**Mechanistic insights into the subversion of the linear ubiquitin chain assembly complex by the E3 ligase IpaH1.4 of Shigella flexneri**

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*Published March 16, 2022.*

**Shigella flexneri**, a gram-negative bacterium, is the major culprit of bacterial shigellosis and causes a large number of human infection cases and deaths worldwide annually. For evading the host immune response during infection, *S. flexneri* secretes two highly similar E3 ligases, IpaH1.4 and IpaH2.5, to subvert the linear ubiquitin chain assembly complex (LUBAC) of host cells, which is composed of HOIP, HOIL-1L, and SHARPIN. However, the detailed molecular mechanism underpinning the subversion of the LUBAC by IpaH1.4/2.5 remains elusive. Here, we demonstrated that IpaH1.4 can specifically recognize HOIP and HOIL-1L through its leucine-rich repeat (LRR) domain by binding to the HOIP RING1 domain and HOIL-1L ubiquitin-like (UBL) domain, respectively. The determined crystal structures of IpaH1.4 LRR/HOIP RING1, IpaH1.4 LRR/HOIL-1L UBL, and HOIP RING1/UBE2L3 complexes not only elucidate the binding mechanisms of IpaH1.4 with HOIP and HOIL-1L but also unveil that the recognition of HOIP by IpaH1.4 can inhibit the E2 binding of HOIP. Furthermore, we demonstrated that the interaction of IpaH1.4 LRR with HOIP RING1 or HOIL-1L UBL is essential for the ubiquitination of HOIP or HOIL-1L in vitro as well as the suppression of NF-kB activation by IpaH1.4 in cells. In summary, our work elucidated that in addition to inducing the proteosomal degradation of LUBAC, IpaH1.4 can also inhibit the E3 activity of LUBAC by blocking its E2 loading and/or disturbing its stability, thereby providing a paradigm showing how a bacterial E3 ligase adopts multiple tactics to subvert the key LUBAC of host cells.

LUBAC | HOIP | HOIL-1L | IpaH1.4 | ubiquitination

Ubiquitination plays pivotal roles in almost every cellular process in mammals, such as protein degradation, gene transcription, autophagy, and immune signaling (1–5). The ubiquitination event involves the covalent conjugation of the target substrate with one or multiple ubiquitin (Ub) catalyzed by a cascade of three enzymes: an E1 Ub-activating enzyme, an E2 Ub-conjugating enzyme, and an E3 Ub ligase (3, 6). In addition to decorating other substrates, Ub itself can be also ubiquitinated to form eight distinct types of poly-Ub chains, which are linked through the extreme N-terminal methionine residue (M1) or any one of the seven lysine residues (K6, K11, K27, K29, K33, K48, and K63) within Ub (4, 7). Different linkage types of poly-Ub chains encode distinct signals and conduct specific cellular functions in mammalian cells (7–9). For instance, the well-known K48-linked poly-Ub chain can serve as a signal for proteosomal degradation, while the M1-linked poly-Ub chain (also named linear poly-Ub chain), in which the backbone carboxyl group of the extreme C-terminal G76 of one Ub is conjugated to the backbone amino group of the M1 residue in the preceding Ub thereby linking multiple Ub molecules together in a “head-to-tail” manner, is widely involved in immune-related signaling pathways (10–14).

The linear Ub chain assembly complex (LUBAC), which consists of a catalytic subunit HOIP and two regulatory subunits HOIL-1L and SHARPIN, is the only currently identified E3 ligase complex capable of catalyzing linear poly-Ub chains on protein substrates (10, 12, 15–19). HOIP is a multidomain-containing RING-between-RING (RBR)–type E3 ligase. It contains an N-terminal region: N-glycanase/Ub-associated domain (UBA) or UBX-containing protein domain for interacting with two crucial deubiquitinases, OTULIN and CYLD, both of which can trim M1-linked Ub chains (20–22), and a B-box–type zinc finger (ZF) followed by a canonical ZF (Fig. 1A). In addition, the middle region of HOIP contains two Nlp4-like ZF (NZF) domains, an atypical UBA, and a following hinge-like domain (Fig. 1A). The C-terminal part of HOIP has a unique linear Ub chain-determining domain (LDD) and a preceding characteristic RBR region, which is composed of a RING1 domain, an in-between-RING...
motif (IBR), and a RING2 domain (Fig. 1A). Notably, the RBR region together with the LDD domain forms the catalytic core of HOIP for assembling linear Ub chains (23–25), while the HOIP/IpaH1.4, HOIL-1L/IpaH1.4, and HOIP/UBE2L3 interactions characterized in this study are further highlighted by red two-way arrows. (B and C) Analytical gel filtration chromatography analysis of the interaction between HOIP RING1 domain and full-length IpaH1.4 (B) or IpaH1.4 LRR domain (C). (D) The summarized mapping results of the interacting regions between IpaH1.4 and HOIP by analytical gel filtration chromatography; aa, amino acids. (E) Superposition plots of the 1H–15N HSQC spectra of 15N-labeled HOIP RING1 domain titrated with increasing molar ratios of unlabeled IpaH1.4 LRR domain. (F) ITC-based measurement of the binding affinity of full-length IpaH1.4 with the HOIP RING1 domain. The $K_D$ error is the fitted error obtained from the data analysis software when using the one-site binding model to fit the ITC data; DP, differential power measured by the ITC machine; $\Delta H$, heat change measured by the ITC machine. (G) Overlay plot of the sedimentation velocity data of the HOIP RING1 domain (red), the IpaH1.4 LRR domain (blue), and the HOIP RING1/IpaH1.4 LRR complex (black); cs, continuous sedimentation coefficient distribution; MW, molecular weight.

