Function and regulation of novel phosphoribosyl-ubiquitination catalyzed by *Legionella pneumophila* effectors

Dissertation

zur Erlangung des Doktorgrades

der Naturwissenschaften

vorgelegt beim

Fachbereich Biochemie, Chemie und Pharmazie (FB14)

der Johann Wolfgang Goethe -Universität

in Frankfurt am Main

von Yaobin Liu

aus Henan, China

Frankfurt am Main 2020

(D 30)

vom Fachbereich 14 der

Johann Wolfgang Goethe - Universität als Dissertation angenommen.

Dekan : Prof. Dr. Clemens Glaubitz

Gutachter : Prof. Dr. Volker Dötsch, Prof. Dr. Ivan Dikic

Datum der Disputation : 11.Jan.2021

Contents

1 Zusammenfassung

Ubiquitinierung ist eine posttranslationale Modifikation, die von Hefe bis zu Säugetieren konserviert ist. Die Katalyse der kanonischen Ubiquitinierung wird über eine dreigliedrige Enzym-Kaskade reguliert: Zunächst wird ein Ubiquitin (Ub) -Molekül durch ein Ub-aktivierendes Enzym (E1) unter ATP-Verbrauch aktiviert; das aktivierte Ub wird über sein C-terminales Glycin an das katalytische Cystein des E1 gebunden und anschließend auf ein Ub-konjugierendes Enzym (E2) übertragen. In einem letzten Schritt verbindet eine Ub-Ligase (E3) die Carboxylgruppe des C-terminalen Glycins des Ubiquitins über eine Isopeptidbindung mit einer ε-Aminogruppe des Ziellysins im Substratprotein (Hershko et al., 2000). Die Proteinubiquitinierung reguliert eine Vielzahl von zellulären Prozessen, einschließlich der Proteinqualitätskontrolle, Proteintransport, Immunantwort und DNA-Reparatur, indem Substrate durch das Proteasom abgebaut oder ihre Funktionen geändert werden (Ben-Neriah, 2002; Dikic, 2017; Donaldson et al., 2003).

Für effiziente Wechselwirkungen mit Wirtszellen haben viele intrazelluläre Pathogene spezielle Proteinsekretionssysteme entwickelt, wie beispielsweise die gut aufgeklärten Typ III-oder IV-Sekretionssysteme (T3SS oder T4SS), um bakterielle in die Wirtszellen abzugeben. Diese beiden Sekretionsmaschinen bestehen aus Proteinkomplexen, die die gesamte Bakterienhülle durchqueren, um einen Kanal für die effektive Abgabe von Effektorproteinen zu garantieren. Umfangreiche Studien zur Aufklärung von biochemischen Funktionen vieler bakterieller Effektoren haben wesentlich zu einem besseren Verständnis der Biologie der Infektion beigetragen (Mattoo et al., 2007). In Wirtszellen injizierte Effektoren können Bakterien das Entkommen aus der Immunantwort der Wirtszellen erleichtern oder die bakterielle intrazelluläre Proliferation fördern, indem sie eine Vielzahl von Signalwegen des Wirts modulieren, einschließlich der Dynamik des Zytoskeletts, des Membrantransports, der Transkription, des Verlaufs des Zellzyklus sowie der Signaltransduktion (Galán, 2009; Hicks und Galán, 2013). In Übereinstimmung mit der kritischen Rolle der Ubiquitinierung in zellulären Prozessen deuten neuere Erkenntnisse darauf hin, dass manche Krankheitserreger die Ubiquitinierungsmaschinerie für eine effiziente Invasion missbrauchen können (Bomberger et al., 2011; Hicks und Galán, 2013; Maculins et al., 2016). Beispielsweise sezerniert der intrazelluläre gramnegative Erreger Legionella pneumophila über sein Typ IV-Sekretionssystem (T4SS) mehr als 300

Effektoren in Wirtszellen (Hubber und Roy, 2010). Viele dieser Toxine fungieren als E3-Ligasen und manipulieren Studien zufolge die Ubiquitinierung des Wirts, einschließlich der Inaktivierung von Ubiquitin, den Missbrauch von E2s, der Nachahmung von Deubiquitinierungsenzymen (DUBs) und E3-Ligasen (Qiu und Luo, 2017).

