA-SopB mobility, which is indicative of ubiquitination, in both anti-HA and anti-FLAG immunoblots. In contrast, although Cdc34 and UbcH5b exhibited a slightly higher level of ubiquitinated 3HA-SopB in the anti-HA immunoblots, they showed no evidence of 3HA-SopB ubiquitination in the anti-FLAG immunoblots. Taken together, these results suggest that UbcH5c is a potent E2 ubiquitin-conjugating enzyme involved in the cascade of SopB ubiquitination. However, these results do not rule out the possibility that other E2 ubiquitin-conjugating enzymes that were not tested in the current study may also contribute to the ubiquitination of SopB.

3.2. Purification of E3 ubiquitin ligase

In step 1, the S100 extraction product was fractionally precipitated by ammonium sulfate. The precipitations with E3 ubiquitin ligase activity after step 1 were subjected to three sequential chromatography columns, namely, phenyl HP, Q HP, and SP HP, as schematically shown in Fig. 2A. The fractions from each column were assayed for the capability to ubiquitinate 3HA-SopB (see "Section 2"). The analysis for E3 ubiquitin ligase activity of the SP HP fraction (Step 4) is shown in Fig. 2B (upper panel). The major peak of ubiquitin ligase activity was found for Fraction 8 and with a minor peak for Fraction 10. The eluted proteins in Fractions 8 and 10 were stained by coomassie R-250 after SDS–PAGE, as shown in the lower panel of Fig. 2B. Through tandem mass spectrometry analysis (MALDI-TOF-TOF), the band migrating between the 55-kDa and 72-kDa markers hinted that it was TRAF6, a type of E3 ubiquitin ligase of *Homo sapiens*. The six bitted peptides in the purified protein are shown in Table 1. Therefore, we hypothesized that TRAF6 may be the E3 responsible for the ubiquitination of SopB.

3.3. TRAF6 ubiquitinates SopB in vitro

To investigate the function of TRAF6, we purified the recombinant TRAF6 in *E. coli* and used it in the *in vitro* assays of SopB ubiquitination. The results shown in Fig. 3A demonstrate that TRAF6 ubiquitinates SopB in a dose-dependent manner. It is worth mentioning that the recombinant TRAF6 also has a His-tag in its N terminus, as described in "Section 2". Thus, it can be pulled down by Ni–NTA beads, similarly to 3HA-SopB. However, only 3HA-SopB could be specifically detected with anti-HA immunoblotting, a process that did not detect TRAF6 at all. Based on these findings, we assured that the detected signal was specific to SopB. In contrast to TRAF6, its C70A mutant, which does not possess E3 ubiquitin ligase activity, completely impaired the ubiquitination of SopB (Fig. 3B). Two UbcH5c activity-deficient mutants, namely F62A and S22R, abolished the TRAF6-catalyzed SopB ubiquitination (Fig. 3C). These findings appear to strongly support the relevance of TRAF6/UbcH5c for SopB ubiquitination. To further confirm the specificity of SopB ubiquitination by TRAF6, we replaced ubiquitin