Fig. 1. Biochemical characterization of the interaction between HOIP and IpaH1.4. (A) A schematic diagram showing the domain organizations of HOIP, HOIL-1L, SHARPIN, IpaH1.4/2.5, and UBE2L3. In this drawing, the intermolecular interactions of HOIP, HOIL-1L, and SHARPIN are indicated by black two-way arrows, while the HOIP/IpaH1.4, HOIL-1L/IpaH1.4, and HOIP/UBE2L3 interactions characterized in this study are further highlighted by red two-way arrows. (B and C) Analytical gel filtration chromatography analysis of the interaction between HOIP RING1 domain and full-length IpaH1.4 (B) or IpaH1.4 LRR domain (C). (D) The summarized mapping results of the interacting regions between IpaH1.4 and HOIP by analytical gel filtration chromatography; aa, amino acids. (E) Superposition plots of the 1H–15N HSQC spectra of 15N-labeled HOIP RING1 domain titrated with increasing molar ratios of unlabeled IpaH1.4 LRR domain. (F) ITC-based measurement of the binding affinity of full-length IpaH1.4 with the HOIP RING1 domain. The $K_D$ error is the fitted error obtained from the data analysis software when using the one-site binding model to fit the ITC data; DP, differential power measured by the ITC machine; $\Delta H$, heat change measured by the ITC machine. (G) Overlay plot of the sedimentation velocity data of the HOIP RING1 domain (red), the IpaH1.4 LRR domain (blue), and the HOIP RING1/IpaH1.4 LRR complex (black); cs, continuous sedimentation coefficient distribution; MW, molecular weight.

motif (IBR), and a RING2 domain (Fig. 1A). Notably, the RBR region together with the LDD domain forms the catalytic core of HOIP for assembling linear Ub chains (23–25), while the RING1 domain is responsible for recruiting Ub-conjugated E2 enzymes, such as UBE2L3 (10, 25, 26). Intriguingly, HOIL-1L is also an RBR-type E3 ligase (Fig. 1A). However, the catalytic activity of HOIL-1L is not directly involved in the linear Ub chain assembly (10). Instead, HOIL-1L can conjugate monoubiquitin onto all LUBAC subunits to attenuate the function of LUBAC by providing preferred monoubiquitinated substrates for HOIP-mediated auto linear ubiquitination of LUBAC (27). In addition, HOIL-1L has an N-terminal LUBAC-tethering motif (LTM), a Ub-like (UBL) domain, and a middle NZF domain that can selectively recognize linear Ub chains (28) (Fig. 1A). As a unique adaptor protein in LUBAC, SHARPIN has an N-terminal pleckstrin homology domain that can mediate the dimerization of SHARPIN (29), an LTM motif, and a UBL domain followed by a Ub-binding NZF domain (16) (Fig. 1A). Previously, studies from our group and other groups revealed that the UBL domains of HOIL-1L and SHARPIN can simultaneously interact with the HOIP UBA domain to assemble the ternary LUBAC (15, 30, 31), which is further stabilized by a cooperative interaction between the two LTM motifs of HOIL-1L and SHARPIN (31). Notably, these mutual interactions among the three subunits of LUBAC are demonstrated to be essential for the stability of the trimeric
LUBAC in cells (15–17, 31, 32). Importantly, previous functional studies established that LUBAC plays crucial roles in NF-kB signaling and antibacterial selective autophagy (xenophagy) (12, 32–35). Not surprisingly, as a key player in immune signaling pathways, LUBAC is targeted by many pathogenic bacteria or viruses to subvert host responses during infections (18), such as S. flexneri (36, 37), Salmonella typhi murium (38), hepatitis B virus (39), hepatitis C virus (40), porcine reproductive and respiratory syndrome virus (41), and Epstein–Barr virus (42). However, until now, many of the detailed molecular mechanisms underlying the subversion of LUBAC by these pathogens are still largely unknown.

S. flexneri is a type of gram-negative enteric pathogen that is capable of invading the intestinal epithelium and replicating rapidly in the cytosol of host cells. It is the leading cause of shigellosis, an acute bloody diarrhea in humans (43). To suppress host defense during infection, S. flexneri secretes dozens of virulent factors into the cytoplasm of infected host cells (44). Several effector proteins have been shown to suppress the innate immune signaling or gene transcription of host cells (36, 45). Among these effector proteins, two highly similar E3 Ub ligases IpaH1.4 and IpaH2.5 were recently uncovered to suppress NF-kB activation by targeting LUBAC (37). Both IpaH1.4 and IpaH2.5 contain an N-terminal substrate-binding leucine-rich repeat (LRR) domain and a C-terminal catalytic E3 ligase (NEL) domain (Fig. 1A). They belong to the bacterial NEL E3 ligase family that can catalyze the K48-linked ubiquitination of HOIP for proteasomal degradation (38). Given that IpaH1.4 and IpaH2.5 share highly similar amino acid sequences, especially in the target-binding LRR domain (~98.83% sequence identity) and the catalytic NEL domain region (SI Appendix, Fig. S1A), we chose IpaH1.4 for further biochemical and structural characterization. Using analytical gel filtration chromatography–based coimmunoprecipitation assays with purified relevant HOIP and IpaH1.4 fragment proteins, we revealed that the full-length IpaH1.4 can specifically recognize the RING1 domain rather than the previously reported hinge region between the UBA and RING1 domains of HOIP (Fig. 1B and D and SI Appendix, Fig. S2). Further reciprocal in vitro binding assays revealed that the LRR domain of IpaH1.4 alone is sufficient for the recognition of HOIP RING1 (Fig. 1C and D and SI Appendix, Fig. S3). We further utilized NMR spectroscopy to characterize the interaction between HOIP RING1 and IpaH1.4 LRR. Titrations of 15N-labeled HOIP RING1 with unlabeled IpaH1.4 LRR proteins showed that many peaks in the 1H,15N heteronuclear single quantum coherence (HSQC) spectra of HOIP RING1 undergo significant peak broadenings or chemical shift changes (Fig. 1E), indicating that HOIP RING1 can directly bind to the LRR domain of IpaH1.4, and the interaction between these two proteins is in an equilibrium with an intermediate exchange rate. Further quantitative isothermal titration calorimetry (ITC) analysis uncovered that IpaH1.4 binds to HOIP RING1 with a binding stoichiometry of 1:1 (N value is about 1.0) and a dissociation constant (Kd) value of ~1.0 μM (Fig. 1F). Finally, using analytical ultracentrifugation-based assays, we elucidated that HOIP RING1 and IpaH1.4 LRR both are monomers in solution and can associate with each other to form a stable 1:1 stoichiometric complex (Fig. 1G), which is in line with our ITC data. Taken together, these biochemical results clearly demonstrate that the recognition of HOIP RING1 by IpaH1.4 LRR is responsible for the specific interaction between HOIP and IpaH1.4.