Verschiedene Studien haben gezeigt, dass Effektoren der SidE-Familie (SdeA, SdeB, SdeC und SidE) eine NAD^+-abhängige, ATP-unabhängige Art der Ubiquitinierung ohne die Notwendigkeit von E2- und E3-Enzymen katalysieren (Bhogaraju et al., 2016; Qiu et al ., 2016). Im Gegensatz zur herkömmlichen Ubiquitinierung, die an Lysinresten von Substratproteinen auftritt, katalysieren Effektoren der SidE-Familie die Konjugation von Ub über die Phosphoribosylgruppe an Serinreste des Wirtssubstratproteins. Dieser Vorgang wird durch ein katalytisches Reaktion von zwei Domänen der SidE-Familienmitglieder bewerkstelligt: eine Mono-ADP-Ribosyltransferase (mART)) Domäne, die ADP-Ribosylate Arg42 von Ub und eine Phosphodiesterase (PDE)-Domäne, die die Phosphodiesterbindung des ADP-ribosylierten Ub (ADPR-Ub) spaltet und das resultierende Phosphoribosylubiquitin (PR-Ub) an den Serinrest eines Substrats konjugiert (Akturk et al., 2018; Dong et al., 2018; Kalayil et al., 2018; Wang et al., 2018). Effektoren der SidE-Familie sind entscheidend für die bakterielle Virulenz. Einem Legionellen Stamm, dem Mitglieder der SidE-Familie fehlen, zeigt eingeschränktes Wachstum in Wirtszellen. Dieser Phänotyp kann durch Ergänzung von exogenem SdeA gerettet werden (Bardill et al., 2005; Qiu et al., 2016). Somit erweitert die SdeA-vermittelte ADP-Ribosylierung von Ubiquitin und die daraus resultierende Substratubiquitinierung das Wissen über die durch Ubiquitin vermittelten Effekte in posttranslationalen Modifikationen und Funktionsregulation. Die Entdeckung dieser All-in-One-Ubiquitin-E3-Ligase aus Bakterien stellt auch einen neuen Mechanismus dar, der sich vollständig von der in eukaryotischen Zellen charakterisierten kanonischen enzymatischen Kaskade unterscheidet. Weitere biochemische Studien sind erforderlich, um den detaillierten Mechanismus der SdeA-vermittelten Ubiquitinierung zu ergründen. Darüber hinaus müssen andere potenzielle Substrate von SidE-Effektoren identifiziert werden, um die Toxizität der SdeA-Expression in eukaryotischen Zellen und die nachfolgenden biologischen Funktionen der SdeA-katalysierten Ubiquitinierung in Wirtszellen zu erklären.

Hier verwendeten wir biochemische und zellbiologische Ansätze, um die biologische Wirkung der durch SdeA verursachten Phosphoribosylierung von Ubiquitin zu untersuchen. Die SdeA PDE-Mutante H277A ADP-Ribosylate ubiquitiniert Ubiquitin, jedoch keine Substratproteine. Ähnlich wie bei Wildtyp-SdeA ist die Expression von SdeA H277A jedoch auch für Hefezellen toxisch, was darauf hinweist, dass die Modifikation von Ubiquitin eine wesentliche Funktion von SdeA sein könnte. Phosphoribosyliertes Ubiquitin ist für E1- und E2-Enzyme der herkömmlichen Ubiquitinierungskaskade nicht verfügbar, wodurch SdeA zahlreiche klassische zelluläre Prozesse im Zusammenhang mit der Ubiquitinierung beeinträchtigen könnte. Unter Verwendung eines Ubiquitin-Antikörpers, der das durch SdeA modifizierte Ubiquitin nicht erkennen kann, haben wir gezeigt, dass die Expression der SdeA WT- oder mART H277A-Mutante in Zellen zur Phosphoribosylierungs- oder ADP-Ribosylierungsmodifikation von Ubiquitin führt, was *in vitro* Beobachtungen von mit SdeA behandelten Ubiquitins ähnlich ist. Angesichts der Tatsache, dass verschiedene Verknüpfungstypen von Polyubiquitin-Ketten unterschiedliche Effekte auf deren Substrate haben können, haben wir versucht, die Details der Interferenz von SdeA bei der herkömmlichen Ubiquitinierung zu entschlüsseln. Unsere Western-Blot Ergebnisse zeigen, dass die SdeA-Expression entweder die K48-verknüpfte oder die K63-verknüpfte Ubiquitinierung reduziert. Darüber hinaus scheint SdeA K63-Ketten gegenüber K48-Ketten zu bevorzugen. Dieses Ergebnis steht im Einklang mit den Beobachtungen, dass die Expression der SdeA WT- oder H277A-Mutante, jedoch nicht der mART-Mutante, zu einer HIF1-α-Stabilisierung in HeLa-Zellen führt. Neben dem Proteinabbau, Ubiquitinierung mehrerer Proteine, die an verschiedenen Stadien der Aktivierung des NF-κB-Signalwegs und der durch Carbonylcyanid m-Chlorphenylhydrazon (CCCP) induzierten Mitophagie beteiligt sind, haben wir durch Expression von SdeA oder SdeA H277A in Zellen und Überwachung der nuklearen Translokation von p65 und Ubiquitinierung an Mitochondrien bestätigt, dass die durch SdeA-Expression verursachte Phosphoribosylierungsmodifikation von Ubiquitin auch diese Ubiquitin-assoziierten Signalwege beeinflusst.