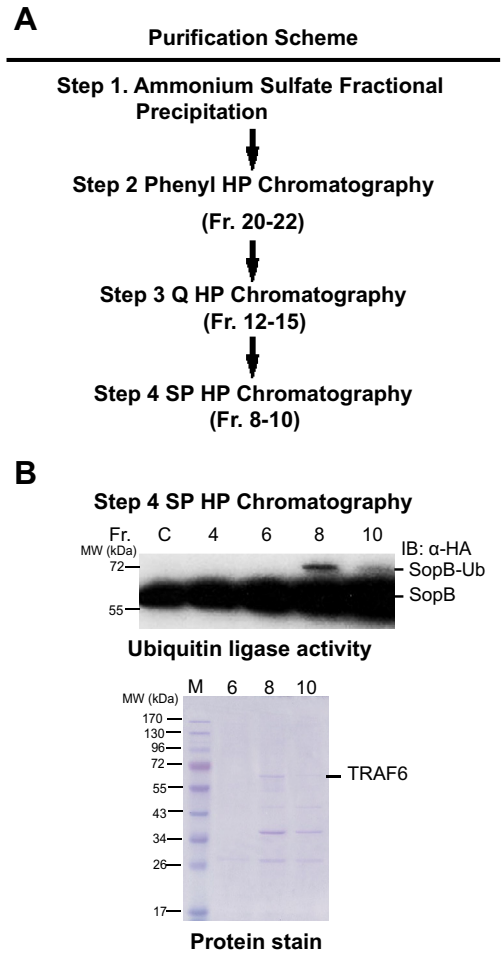

Fig. 2. Purification of the E3 ubiquitin ligase responsible for SopB ubiquitination from the Henle-407 S100 extraction. The starting material was the S100 extraction product obtained from Henle-407 cells. The fractions (Fr.) from each column were assayed for ubiquitin ligase activity; "C" refers to a control without added fraction. (A) Purification scheme for the identification of ubiquitin ligase activity against 3HA-SopB. (B) Upper panel, assay of the column fractions from step 4 (SP cation exchange chromatography) for ubiquitin ligase activity. Lower panel, coomassie R-250 staining of the proteins in the column fractions with ubiquitin ligase activity.

with a ΔGG variant that lacked the extreme C-terminal Gly-Gly dipeptide. As expected, the ubiquitinated SopB was no longer detected with ΔGG ubiquitin (Fig. 1D). The replacement of ubiquitin two other variants, namely K63 (a ubiquitin mutant in which lysine 63 is the only residue available for conjugation) or K0 (in which no lysine residue is available for conjugation) resulted in the normal detection of the ubiquitination of SopB, similarly to that obtained with wild-type ubiquitin (Fig. 3E), and these results are in accordance with the reported characteristics of SopB ubiquitina-