The Crystal Structure of the IpaH1.4 LRR/HOIP RING1 Complex. To further gain mechanistic insights into the recognition of HOIP RING1 by IpaH1.4, we solved the atomic structures of the apo form IpaH1.4 LRR and IpaH1.4 LRR in complex with HOIP RING1 using X-ray crystallographic methods (SI Appendix, Table S1). The apo form structure of IpaH1.4 LRR reveals that IpaH1.4 LRR has a canonical horseshoe shape, which is composed of nine units of LRR capped by two N-terminal α-helices (α1 and α2) and a C-terminal α4-helix together with a short β10-strand that directly augments the β-sheet of LRR in a parallel manner (Fig. 2A). Further structural analyses revealed that IpaH1.4 LRR contains a positively charged groove located at its potential substrate-binding concave side (Fig. 2B). The overall architecture of IpaH1.4 LRR is highly similar to that of the LRR domain of IpaH3 from S. flexneri (SI Appendix, Fig. S4A). As expected, in the IpaH1.4 LRR/HOIP RING1 complex, IpaH1.4 LRR binds to HOIP RING1 through its concave side (Fig. 2C). No significant structural changes in the IpaH1.4 LRR domain can be observed upon its binding to HOIP (SI Appendix, Fig. S4B). Concurrently, HOIP RING1 is composed of two short N-terminal antiparallel β-strands together with three α-helices and forms a unique architecture coordinating two Zn2+ ions (Fig. 2C). In the complex structure, HOIP RING1 packs extensively with the solvent-exposed and highly positively charged groove of IpaH1.4 LRR mainly through its β-sheet and two Zn2+-binding regions, burying a total surface area of ~614 Å2 (Fig. 2C and D). Detailed structural analysis of the binding interface in the IpaH1.4 LRR/HOIP RING1 complex revealed that the specific interaction between IpaH1.4 LRR and HOIP RING1 is mainly mediated by polar interactions (Fig. 2E). Particularly,
the positively charged side chains of R99 and K100 in IpaH1.4 LRR form two salt bridges with the negatively charged side chain of D724 in HOIP RING1 (Fig. 2E). In parallel, the side chains of E97 and D140 in IpaH1.4 LRR form two hydrogen bonds with the side chains of W704 and Q728 in HOIP RING1 (Fig. 2E). Meanwhile, the side chains of Q119, R157, and N240 in IpaH1.4 LRR directly couple with the backbone carbonyl groups of C699, C702, G703, and C747 in HOIP RING1 to form five specific hydrogen bonds (Fig. 2E). In addition, the IpaH1.4 LRR/HOIP RING1 complex is further stabilized by a cation–π interaction between the positively charged guanidyl moiety of IpaH1.4 R99 and the aromatic side chain of HOIP W704 (Fig. 2E). Notably, the key interface residues in IpaH1.4 for interacting with HOIP RING1 also can be found in IpaH3, IpaH7, and IpaH9.8 (SI Appendix, Fig. S1B), suggesting that HOIP is likely a specific target for IpaH1.4/2.5. Importantly, in accordance with their important structural roles, all the key interface residues of HOIP RING1 involved in the interaction with IpaH1.4 LRR are highly conserved across different eukaryotic species (SI Appendix, Fig. S5). Using site-directed mutagenesis together with comigration and ITC-based assays (SI Appendix, Figs. S6–S8), we further verified the interaction between IpaH1.4 LRR and HOIP RING1 observed in the complex structure. In agreement with our structural data, point mutations of key interface residues, such as the W704A, D724A, and Q728A mutations in HOIP RING1 or the R99A, K100A, Q119A, R157A, and N240A mutations in IpaH1.4 LRR, all largely attenuate or essentially disrupt the specific interaction between HOIP RING1 and IpaH1.4 LRR (Fig. 2F and SI Appendix, Figs. S6–S8).

**The Structure of the UBE2L3/HOIP RING1 Complex.** As an RBR-type E3 protein, HOIP relies on its RING1 domain to specifically recognize E2 proteins for catalyzing the linear Ub chain assembly. Previous studies have systematically characterized the efficiency of different E2 proteins in HOIP-catalyzed linear ubiquitination (10, 26), and it is well demonstrated that UBE2L3 is the cognate E2 species for HOIP with the highest catalytic efficiency (26). Indeed, our analytical gel filtration chromatography-based comigration results confirmed that UBE2L3 can effectively bind to the HOIP RING1 domain (Fig. 3A). Given that both UBE2L3 and IpaH1.4 can directly bind to HOIP RING1, it is intriguing to know what the relationship is between IpaH1.4 and UBE2L3 in binding to HOIP. Therefore, we solved the crystal structure of the UBE2L3/HOIP RING1 complex (SI Appendix, Table S2). In the complex structure, UBE2L3 features an architecture assembled by a four-stranded antiparallel β-sheet packing with four α-helices (Fig. 3B) and specifically binds to HOIP RING1 with an overall binding mode like that of the UBE2D2/HOIP RING1 complex structure.
IpaH1.4 LRR Can Inhibit the E3 Activity of HOIP through Blocking its E2 Binding. Structural comparison analysis showed that some key interfacial residues of HOIP RING1, such as W704 and D724, are both involved in the interactions with IpaH1.4 LRR and UBE2L3 (Figs. 2E and 3C and SI Appendix, Fig. S5), and the UBE2L3-binding and IpaH1.4-binding sites on HOIP RING1 are heavily overlapped (Fig. 3D), suggesting a direct competition between E2 UBE2L3 and IpaH1.4 in binding to HOIP. We sought to measure the binding affinity between UBE2L3 and HOIP RING1 using the ITC method. However, the enthalpy change of the ITC titration assay of UBE2L3 with HOIP RING1 is too small to calculate a reliable $K_D$ value for the UBE2L3/HOIP RING1 interaction (SI Appendix, Fig. S10 A and B). Fortunately, as an alternative, we successfully measured the binding affinities of the HOIP UBA-RBR-LDD/HOIL-1L UBL complex with UBE2L3 and IpaH1.4 LRR (SI Appendix, Fig. S10 C and D). The ITC results revealed that IpaH1.4 LRR has a higher affinity ($K_D = 2.3 \pm 0.4 \mu M$) than UBE2L3 ($K_D = 5.8 \pm 0.6 \mu M$) for binding to HOIP RING1 (SI Appendix, Fig. S10 C and D). Consistent with our structural and ITC data, the analytical gel filtration chromatography coupled with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) assays showed that IpaH1.4 can readily compete with UBE2L3 for interacting with the wild-type IpaH1.4 LRR, while the R157A mutant of IpaH1.4 LRR, which cannot interact with HOIP RING1, loses the ability to inhibit the activity of HOIP.