Die zeitliche Regulierung der Effektorfunktionen durch Effektoren mit antagonisierenden Aktivität sind bei Infektionen mit *L. pneumophila* gut untersucht. Die durch Effektoren der SidE-Familie katalysierte PR-Ubiquitinierung ist ebenfalls reversibel. Es ist seit langem bekannt, dass SidJ die Toxizität von SdeA gegenüber Hefezellen reduzieren kann. Die SidJ-Sekretion in Wirtszellen nimmt mit

fortschreitender Legionellen Infektion zu, was zu einer Hemmung der Wirkungen von SdeA im späten Stadium der Infektion führt. Durch Durchführen eines In-vitro-Reaktionsexperiments mit gereinigtem rekombinantem SidJ oder Säugetierzelllysat, das SidJ enthält, haben wir gezeigt, dass SidJ zwar SdeA Aktivität hemmt, dies aber nicht durch Spaltung von PR-ubiquitinierten Substraten geschieht. Jüngste Studien zeigten, dass die SdeA-Ligaseaktivität durch direkte Glutamylierung von SdeA an E820, katalysiert durch SidJ, gehemmt wird. Interessanterweise fanden wir, dass Legionellenlysat ohne SidJ Ubiquitin von PR-ubiquitinierten Substraten entfernte, was darauf hinweist die Existenz von Deubiquitinase, die auf die neuartige PR-Ubiquitinierung hinweißt. Unter Verwendung eines biochemischen Ansatzes identifizierten wir DupA und DupB, zwei bakterielle Legionella-Effektoren, die die durch SdeA katalysierte neue Serin-PR-Ubiquitinierung umkehren. DupA und DupB besitzen eine hoch homologe katalytische Phosphodiesterase (PDE) -Domäne, die Ubiquitin von modifizierten Substraten, durch Spaltung der Phosphodiester-Bindung zwischen dem phosphoribosylierten Ubiquitin und dem Serin des Proteinsubstrates, entfernt.
Diese neuen Deubiquitinasen DupA/B katalysieren die entgegengesetzte Modifikation mit SdeA-PDE in einem Mechanismus, der einheitlich zu sein scheint. Um die Unterschiede zwischen SdeA-PDE und DupA zu unterscheiden, haben wir die PDE-Aktivitäten von DupA mit der SdeA-PDE-Domäne verglichen.
Unsere Daten zeigen, dass SdeA PDE die von sich selbst modifizierte Ubiquitinierung nicht rückgängig machen konnte und darüber hinaus weniger Aktivität als DupA / B zeigte, um die ADP-Ribosylierung von Ubiquitin zu eliminieren. Im Gegensatz zu der funktionierenden Ubiquitin-Ligase SdeA, die eine PR-Ubiquitinierung von Substraten katalysiert, zeigten DupA und DupB keine Aktivität zur Übertragung von PR-Ub auf Substrate. Weitere *In-vitro*-Reaktionsexperimente legen nahe, dass DupA nach kurzer Inkubationszeit, von 5 Minuten, die Ubiquitinierung des Substrats Rab33b vermittelt. Diese Ubiquitinierung verschwindet jedoch sehr schnell (Shin et al., 2020). Die Bestimmung der Struktur des DupA H67A- und PR-Ub-Komplexes und biochemische Experimente zeigten, dass DupA aufgrund ausgedehnter elektrostatischer Wechselwirkungen eine stärkere Bindungsaffinität zu Ubiquitin, ADPR-Ub und PR-ubiquitinierten Substraten aufweist. Diese Unterschiede erklären gut, warum SdeA PDE nicht die Deubiquitinase-Aktivität von DupA aufweist und warum DupA ADPR-Ub effektiv spaltet, PR-Ub jedoch nicht auf Substrate überträgt.