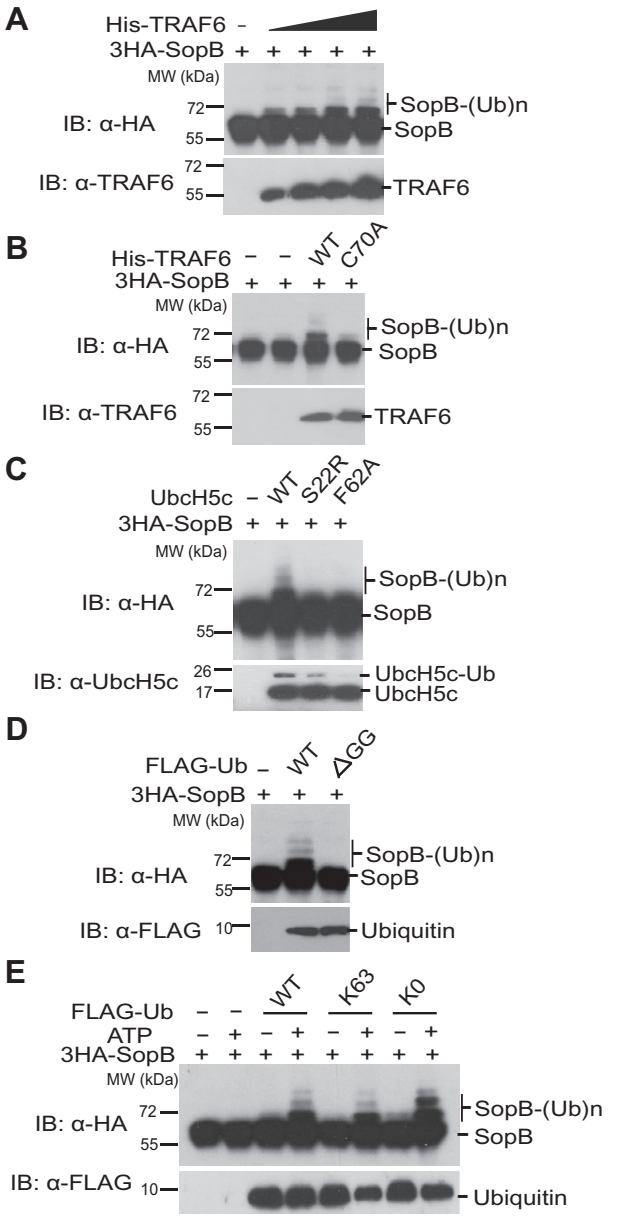

Fig. 3. Properties and mechanisms of the TRAF6-catalyzed SopB ubiquitination *in vitro*. (A) TRAF6 catalyzes the ubiquitination of SopB in a dose-dependent manner. (B) TRAF6 but not its C70A activity mutant ubiquitinates SopB. (C) UbcH5c but not its S22R or F62A mutants conjugates the ubiquitination of SopB by TRAF6. (D) The disability of the ubiquitin ΔGG mutant to modify SopB confirms the specificity of the ubiquitination of SopB by TRAF6. (E) Capability of the TRAF6-catalyzed ubiquitination of SopB with ubiquitin and its variants, including K63 and K0. Besides SopB, TRAF6 and its mutant, UbcH5c and its mutants, and ubiquitin and its mutants were all shown by separating the whole reaction with SDS–PAGE followed by immunoblotting for TRAF6, UbcH5c, or FLAG, respectively.

Table 1
Peptide summary report of the protein analyzed through MALDI-TOF-TOF.

Query	Observed	Mr. (expt)	Mr. (calc)	ppm	Miss	Score	Expect	Rank	Unique	Peptide
2	1341.8661	1340.8588	1340.7300	96.1	0	83	8.2e−07	1	U	R.LTILDQSEAPVR.Q
4	1471.8705	1470.8632	1470.7215	96.3	0	102	3.4e−08	1	U	R.NFQETIHQLEGR.L
5	1601.9113	1600.9040	1600.7589	90.7	1	109	9e−09	1	U	K.METQSMYVSELKR.T
6	1640.9656	1639.9583	1639.8181	85.5	0	82	3e−06	1	U	K.GFGYVTFMHLEALR.Q
9	2372.4304	2371.4231	2371.2206	85.4	0	176	6.5e−16	1	U	R.MLAQAVHSLSVIPDSGYISEVR.N
12	2733.6526	2732.6453	2732.4068	87.3	0	121	1.6e−10	1	U	R.QNHEEIMDAKPELLAFQRPTIPR.N

Mascot score histogram of the purified protein analyzed by MALDI-TOF-TOF. The ion score is $-10 * \mathrm{Log}(P)$, where P is the probability that the observed match is a random event. Individual ion scores greater than 38 indicate identity or extensive homology ($p < 0.05$). The protein scores are derived from the ion scores as a non-probabilistic basis for the ranking of the protein hits.

tion [8]. However, to date, we cannot determine the type of ubiquitination catalyzed by TRAF6 in SopB, i.e., if it is multi-mono-ubiquitination, linear ubiquitination, or a mix of these two forms. Distinct with the monouniquitination of SopB by S100 or fractions as shown in Figs. 1 and 2, TRAF6 was found to catalyze the multiubiquitination of SopB. Moreover, the level of ubiquitinated SopB was higher than those by S100 or fractions, implying that a rise of TRAF6 amount results in the elevation of ubiquitinated SopB and a pattern of multiubiquitination as previously reported [8,9].

3.4. Functional verification of UbcH5c/TRAF6 relevance for SopB

To investigate the role of TRAF6 in SopB ubiquitination during infection, *Traf6*$^{+/+}$ and *Traf6*$^{-/-}$ MEFs were used. As shown in Fig. 4A, the translocated 3HA-SopB, which is expressed by pBAD24 in *S. typhimurium*, was found to be rapidly ubiquitinated within 30 min and sustained up to 60 min and 120 min post-infection in *Traf6*$^{+/+}$ MEFs, as detected by anti-HA and anti-FLAG immunoblottings, which were consistent with previous results [8,9]. Distinctly, the translocated SopB only migrated as a single unmodified band in the *Traf6*$^{-/-}$ MEFs. This disappearance of ubiquitinated SopB in *Traf6*$^{-/-}$ MEFs verified the *in vivo* function of TRAF6 observed *in vitro*. We then ectopically expressed FLAG-tagged TRAF6 in *Traf6*$^{-/-}$ MEFs to determine whether TRAF6 expression can rescue the disappearance of ubiquitinated SopB. As shown in Fig. 4B, ectopically expressed TRAF6 in *Traf6*$^{-/-}$ MEFs rescued two species of ubiquitinated SopB. Moreover, it could be seen that the level of ubiquitinated SopB was quantitative as previously reported [8,9,16], verifying the assumption that the marginal degree of ubiquitinated SopB by Henle-407 S100 *in vitro* results from the minor amount of TRAF6 in S100. The recovery of TRAF6 in *Traf6*$^{-/-}$ MEFs can fully reconstitute the ubiquitination of SopB. However, the TRAF6 C70A mutant cannot reconstitute the SopB ubiquitination, suggesting that the RING-type ubiquitin ligase activity and not its function as an adaptor [20] of TRAF6 is pivotal for SopB ubiquitination.