Fig. 3. IpaH1.4 can inhibit the E3 catalytic activity of HOIP through blocking its E2 binding. (A) Analytical gel filtration chromatography analysis of the interaction between the HOIP RING1 domain and UBE2L3. (B) Ribbon diagram showing the overall structure of the HOIP RING1/UBE2L3 complex. (C) The ribbon–stick–ball representation showing the detailed binding interface in the HOIP RING1/UBE2L3 complex structure. In this drawing, the side chains as well as relevant backbone groups of the key binding interface residues are shown in the stick-ball mode, and the hydrogen bonds involved in HOIP RING1/UBE2L3 binding are shown as dotted lines. (D) Ribbon representations showing the structural comparison of the HOIP RING1/IpaH1.4 LRR complex (slate/green) with the HOIP RING1/UBE2L3 complex (blue/magenta). In this drawing, these two structures are overlaid by aligning the HOIP RING1 domain in these two complex structures. (E) Analytical gel filtration chromatography analyses of the purified HOIP RING1/UBE2L3 complex titrated with increasing molar ratios of full-length IpaH1.4 proteins. (F) SDS-PAGE combined with Coomassie blue staining analyses showing the protein components of the corresponding fractions collected from the analytical gel filtration chromatography experiments of the HOIP RING1/UBE2L3 complex titrated with different molar ratios of full-length IpaH1.4 LRR. (G) In vitro linear Ub chain assembly assays showing that the catalytic activity of HOIP (RBR-LDD region) can be efficiently blocked by the wild-type IpaH1.4 LRR, while the R157A mutant of IpaH1.4 LRR, which cannot interact with HOIP RING1, loses the ability to inhibit the activity of HOIP.
with HOIP RING1 (Fig. 3E and F). Since the recognition of E2 by HOIP RING1 is essential for the E3 activity of HOIP, we wondered whether IpaH1.4 LRR alone could inhibit the catalytic activity of HOIP for assembling linear Ub chains by binding to the HOIP RING1 domain. As expected, in our reconstituted in vitro linear Ub chain assembly assay, the isolated IpaH1.4 LRR domain can efficiently block the enzymatic activity of the HOIP RBR-LDD catalytic fragment to assemble linear Ub chains (Fig. 3G). In contrast, the R157A mutant of IpaH1.4 LRR, which loses the ability to interact with HOIP RING1 (Fig. 2F), has negligible inhibitory effects on the assembly of linear Ub chains mediated by HOIP RBR-LDD (Fig. 3G). Collectively, all these biochemical observations together with our structural analyses clearly demonstrated that IpaH1.4 can efficiently suppress the E3 activity of HOIP by disturbing its E2 binding in vitro.

Biochemical Characterization of the Interaction between IpaH1.4 and HOI-1L. A previous study also showed that IpaH1.4 can directly target the HOI-1L subunit of LUBAC (37). Using copurification assays with recombinant proteins from Escherichia coli, we first confirmed that IpaH1.4 can directly but weakly bind to HOI-1L (Fig. 4A and SI Appendix, Fig. S11A). Subsequently, we carefully mapped the binding regions between HOI-1L and IpaH1.4 and uncovered that the IpaH1.4/HOI-1L interaction is mainly mediated by the specific binding between IpaH1.4 LRR and HOI-1L UBL (Fig. 4A and SI Appendix, Fig. S11). Further NMR titration analyses using 15N-labeled HOI-1L UBL titrated with unlabeled IpaH1.4 LRR confirmed that HOI-1L UBL can directly interact with IpaH1.4 LRR (Fig. 4B). Intriguingly, an ITC-based quantitative analysis revealed that the interaction between IpaH1.4 LRR and HOI-1L UBL has a $K_D$ value of $\sim10.7 \mu M$ (Fig. 4C), which is much weaker than that of the IpaH1.4 LRR and HOIP RING1 interaction (Fig. 1F).

Structure of the IpaH1.4 LRR/HOIL-1L UBL Complex. To reveal the mechanistic basis for the recognition of HOI-1L by IpaH1.4, we determined the crystal structure of the HOI-1L UBL/IpaH1.4 LRR complex (SI Appendix, Table S2). In the final complex structure model, an asymmetric unit contains two HOI-1L UBL and two IpaH1.4 LRR molecules (SI Appendix, Fig. S12A), and two distinct contacting sites (site 1 and site 2) between IpaH1.4 LRR and HOI-1L UBL can be

![Image](https://doi.org/10.1073/pnas.2116776119)
identified (SI Appendix, Fig. S12B). Particularly, the second interface (site 2) is mainly maintained by polar interactions, including salt bridges and hydrogen bonds (SI Appendix, Fig. S12C). However, point mutations of key interface residues of IpaH1.4 involved in site 2 do not affect the specific interaction between IpaH1.4 and HOIL-1L in solution (SI Appendix, Fig. S12 D–H). Therefore, the site 2 interface is merely induced by crystal packing, and only the site 1 interface is a real binding interface between IpaH1.4 and HOIL-1L in solution.

Further structural analyses of the interaction between IpaH1.4 LRR and HOIL-1L UBL in site 1 showed that HOIL-1L UBL adopts a Ub-like fold and specifically binds to the concave side of IpaH1.4 LRR via its α1 and β2 regions (Fig. 4D). Unsurprisingly, the binding of HOIL-1L UBL to IpaH1.4 LRR does not induce large conformational changes in IpaH1.4 LRR (SI Appendix, Fig. S13A), and the overall structure of HOIL-1L UBL in the IpaH1.4 LRR/HOIL-1L UBL complex is highly similar to that of HOIL-1L UBL in complex with HOIP UBA (SI Appendix, Fig. S13B). Detailed structural analyses of the binding interface between IpaH1.4 LRR and HOIL-1L UBL revealed that the side chains of V69, Y92, F94, and L130 from HOIL-1L UBL make hydrophobic contacts with the hydrophobic side chains of the F238 and F269 residues of IpaH1.4 LRR (Fig. 4E and F). Concurrently, the hydrophobic side chain of HOIL-1L L90 packs against a hydrophobic pitch formed by the side chains of V177 and A197 residues of IpaH1.4 LRR (Fig. 4E and F). Furthermore, the imidazole ring of His77 and the backbone carboxyl group of the Y92 residue of HOIL-1L respectively interact with the I268 and N240 residues of IpaH1.4 LRR to form two hydrogen bonds, and the negatively charged HOIL-1L D91 forms specific charge–charge and hydrogen-bonding interactions with the positively charged R215 residue of IpaH1.4 LRR (Fig. 4F).

In line with their critical structural roles, all these key binding interface residues of HOIL-1L are highly conserved during evolution (SI Appendix, Fig. S14). Notably, the key interfacial residues of IpaH1.4 LRR for interacting with HOIL-1L UBL can also be found in IpaH2.5 but are missing in other S. flexneri IpaH family members (SI Appendix, Fig. S1B), implying that HOIL-1L is also a likely specific target of IpaH1.4/2.5. Using comigration and ITC analyses, we further validated the specific interactions between IpaH1.4 LRR and HOIL-1L UBL observed in the complex structure. Consistent with our structural results, mutations of key interfacial residues from IpaH1.4, including the R215E, F238E/N240D, F269E, and R215E/F238E/N240D/F269E (hereafter referred to as 4M) mutations of IpaH1.4 LRR, all essentially abolish the specific interaction between IpaH1.4 and HOIL-1L in solution (SI Appendix, Figs. S15 and S16 and Fig. 4G).