In Anbetracht der kritischen Rolle von Effektoren der SidE-Familie bei der Infektion und Proliferation von Bakterien ist es notwendig, die PR-Ubiquitinierungssubstrate von Effektoren der SidE-Familie zu identifizieren. DupA H67A, die katalytisch inaktive Mutante von DupA, bindet stark an PR-ubiquitinierte Proteine, ist jedoch nicht in der Lage, PR-Ubiquitin zu spalten. Wir haben diese als Protein-Köder verwendet und über 180 Substrate der PR-Ubiquitinierung aus Lysaten von mit Legionellen infizierten Zellen per Massenspektrometrieanalyse identifiziert. Darunter sind eine Reihe von ER- und Golgi-Proteinen.

Frühere strukturelle und biochemische Studien haben gezeigt, dass die Struktur des katalytischen SdeA-Kerns, die Funktion der Carboxyl-terminalen (CT) Region, von der vorhergesagt wird, dass sie Coiled-Coil ist, unbekannt blieb. Angesichts der Tatsache, dass Coiled-Coil-Domänen als für die Membranlokalisierung vieler Salmonella-Typ-III-Effektoren erforderlich gemeldet wurden, stellten wir die Hypothese auf, dass die CT-Domäne von SdeA für die Membranassoziation verantwortlich ist. Die Färbung mit dem ER-residenten Calnexin- und Golgi-Marker GM130 ergab, dass SdeA zusammen mit ER und auch dem Golgi-Apparat lokalisiert war, während die verkürzte Mutante SdeA1-972 dies nicht tat, was darauf hindeutet, dass SdeA über seine C-Terminus-Region auf den Membranen lokalisiert ist. Interessanterweise fanden wir, dass die Expression von SdeA in voller Länge in Zellen oder die Infektion von Wildtyp-Legionellen, jedoch nicht der Stamm ohne Effektoren der SidE-Familie, eine Golgi-Störung induzierte. Diese Ergebnisse stimmen mit der Beobachtung überein, dass SdeA auf ER- und Golgi-Proteine.

Es wurden umfangreiche Anstrengungen unternommen, um die Funktion von SdeA und den Mechanismus der SdeA-vermittelten PR-Ubiquitinierung aufzuklären, trotzdem sind Details der zellulären Auswirkungen dieser neuartigen Ubiquitinierung bisher unbekannt. Unter den PR-ubiquitinierten Proteine, die unter Verwendung des DupA-Köders identifiziert wurden, waren die Golgi-Tethering-Proteine GRASP55 und GCP60 stark angereichert. Durch In-vitro-Reaktion und In-vivo-Expression bestätigten wir, dass die Golgi-Tethering-Proteine GRASP55, GCP60 und GRASP65, das Homologprotein von GRASP55, PR-ubiquitiniert sind. Mittels Massenspektrometrie identifizierten wir vier Serine (S3, S408, S409, S449) von GRASP55 PR, welche *in vitro* durch SdeA ubiquitiniert wurden. Diese Identifizierung wurde weiter bestätigt, indem die PR-Ubiquitinierung der GRASP55 7S*-Mutante in der diese Serine und benachbarte Serine ausgetauscht wurden in Gegenwart von SdeA bewertet wurde.