To determine the UbcH5c/TRAF6 relevance *in vivo*, we knocked down UbcH5c with shUbcH5c and then detected the status of SopB. As shown in Fig. 4C, the knockdown of UbcH5c showed a robust inhibition of ubiquitinated SopB after *S. typhimurium* infection compared with that obtained with control shRNA, implying that UbcH5c has a coupling function with TRAF6 during infection in accordance with the conclusions obtained *in vitro*. It is known that Ubc13/Uev1a is the most widely reported E2 associated with TRAF6 [21]. Inevitably, we investigated whether Ubc13/Uev1a also participates in the TRAF6-catalyzed SopB ubiquitination. As shown in Fig. 4C, the silencing of Ubc13 only exerted a negligible effect on the level of ubiquitinated SopB in contrast to the sharp role of UbcH5c. Collectively, these results demonstrate that it is UbcH5c and not the routine Ubc13/Uev1 that mediates the TRAF6-catalyzed ubiquitination of SopB. Despite the argument of UbcH5 a rather unspecific and therefore likely to light up in *in vitro* ubiquitination assays [22], the conjugation to TRAF6 is specific to UbcH5c, but not other members of the UbcH5 family, is highlighting the unique critical role of UbcH5c. TRAF6 is known a eukaryotic really interesting new gene (RING) type E3 ubiquitin ligase [23]. Therefore, we speculated that TRAF6 might use zinc chelation and hydrogen bonds/salt bridges to transfer Ub from the UbcH5c-Ub intermediate to SopB. Not surprisingly, this finding is not the first illustration of the relevance of UbcH5c/TRAF6. A new mechanism of IκB kinase activation has uncovered an analogous coupling of UbcH5c/TRAF6, which act in concert toward the synthesis of unanchored polyubiquitin chains [24]. Previous reports have revealed that TRAF6 usually catalyzes K63-linked polyubiquitination that is discovered to function as a signaling moiety rather than hallmark for proteasomal degradation [25]. The signaling role