### Relationship between IpaH1.4, HOIP, and HOIL-1L in Binding to Each Other

**Based on our study, both HOIL-1L UBL and HOIP RING1 can directly interact with IpaH1.4 LRR (Figs. 2E and 4B). However, further structural comparison analysis showed that HOIL-1L UBL and HOIP RING1 are unable to simultaneously bind IpaH1.4 LRR due to potential steric exclusion (Fig. 4H). Considering that the binding affinity between IpaH1.4 LRR and HOIP RING1 is roughly tenfold stronger than that of the IpaH1.4 LRR/HOIL-1L UBL interaction (Figs. 1F and 4C), IpaH1.4 should preferentially bind to HOIP rather than HOIL-1L when targeting LUBAC. In addition to interacting with IpaH1.4 LRR, HOIL-1L UBL can also directly bind to HOIP UBA through its α1 and β2 regions (SI Appendix, Fig. S17A), and the IpaH1.4-binding and HOIP-binding sites on HOIL-1L UBL are highly overlapped (Fig. 4I and SI Appendix, Fig. S14). Therefore, IpaH1.4 and HOIP should be competitive in binding to HOIL-1L. Interestingly, ITC-based analyses revealed that IpaH1.4 LRR has a weaker binding affinity (K_D = 10.7 ± 0.1 μM) than HOIP UBA (K_D = 3.9 ± 0.4 μM) for interacting with HOIL-1L UBL (Fig. 4C and G and SI Appendix, Fig. S17C). Consistent with our structural and ITC results, further analytical gel filtration chromatography coupled with SDS-PAGE assays showed that IpaH1.4 is unable to form a ternary complex with the purified HOIP UBA/HOIL-1L UBL complex and is ineffective in destabilizing the HOIP UBA/HOIL-1L UBL interaction (SI Appendix, Fig. S17 D and E).

### In Vitro Ubiquitination of HOIP and HOIL-1L by IpaH1.4 Relies on the Specific Interactions of IpaH1.4 LRR with HOIP RING1 and HOIL-1L UBL

Our biochemical and structural data clearly demonstrated that IpaH1.4 can directly recognize HOIP and HOIL-1L through its LRR domain (Figs. 2 and 4). It is intriguing to know whether HOIP and HOIL-1L in the context of LUBAC can be recognized and ubiquitinated by IpaH1.4. Using a reconstituted in vitro ubiquitination assay with purified LUBAC core fragments together with relevant E1, E2, and Ub proteins, we revealed that IpaH1.4 can readily and preferentially catalyze the poly-ubiquitination of HOIP and very weakly ubiquitinates HOIL-1L (Fig. 5A and SI Appendix, Fig. S18A). In contrast, the SHARPIN component in the mini-LUBAC complex cannot be ubiquitinated by IpaH1.4 (Fig. 5A and SI Appendix, Fig. S18A), suggesting that IpaH1.4 only ubiquitinitates directly bound substrate but cannot modify indirectly associated proteins, which might be out of the accessibility of the IpaH1.4 catalytic site. Importantly, the R157A mutation of IpaH1.4 that specifically disrupts the IpaH1.4/HOIP RING1 interaction but does not affect the IpaH1.4/HOIL-1L UBL binding (Fig. 2F and SI Appendix, Fig. S19A) essentially abolishes the poly-ubiquitination of HOIP mediated by IpaH1.4 (Fig. 5B). Interestingly, likely due to the elimination of the competitive binding from HOIP RING1, HOIL-1L can be more easily modified by the IpaH1.4 R157A mutant than the wild-type IpaH1.4 (Fig. 5A and B). Furthermore, consistent with our aforementioned biochemical and structural results (Fig. 4), IpaH1.4 mutations that specifically disrupt the interaction between IpaH1.4 LRR and HOIL-1L UBL, such as the R215E and F238E/N240D mutations of IpaH1.4 (Fig. 4G), completely eliminate the ubiquitination of HOIL-1L conducted by IpaH1.4 (Fig. 5C).

### Recognition of HOIP and HOIL-1L by IpaH1.4 Is Required for the IpaH1.4-Mediated Suppression of NF-κB Activation in Cells

Previous studies demonstrated that the overexpression of LUBAC in HEK293T cells can induce a strong activation of the NF-κB pathway that depends on the linear Ub assembly activity of LUBAC (15–17, 35), and IpaH1.4 can directly target LUBAC for proteasomal degradation, especially the catalytic HOIP subunit (37). To further evaluate the recognition of HOIP and HOIL-1L by IpaH1.4 on the NF-κB activation in cells, we established a reliable NF-κB reporter dual-luciferase assay to measure the activities of LUBAC in the presence of wild-type IpaH1.4 or different IpaH1.4 variants. As expected, overexpression of LUBAC alone in HEK293T cells can promote the NF-κB reporter luciferase level over 200-fold compared with the control, while the coexpression of LUBAC with the wild-type IpaH1.4 can effectively abolish the activation effect induced by LUBAC (Fig. 5D). Interestingly, the catalytic-dead C368A mutant of IpaH1.4...
does not completely lose the inhibitory activity toward LUBAC but still retains about half of the inhibitory activity of the wild-type IpaH1.4 (Fig. 5D), suggesting that even without its E3 ubiquitination activity, the sole interaction of IpaH1.4 with LUBAC should contribute to the IpaH1.4-mediated suppression of NF-κB activation induced by LUBAC. Meanwhile, the IpaH1.4 R157A mutant and 4M mutant, which lose the HOIP-binding ability and the HOIL-1L-binding ability, respectively (Figs. 2F and 4G and SI Appendix, Fig. S19 A and B), both retain similar inhibitory activity as the IpaH1.4 C368A mutant (Fig. 5D), indicating that the recognition of HOIP and HOIL-1L by IpaH1.4 is required for the effective IpaH1.4-mediated inhibition of NF-κB activation induced by LUBAC. In line with our aforementioned biochemical results (Fig. 3 E–G), the IpaH1.4 4M/C368A mutant, which loses ubiquitination activity as well as HOIL-1L binding ability but retains the ability to interact with the HOIP RING1 for interfering with the E2 binding of HOIP, still has weak inhibitory effects on NF-κB activation induced by LUBAC (Fig. 5D). These observations demonstrated that the abilities for
interacting with HOIP and HOIL-1L as well as the E3 ubiquitin-
tation activity of IpaH1.4 are required for the IpaH1.4-mediated
suppression of NF-κB activation induced by overexpressed
LUBAC in cells.