Wir beobachteten, dass die Ubiquitinierung der GRASP55 7S*-Mutante in Zellen, die SdeA co-exprimieren oder mit Legionellen infiziert waren, im Vergleich zu der des Wildtyp-GRASP55 deutlich verringert war. Insbesondere hat die Mutation dieser Serine das Ubiquitinierungssignal von gereinigtem GRASP55 nicht vollständig aufgehoben, was darauf hindeutet, dass weitere Reste in GRASP55 auch durch SdeA modifiziert werden könnten. Die Golgi-Zisternen-Tethering-Funktion von GRASP55 wird durch Phosphorylierung der C-terminalen Serin- und Prolin-reichen (SPR) Domäne durch mitotische Kinasen reguliert (Feinstein und Linstedt, 2008; Wang et al., 2005). Es wurde außerdem berichtet, dass mehrere Serine in dieser C-terminalen Region von GRASP55, einschließlich S408, S409, S441, S449, welche in dieser Studie als PR-Ubiquitiniert identifiziert wurden, phosphoryliert werden können (Bian et al., 2014; Kim et al., 2016). Phosphorylierungsnachahmer an diesen Stellen stören die Homodimerisierung von GRASP, möglicherweise durch eine Änderung der Proteinkonformation (Kim et al., 2016; Truschel et al., 2012). Wir stellten die Hypothese auf, dass die PR-Ubiquitinierung von GRASP-Proteinen die Homodimerisierung und damit die Golgi-Integrität beeinflusst. Co-IP-Analysen zeigten, dass PR-ubiquitiniertes GRASP55 im Vergleich zu unmodifiziertem GRASP55 eine verringerte Selbstinteraktion zeigt und die Fähigkeit von PR-ubiquitiniertem GRASP55 zur Selbstinteraktion mit GRASP55 im Vergleich zu SdeA-resistentem HA-GRASP55 7S* verringert ist. Durch Überwachung der strukturellen Stabilität des Golgi von Zellen, die SdeA exprimieren, fanden wir, dass die GRASP55 7S*-Mutante die durch SdeA verursachte Golgi-Störung teilweise rettete. Diese Daten deuten darauf hin, dass die SdeA-verursachte Golgi-Störung das Ergebnis einer Modifikation von GRASP-Proteinen ist, welche die Verbindung zwischen Golgi-Stapeln.

Intrazelluläre Pathogene neigen dazu, sich eine von Membranen umgebene Nische für Reifung, Proliferation und Flucht vor Abwehrmechanismen wie der selektiven Autophagie innerhalb der Wirtszelle zu nutzen zu machen. Deshalb erzeugt zum Beispiel eine Chlamydia-Infektion eine Golgi-Fragmentierung, um Golgi-Ministacks für bakterielle Einschlüsse zu erzeugen (Heuer et al., 2009). Legionellen, die Vakuolen (LCVs) enthalten, rekrutieren ER-Membranen und wandeln so das Phagosom in ein spezifisches Kompartiment um, das Merkmale des ER aufweist (Kotewicz et al., 2017; Shin et al., 2020; Xu und Luo, 2013). Wir stellen die Hypothese auf, dass eine Legionellen Infektion die Golgi-Störung induziert, um die Fusion von Vesikeln aus dem Golgi mit LCV zu erleichtern und die Bildung von LCV und letztendlich die intrazelluläre

Replikation zu fördern. Die Ergebnisse der Immunfärbung deuteten jedoch darauf hin, dass weder endogener cis-Golgi-Marker noch trans-Golgi auf LCV akkumulierten, was darauf hinweist, dass die funktionelle Konsequenz der Golgi-Störung nicht mit der Rekrutierung von Golgi-Membranen in den wachsenden Legionellen-haltigen Vakuolen zusammenhängt.