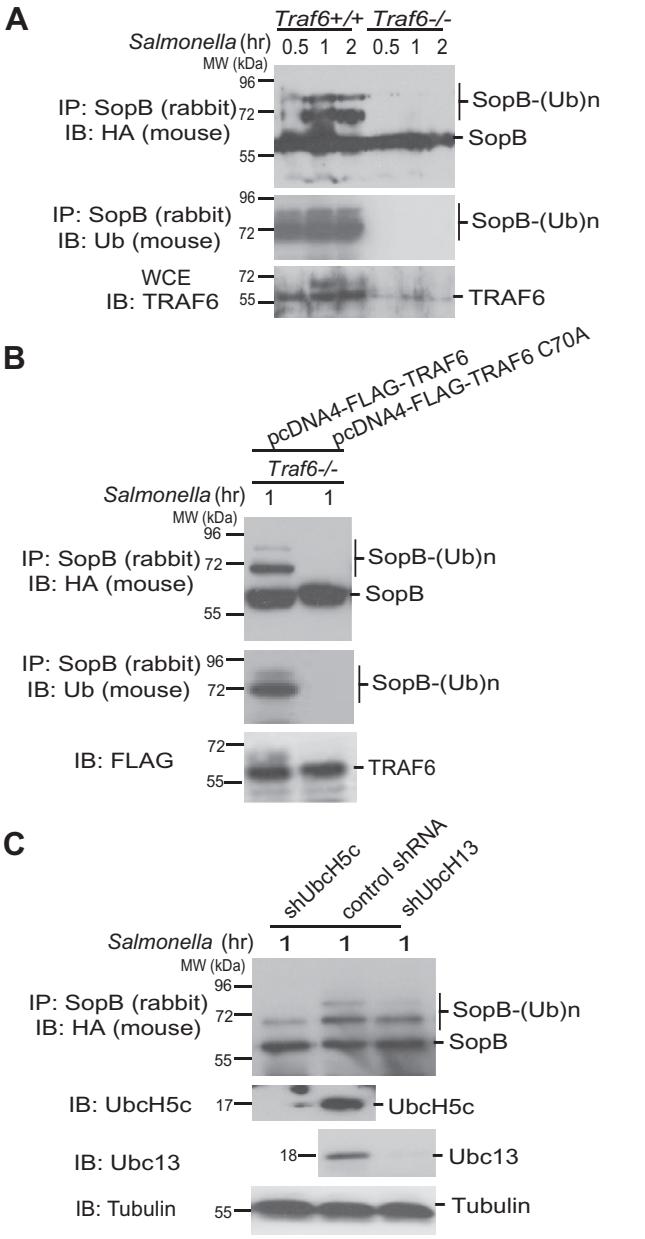

Fig. 4. Functional verification of UbcH5c/TRAF6 relevance for SopB. (A) Presence of SopB and ubiquitinated SopB in *Traf6*$^{+/+}$ and *Traf6*$^{-/-}$ MEFs during *S. typhimurium* infection. "WCE" refers to whole-cell extract. (B) Ectopically expressed wild-type TRAF6 but not its C70A mutant rescues the ubiquitination of SopB in *Traf6*$^{-/-}$ MEFs. pcDNA4-FLAG-TRAF6 or its C70A mutant, was transfected into *Traf6*$^{-/-}$ MEFs for 24 h, and the *Traf6*$^{-/-}$ MEFs were then infected with *S. typhimurium* for 60 min and harvested for the detection of SopB ubiquitination. (C) UbcH5c but not Ubc13/Uev1a mediates the role of TRAF6 in SopB ubiquitination upon infection. The Henle-407 cells were incubated with the control shRNA, shUbcH5c, or shUbc13 lentivirus particles for 24 h. The cells were then infected with *S. typhimurium* for 60 min and harvested for the detection of SopB ubiquitination. The RNAi efficiency of shUbcH5c and shUbc13 were determined by anti-UbcH5c and anti-Ubc13 immunoblots, respectively.

of SopB ubiquitination during infection further confirmed the mode of TRAF6 catalyzation. To the aspect of UbcH5c, it has also been usually found an active E2 coordinated with the E3 ubiquitin ligase secreted by bacteria, such as the SopA from *Salmonella* [26] and the family of IpaH9.8, including IpaH3, IpaH4.5, and IpaH7.8, from *Shigella* [27]. The frequent cooperation of UbcH5c with the effectors secreted by bacteria may imply an unknown mechanism of UbcH5c upon bacterial invasion.

In conclusion, our results suggest the relevance of UbcH5c/TRAF6 for SopB during *S. typhimurium* infection by implying that *S. typhimurium* has evolved a mechanism of utilizing the host's E3 ubiquitin ligase to modify and modulate the function of its effector proteins in order to secure pathogen and host cell survival.

Acknowledgments

This work was supported by the National Natural Science Foundation of China (Grant No. 81101220 to HHR and 81172837 to HZ) and Tianjin Municipal Science and Technology Commission (Grant 12JCQNJC08100 to HHR). We are grateful to Dr. Jun-ichiro Inoue and Dr. Jin Gohda from the University of Tokyo for the gift of *Traf6*$^{+/+}$ and *Traf6*$^{-/-}$ MEFs. We thank Dr. Hongtao Li from Xinan University for gifting the series plasmids that were used in this study.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.03.126.

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