To further validate our structural conclusions under more
physiological conditions, we generated an *ipaH1.4/ipaH2.5*
double-knockout (Δ*ipaH1.4ΔipaH2.5) S. flexneri strain and reconstituted it with a wild-type *ipaH1.4* gene or different
*ipaH1.4* mutant genes (R175A, C368A, R175A/C368A, 4M, and
4M/C368A) in the original *ipaH1.4* locus by the lipopase-
mediated recombination method. Notably, all the wild-type
*ipaH1.4* and different *ipaH1.4* mutant genes that were used to
reconstitute the *ipaH1.4/ipaH2.5* double-knockout *S. flexneri*
strain are C-terminally fused with a His tag sequence gene, and
the proper protein expression levels of these *ipaH1.4* variants in
mutant strains were further confirmed by Western blotting using
a specific anti-His antibody (*SI Appendix, Fig. S20*). Then, we
used these engineered *S. flexneri* strains and the wild-type *S.
flexneri* M90T strain to infect cultured HeLa cells. Subsequently,
the nuclear localization of p65 in infected HeLa cells was quanti-

dified by immunofluorescence. The results showed that the wild-type
*S. flexneri* M90T strain can effectively block p65 nuclear translocation
in ~70% of cells with bacterial infection, while the *ipaH1.4/
ipaH2.5* double-knockout *S. flexneri* (Δ*ipaH1.4ΔipaH2.5) strain
has a poor ability to block the nuclear translocation of p65 (Fig. 5
E and F). In line with a previous study (37), the reconstitunent
of the double-knockout strain with the wild-type *ipaH1.4*
confers the bacteria with a comparable ability as that of the wild-
type strain to inhibit p65 nuclear translocation in infected cells
(Fig. 5 E and F). In contrast, the *S. flexneri* strain reconstituted with the
*ipaH1.4* R157A mutant or the *ipaH1.4* 4M mutant is unable to inhibit p65 nuclear translocation (Fig. 5 E and F), sug-
gesting that the binding abilities of IpaH1.4 to HOIP and HOIL-
1L are critical for *S. flexneri* to suppress the nuclear translocation of p65. Interestingly, the strain reconstituted with the
*ipaH1.4* C368A (catalytic-dead) mutant can still inhibit p65 nuclear translocation (Fig. 5 E and F). As expected, the *S.
flexneri* strain reconstituted with the *ipaH1.4* R157A/C368A or 4M/C368A mutant has little inhibition on the nuclear translocation of p65, similar to that of the double-knockout strain (Δ*ipaH1.4ΔipaH2.5*) (Fig. 5 E and F). In all, these data clearly indicated that *S.
flexneri* strains with mutants of IpaH1.4 defective in binding to
HOIP RING1 or HOIL-1L UBL are attenuated in suppression of
host immunity.

Discussion

The secretion of special E3 Ub ligases to target key proteins of
infected host cells for proteasomal degradation is an efficient and
smart strategy adopted by some deleterious bacteria to subvert
the host antibacterial immune response for facilitating their sur-
vival and replication in host cells. For successful proliferation in
infected human cells, *S. flexneri* uses two highly similar E3 effec-
tor proteins, IpaH1.4 and IpaH2.5, to subvert LUBAC through
proteasomal degradation (37). In this study, we uncovered the
molecular mechanism as well as the related downstream con-
sequences for the recognition between IpaH1.4 and LUBAC
through systematic biochemical and structural characterization.
Particularly, we have elucidated that IpaH1.4 directly binds to
the RING1 domain of HOIP and the UBL domain of HOIL-1L
by different but partially overlapped surfaces of its N-terminal
LRR domain (Figs. 2 and 4). Importantly, the interaction
between IpaH1.4 and HOIP or HOIL-1L is indispensable for
the poly-ubiquitination of HOIP or HOIL-1L by IpaH1.4
in vitro (Fig. 5). Interestingly, our structural and biochemical
assays also revealed that the binding of IpaH1.4 to HOIP masks
the E2-interacting surface in the RING1 domain of HOIP (Fig.
3), thus inhibiting the linear Ub chain assembly activity of
LUBAC by blocking the E2 binding to HOIP in addition to
inducing Ub–proteasome-dependent HOIP degradation. Nota-

tively, blocking the RING domain of a targeting E3 ligase is a com-
mon strategy adopted by many other E3 ligase inhibitors, such as
glomulin (48), Em11 (49), and Salmonella effector SopA (50).

Intriguingly, the determined IpaH1.4/HOIL-1L complex struc-
ture uncovered that IpaH1.4 binds to a hydrophobic surface in
the HOIL-1L UBL domain (Fig. 4 F). This also mediates
the HOIL-1L/HOIP interaction (*SI Appendix, Fig. S17 A and
B*). Therefore, in addition to mediating the poly-ubiquitination of
HOIL-1L, IpaH1.4 might compete with HOIP for binding to
HOIL-1L, thereby destabilizing the HOIL-1L/HOIP interaction
in the LUBAC that is crucial for the stability of HOIP in cells
(15, 16, 32). However, based on our competition binding assay
and quantitative ITC results (*SI Appendix, Fig. S17 C–E and Fig.
4 C), under a condition with limited IpaH1.4 molecules, IpaH1.4
is unlikely to effectively impair the integrity of LUBAC by bind-
ing to HOIL-1L UBL. Therefore, when targeting LUBAC,
IpaH1.4 should preferentially attack HOIP rather than HOIL-
1L. However, when the HOIP level drops by proteasomal degra-
dation as infection time progresses, it is likely that the exposed
HOIL-1L becomes a follow-up substrate for IpaH1.4. Further-
more, considering that HOIL-1L alone has specific cellular func-
tions (51), the recognition of HOIL-1L by IpaH1.4 might also
affect the function of HOIL-1L in a LUBAC-independent sce-
nario. Therefore, the crystal structures of the IpaH1.4/HOIP and
IpaH1.4/HOIL-1L complexes solved in this study may provide
potential drug targets for the future development of innovative
therapeutic strategies against *S. flexneri* infection.