Der Golgi-Apparat spielt eine zentrale Rolle im Sekretionsweg. Mit VSVG als Marker konnten wir den Effekt der SidE-vermittelten PR-Ubiquitinierung auf den Proteintransport untersuchen. Wir haben gezeigt, dass die PR-Ubiquitinierung den VSVG-Transport über den Golgi mithilfe von Mikroskopie und EndoH-Verdauungstests verlangsamt. Als Teil der Immunantwort sezernieren Makrophagenzellen bei bakterieller Infektion Zytokine. Da der ER-zu-Golgi-Transportweg eine wichtige Rolle beim konventionellen Transport der meisten Zytokine spielt und die Aufrechterhaltung der Golgi-Struktur für die Sekretion einiger Zytokine wie TNFα entscheidend ist (Micaroni et al., 2013), haben wir den Effekt untersucht der PR-Ubiquitinierung auf die Zytokinsekretion von Makrophagenzellen nach Legionellen Infektion hat. Die ELISA-Ergebnisse zeigten, dass mit Legionellen infizierte Zellen, denen Effektoren der SidE-Familie fehlen, mehr TNFα freisetzen als Zellen, die mit Wildtyp-oder dupA/B-Legionellastämmen infiziert waren. Interleukin IL-1β ist eines der Zytokine, die unabhängig vom konventionellen ER-Golgi-Handel ausgeschieden werden. Ähnlich wie bei TNFα, war die Sekretion von IL-1β in THP-1-Zellen, die mit Legionellen infiziert waren, denen Effektoren der SidE-Familie fehlten, im Vergleich zu Zellen, die mit Wildtyp- oder ΔDupA/B-Legionellenstämmen infiziert waren, erhöht. Dies deutet darauf hin, dass sowohl konventionelle als auch unkonventionelle Sekretionsprozesse von Effektoren der SidE-Familie betroffen sein können. Es wurde gezeigt, dass die unkonventionelle Sekretion von IL-1β von GRASP55 und GRASP65 abhängt (Chiritoiu et al., 2019). Basierend auf den Ergebnissen einer Studie mit In-vivo-GRASP55/66-Depletion haben Grond et al. vorgeschlagen, dass GRASP-Proteine anstelle des Stapelns des Golgi-Zisternenkerns die Ränder der Golgi-Zisternen verbinden, die die Quelle für Membranen sein könnten, die für eine unkonventionelle Sekretion erforderlich sind (Grond, R., et al., 2020). Es ist möglich, dass die PR-Ubiquitinierung die Oligomerisierung von GRASP-Proteinen verhindert, zum Lösen des Golgi-Bandes und zur Vesikulation von Golgi-Zisternenrändern führt, was zu dispergiertem Golgi und einer Hemmung der unkonventionellen Sekretion führt.

Zusammengenommen deckte unsere Studie die Wirkung der SdeA-katalysierten Phosphoribosylierung von Ubiquitin auf Signalwege auf und identifizierte Legionella-PR-Deubiquitinasen. Wir haben gezeigt, dass SdeA im ER und teilweise an Golgi-Membranen lokalisiert ist und somit die Golgi-Tethering-Proteine GRASP55 und GRASP65 PR-ubiquitiniert, was zur Golgi-Störung und Hemmung des Sekretionsweges führt. Unsere Studie enthüllt biologische Konsequenzen der PR-Ubiquitinierung auf Golgi-Proteine und deckt eine von Legionellen genutzte Golgi-Manipulationsstrategie auf. Dies führt zu einer erhöhten bakteriellen Infektionsrate und Replikation in Wirtszellen. Es wird interessant sein herauszufinden, ob die PR-Ubiquitinierung zusätzliche Mechanismen zur Erleichterung der bakteriellen Infektion bietet, indem in Zukunft andere Substrate von SidE-Effektoren untersucht werden.

2 Abstract

Protein ubiquitination is a post-translational modification that typically involves the conjugation of ubiquitin to substrate proteins via a three-enzyme cascade and regulates a wide variety of cellular processes. Recent studies have revealed that SidE family of *Legionella* effectors such as SdeA catalyzes novel phosphoribosyl-linked ubiquitination (PR-ubiquitination) of serines in host substrate proteins utilizing NAD^+, without the need of E2, E3. The catalytic core of SdeA comprises a mono-ADPribosyltransferase (mART) domain that functions to ADP-ribosylate ubiquitin, and a phosphodiesterase (PDE) domain that processes ADP-ribosylated ubiquitin and transfers the resulting phosphoribosylated ubiquitin to serines of substrates.

To date, extensive efforts have been made to study the function of SdeA and mechanism of SdeA mediated PR-ubiquitination, however, the cellular effects of this novel ubiquitination and phosphoribosylation of ubiquitin remained poorly understood. In our study, using biochemical and cell biological approaches, we explored the biological effect of phosphoribosylation of ubiquitin caused by SdeA in cells. We found that phosphoribosylated ubiquitin is not available for conventional ubiquitination, thereby phosphoribosylation of ubiquitin impairs numerous classical ubiquitination related cellular processes including mitophagy, TNF-α signaling and proteasomal degradation.