IpaH1.4 and IpaH2.5 share almost identical amino acid sequences in their LRR and NEL domains (*SI Appendix, Fig.
S1A*); therefore, the binding mechanism of IpaH1.4 with
LUBAC uncovered in this study should be also applicable to
IpaH2.5. Indeed, in the NF-κB reporter dual-luciferase assays,
similar phenomena were observed for IpaH1.4 and IpaH2.5
(Fig. 5D and *SI Appendix, Fig. S1B*). Interestingly, besides
IpaH1.4/2.5, *S. flexneri* also secretes other E3 ligases of
the NEL family, such as IpaH3, IpaH4.5, IpaH7.8, and IpaH9.8,
which share almost identical amino sequences in their catalytic
NEL domains but have distinct N-terminal substrate-binding
LRR domains (*SI Appendix, Fig. S21*) (52). Sequence align-
ment analyses of these homologs uncovered that the critical res-
ides for HOIP or HOIL-1L interaction are conserved in
IpaH1.4 and IpaH2.5 but are absent in other IpaH members
(*SI Appendix, Fig. S1B*). For example, the key HOIP-binding
R157 residue of IpaH1.4/2.5 is replaced by an Asp, Ser, or Phe
residue in other IpaH members; the hydrophobic HOIL-1L-
binding F238 residue is substituted by a polar Ser, Thr, Asp, or
Arg residue (*SI Appendix, Fig. S1B*). Therefore, only IpaH1.4/
2.5 can specifically target HOIP and HOIL-1L, in line with a
previous study (37). Apparently, the sequence diversities of the
LRR domains confer NEL family proteins with different spec-
tra of substrate specificities. Through the modular combination
of a highly variable N-terminal substrate-binding domain and
a highly conserved C-terminal NEL domain for catalyzing K48-
conjugated Ub chain formation, the NEL family proteins
evolve to become a wide-spread type of effector protein deliv-
ered by many bacterial pathogens, including *Shigella*, *Salmo-
nella*, *Yersinia*, and *Pseudomonas*, to modulate essential host cell
processes by targeting relevant host proteins for proteasomal
degradation. Intriguingly, we elucidated that in addition to mediating K48-ty peptide ubiquitination as well as the subsequent proteasomal degradation of the substrate, IpaH1.4/2.5 can disturb the function of LUBAC by solely interacting with HOIP and/or HOIL-1L through its substrate-binding LRR domain. The ability of the IpaH1.4 LRR domain to directly inhibit the function of host LUBAC implies that the LRR domains of IpaH family proteins may have evolved to disrupt host signaling pathways before they fuse with the NEL domain. Apparently, the later addition of an LRR domain with a catalytic NEL domain that has an E3 ligase activity can further augment the inhibitory function on host client proteins of the LRR domain through proteasomal degradation.

Notably, our observations on the suppression of NF-κB signaling by IpaH1.4 mutants in the S. flexneri infection assay are somewhat different from that of the IpaH1.4 overexpression luciferase assay (Fig. 5 D–F). In the luciferase assay, the single disruption of IpaH1.4’s binding to either HOIP or HOIL-1L cannot completely abolish IpaH1.4’s inhibition of NF-κB signaling (Fig. 5D), likely due to the high concentration of overexpressed IpaH1.4 protein. While both assays agree that the catalytic-dead (C368A) IpaH1.4 mutant can still inhibit NF-κB activation, the binding ability to either HOIP or HOIL-1L is crucial for IpaH1.4 to suppress host NF-κB signaling (Fig. 5 D–F). Importantly, the fact that the catalytic-dead IpaH1.4 C368A mutant can still effectively suppress host NF-κB signaling undermines the direct interference on its host client protein of an IpaH E3 ligase through its LRR binding and reminds us that functional disruption of an IpaH E3 ligase by solely mutating its catalytic residue is likely to be insufficient. Thus, functional assays with a catalytic-dead mutant of IpaH E3 ligase might not give strong signals, and the relevant experimental data should be interpreted with caution.

Finally, based on our study together with other groups’ reports (30, 31, 37), we proposed a working model to depict the formation of K48-linked poly-Ub chains on HOIP and subsequently leads to the proteasomal degradation of HOIP (SI Appendix, Fig. S22). Second, in the absence of HOIP, HOIL-1L exposes its UBL domain to IpaH1.4/2.5, which further induces the K48-linked poly-ubiquitination of HOIL-1L and promotes its proteasomal degradation, thereby eventually resulting in the subversion of LUBAC.

Materials and Methods

Plasmids and Mutagenesis. For recombinant protein expression in E. coli, the DNA fragments encoding desired proteins were cloned into a modified pET32M (Novagen) or pGEX-4T-1 (GE Healthcare) vector, which encodes a Trx-6xHis or glutathione-S-transferase (GST) tag followed by an HRV 3C protease cutting sequence before the multiple cloning sites. We also constructed a modified pET32M vector in which the Trx-6xHis tag is replaced by a maltose-binding protein (MBP)-6xHis tag to produce MBP fusion protein. All the point mutations in constructs were introduced by standard PCR protocol. For overexpression in HEK293T cells, the coding sequences for full-length HOIL-1L, HOIP, and SHARPIN were inserted into pFLAG-CMV-1 (Sigma), resulting in a fusion protein with an N-terminal FLAG tag. The IpaH1.4 wild type or mutants were cloned into a modified version of pFLAG-CMV-1, in which a FLAG-mCherry tag was fused to the N terminus of IpaH1.4 or IpaH2.5. All point mutations were introduced by standard PCR methods and verified by DNA sequencing.

Protein Expression and Purification. All the proteins used in structural studies and biochemical assays were expressed in E. coli BL21(DE3) as N-terminal Trx-6xHis, MBP-6xHis, or GST fusion proteins. If desired, the tag was cut by homemade HRV 3C protease, which recognizes a cutting site in the fusion protein following the tag. Protein expression in E. coli was induced by 0.1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) when the optical density at 600 nm reached 0.6−0.8, and E. coli was further cultured at 16 °C for 16 h. For the ZF-containing proteins, 0.1 mM ZnCl2 was added into culture to improve protein folding before IPTG induction. Trx/MBP-6xHis or GST fusion proteins were affinity purified by Ni2+-NTA or glutathione resin (GE Healthcare), respectively, and further polished by size-exclusion column HLoad Superdex 26/60 (GE Healthcare) in buffer A (20 mM Tris-HCl pH 7.5, 100 mM NaCl, and 1 mM dithiothreitol [DTT]). Proteins were concentrated to the desired concentration by ultrafiltration. Uniformly 15N-labeled HOIP RING1 or IpaH1.4 LRR protein was expressed in E. coli BL21(DE3) cells cultured with M9 minimal medium using 15NH4Cl (Cambridge Isotope Laboratories Inc.) as the sole nitrogen source and was purified similar to unlabeled proteins.

Size-Exclusion Chromatography and Comigration Assay. Individual and mixed protein samples (500 μL) were loaded onto a HLoad Superdex 200 Increase 10/300 column (GE Healthcare) and eluted in buffer A; absorbance was monitored at 280 nm.