The precise temporal regulation of the functions of bacterial effectors during *Legionella* infection by other effectors with antagonizing activities has been well studied so far. Not surprisingly, PR-ubiquitination catalyzed by SidE family effecters is tightly controlled as well, it has been long known that effector SidJ counteracts the toxicity of SdeA to yeast cells. Interestingly, in an experiment for verifying the activity of SidJ, we found that *Legionella* lysate lacking SidJ was still able to remove ubiquitin from PR-ubiquitinated substrates. Using biochemical approach we identified DupA and DupB, two *Legionella* bacterial effectors that specifically reverse the novel serine PR-ubiquitination catalyzed by SdeA. We found that DupA and DupB possess a highly homologous PDE domain that removes ubiquitin from PR-ubiquitinated substrates by cleaving the phosphodiester bond between the phosphoribosylated-ubiquitin and serines of substrates. Catalytically deficient mutant DupA H67A strongly binds to PR-ubiquitinated proteins but not capable of cleaving PR-ubiquitin, using it as a trapping bait we identified over 180 substrates of PR-ubiquitination, including a number of ER and Golgi proteins.

In particular, we found that exogenously expressed SdeA localizes to the Golgi apparatus via its C-terminal region and disrupts the Golgi. We validated the identified potential substrates of SidE effectors and found that SdeA modifies Golgi tethering proteins GRASP55 and GRASP65. Using mass spectrometry analyses we identified four serine targets (S3, S408, S409, S449) of GRASP55 PR-ubiquitinated by SdeA *in vitro*. Ubiquitination of GRASP55 serine mutant in cells co-expressing SdeA or infected with *Legionella* was markedly decreased, compared with that of the wild-type GRASP55. In addition, with co-immunoprecipitation analyses we found that SdeA-catalyzed ubiquitination regulates the function of GRASP55. PR-ubiquitinated GRASP55 exhibited reduced self-interaction compared to unmodified GRASP55, expression of GRASP55 serine mutant in cells in part rescued Golgi damage caused by SdeA. Furthermore, our study reveals that Golgi structure disruption caused by SdeA does not result in the recruitment of Golgi membranes to the *Legionella*-containing vacuoles. Instead, it affects cellular secretory pathway including cytokine secretion in cells.

Taken all together, this work expands the understanding of this unconventional PR-ubiquitination catalyzed by *Legionella* effectors and sheds light on the functions of PR-ubiquitination by which *Legionella* regulates the Golgi function and secretion pathway during bacterial infection.

3 Abbreviations

ABC	Ammonium Bicarbonate
ADPR	ADPR-ribosylation
ATP	Adenosine triphosphate
BLAST	Basic Local Alignment Search Tool
BSA	Bovine serum albumin
cDNA	Complementary DNA
Co-IP	Co immunoprecipitation
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide Triphosphates
DUB	Deubiquitinating enzyme
ER	Endoplasmic reticulum
ETD	Electron-transfer dissociation
GFP	green fluorescent protein
GST	Glutathione S-transferase
HA tag	Hemagglutinin
HEK293T	Human Embryonic Kidney 293T
LCV	Legionella-containing vacuole
mART	mono-ADPribosyltransferase
MOI	Multiplicity of infection
MS	Mass spectrometry
NAD	Nicotinamide adenine dinucleotide
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PDE	Phosphodiesterase
PEI	Polyethylenimine
PFA	Paraformaldehyde
PR-Ub	Phosphoribosyl ubiquitin
PTM	Posttranslational modification
SDS-PAGE	SDS polyacrylamide gel electrophoresis
T4SS	Type 4 secretion system
Ub	Ubiquitin
UPS	Ubiquitin-proteasome system
WT	Wild type

4 Introduction

4.1 Ubiquitination

Post-translational modification (PTM) refers to biomedical changes that occur on proteins synthesized by the ribosome. Protein phosphorylation is one the most common and studied post-translational modifications. Other well-studied modifications include precursor protein cleavage, disulfide bond formation, and covalent additions of small groups that achieve modifications such as acetylation, biotinylation, methylation, glycosylation, mono-ADP-ribosylation. Post-translational modifications play critical roles in protein folding, protein trafficking to appropriate subcellular destinations, and regulation of protein activities (Bode and Dong, 2004; Doyle and Mamula, 2001). In addition to the modifications with small chemical groups, cellular proteins can also be covalently decorated with small proteins. Ubiquitination, a post-translational modification that is conserved in eukaryotes from yeast to mammals, is involving in the addition of a 76 amino acids small protein ubiquitin (Ub) to substrate proteins.