ITC Assay. Generally, proteins for titration were concentrated to about 500 μM (in syringe) or 50 μM (in cell) in buffer A. Titration was conducted at 25 °C with a PEAQ-ITC machine (Malvern Instrument). The dissociation constant and number of binding sites were deduced by fitting titration curves with a model for one set of binding sites in the PEAQ-ITC analysis software (Malvern Instrument).

Analytic Ultracentrifugation Assay. Protein for analysis was collected from the peak fraction of a size-exclusion chromatography separation. The absorbance at a wavelength of 280 nm (A280) value was adjusted to −0.5 with buffer A. The same batch of buffer A was used as a reference for absorbance monitoring during ultracentrifugation. Sedimentation velocity experiments were conducted at 142,500 × g with a Beckman XL-A analytical ultracentrifuge operated at 20 °C. The sedimentation velocity data were analyzed in the software SEDFIT (53) and fitted with a continuous sedimentation coefficient distribution c(s) model with parameters such as partial specific volume of protein samples and buffer density calculated in the program SEDNTERP (http://www.rasmb.org/).

NMR Spectroscopy. The 15N-labeled protein for NMR studies was concentrated to ~0.2 mM in a buffer containing 50 mM NaH2PO4/Na2HPO4 (pH 6.5), 50 mM NaCl, and 1 mM DTT. All 15N-HSQC spectra were collected at 25 °C with an 800-MHz spectrometer (Agilent Technologies) equipped with an actively z gradient-shielded triple resonance probe.

Crystal Growth. Protein in buffer A was concentrated to about 10−20 mg/mL for crystallization condition screening with commercial buffer kits. Briefly, 1 μL of protein and an equal volume of reservoir buffer were mixed for a crystallization trial in a sitting drop manner. IpaH1.4 LRR was crystallized in a buffer containing 0.1 M sodium citrate (pH 4.0) and 0.8 M (NH4)2SO4. The IpaH1.4 LRR/HOIP RING1 complex was crystallized in a buffer containing 2% (vol/vol) 1, 4-dioxane, 0.1 M Tris–HCl (pH 8.0), and 15% (vol/vol) PEG3350. The UBE2L3/HOIP RING1 crystal was grown in a buffer containing 0.06 M MgCl2, 0.06 M CaCl2, 0.1 M Tris–HCl (pH 8.1), 12% (vol/vol) MPD, 12% (vol/vol) PEG10000, and 12% (vol/vol) PEG3350. The IpaH1.4 LRR/HOIL-1L complex crystal grew in a buffer...
containing 0.2 M (NH₄)₂SO₄, 0.1 M Tris-HCl (pH 8.5), and 25% (wt/vol) PEG3350.

X-ray Diffraction and Structure Determination. Single crystals were equilibrated with mother liquor containing 20% (vol/vol) glycerol as cryoprotectant and flash-frozen in liquid nitrogen. Diffraction data sets were collected at the beamline BL17U1 or BL19U1 of the Shanghai Synchrotron Radiation Facility (SSRF) (54). Data sets were processed with XDS and autoPROC software suite (55, 56). The apo form structure of IpaH1.4 LRR was solved by molecular replacement using the ipaH1880 structure (PDB: 5KH1) as the searching template. The refined IpaH1.4 LRR, HOIP RING1 (PDB: 5EDV), and HOIL-1L UBL (PDB: 4DBG) structures were used as searching templates to solve the IpaH1.4 LRR/HOIP RING complex and the IpaH1.4 LRR/HOIL-1L UBL complex structures. Similarly, the UBE2L3/HOIP RING1 complex structure was solved by molecular replacement using the structure of UBE2L3 from PDB entry 4Q5H. All structure models were manually adjusted in Coot (57) and re

In Vitro Ubiquitylation Assay. Recombinant murine UBA1 (E1), human UBE2L3 (E2), and Ub expressed in E. coli cells were used for the assay. The typical reaction system contained 1 μL of E1, 2 μM E2, 100 μM Ub, and 1 μM HOIP or IpaH1.4 in a buffer with 20 mM Tris-HCl (pH 7.5), 100 mM NaCl, 1 mM DTT, 10 mM ATP, and 10 mM MgCl₂. Reaction mixtures were incubated at 37 °C for the desired time, and aliquots of sample were immediately denatured by mixing with 2× SDS-PAGE sample buffer.

Luciferase Assay. HEK293T cells were cultured in 12-well plates to a confluency of ~60% in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% (vol/vol) fetal bovine serum before transfection. Each well was transfected with 200 ng of pGL-SV40, 20 ng of pRL-NF-κB, and transfected with 10% (vol/vol) fetal bovine serum before transfection. Each well was transfected with 200 ng of pGL-SV40, 20 ng of pRL-NF-κB, and 10% (vol/vol) fetal bovine serum (BSA) for 45 min. The number of cells with p65 translocation into the nucleus was counted manually. Only infected cells (with presence of DAPI stained bacteria in the cytoplasm of cells) were included in the calculation of percent of p65 nuclear enrichment. A p65 signal higher in the nucleus than in the cytoplasm was scored as nuclear translocation.

Data Availability. The atomic coordinates and structure factors of the crystal structures of the apo form IpaH1.4 LRR, the IpaH1.4 LRR/HOIP RING1 complex, and the IpaH1.4 LRR/HOIL-1L UBL complex have been deposited in the PDB under the accession codes 7V6H, 7V8G, 7VBF, and 7VBE, respectively. All other data study are included in the article and/or SI Appendix.

Acknowledgments. We thank SSRF BL19U1, BLU2U1, and BL10U2 for X-ray beam time, Professor Jiahui Han for the HOIP, SHARPIN, HOIL-1L, and UBE2D1 cDNA, Professor Yufeng Yao for the IpaH1.4 and IpaH2.5 cDNA, and Professor Ronggui Hu for the E1 (mouse UBA1) plasmid used in this study. This work was supported by grants from the National Natural Science Foundation of China (21822705, 91753113, 21621002, and 2021219, the National Key R&D Program of China (2016YFA0501903), the Science and Technology Commission of Shanghai Municipality (20XD1425200), the Strategic Priority Research Program of the Chinese Academy of Sciences (XDB20000000), the start-up fund from National Key Laboratory of Bioorganic and Natural Products Chemistry and Chinese Academy of Sciences (to L.P.), and grants from the National Natural Science Foundation of China (202701297, to J.L.), National Natural Science Foundation of China (31900111) (to D.W.), and National Natural Science Foundation of China (31800646) (to Yingli Wang).

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