4.1.1 Ubiquitination process

The ubiquitination process is catalyzed by a three-enzyme cascade, containing activation conjugation, ligation, which has been studied well (Hershko et al., 2000; Pickart, 2001). Firstly, Ub is activated by an Ub-activating enzyme (E1) consuming ATP to adenylate the C-terminus of Ub and then forms a thioester linkage between the Ub C-terminal carboxyl group with the E1 cysteine sulfhydryl group. So far, of the human genome, 2 genes that encode E1 enzymes: UBA1 and UBA6, have been identified to initiate the process (Schulman and Wade Harper, 2009). This step of ubiquitin activation is followed by the transfer of activated Ub from E1 to catalytic cysteine of an Ub-conjugating enzyme (E2) via a transesterification reaction. Over 35 different enzymes are characterized as E2 according to their highly conserved structures. Ubiquitin attached to E2 is ultimately transferred to substrate lysines in the presence of E3 ubiquitin ligases, resulting in the formation of an isopeptide bond between the ε-amino group of a substrate lysine and the C-terminal carboxylate of Ub (Hershko et al., 2000). E3 ligases are capable of interacting with both E2 and the substrates and function as specific substrate recognition enzymes of the system. To date, hundreds of enzymes have been identified as E3 ligases in eukaryotic cells, which ensure the specificity of substrate targeting (Medvar et al., 2016). According to

their mechanisms in ubiquitination of substrates, these E3 ligases can be divided into three main types of E3 ligases: (1) E3 enzymes that possess a the really interesting new gene (RING) domain that functions as scaffold. This family of E3 ligases bring Ub-charged E2 into the proximity of substrates recognized by the substrate binding domain (SBD) and catalyze the direct transfer of Ub from E2 to a lysine of substrate protein; (2) E3 enzymes that possess a homologue to the E6-AP C terminus (HECT) domain. Unlike RING-E3 ligases, HECT family E3 ligases catalyze the transfer of ubiquitin to substrates through a 2-step reactions. The N-terminus (N-lobe) of HECT domain interacts with E2 enzymes, while the C-terminus (C-lobe) contains a catalytic cysteine residue and forms a thioester intermediate with Ub before transferring it to the substrates; and (3) RING-in-between-RING (RBR)-type E3s working via a hybrid mechanism (Vittal et al., 2015) (Fig. 1).

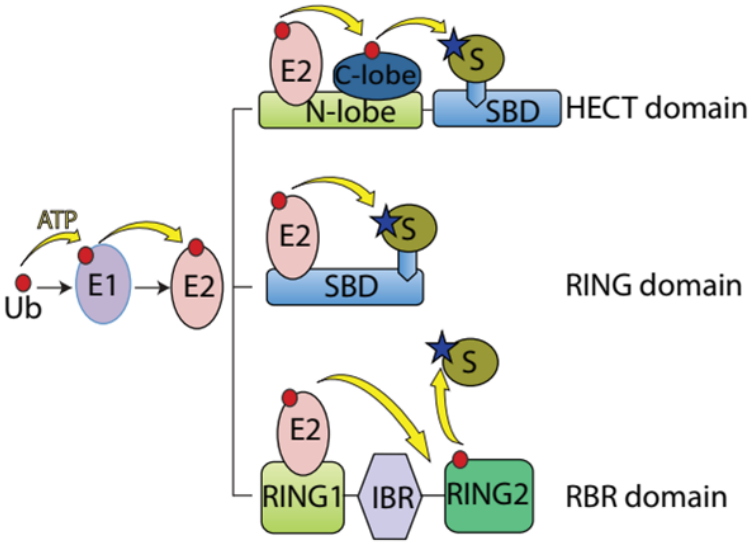


Figure 1. Schematic diagram of the catalysis process of ubiquitination.

The transfer of ubiquitin from E2 to substrate lysines by E3 ligases containing different domains. (Adapted from *Bracco, 2018*)

Ubiquitin contains 7 lysine residues (Lys6, Lys11, Lys27, Lys29, Lys33, Lys48, Lys63) that can be covalently bound to substrate lysines to form monoubiquitination or polyubiquitination (Yau and Rape, 2016). Monoubiquitination or multi-monoubiquitination refers to the addition of ubiquitin to one substrate residue or multiple substrate residues, respectively. Ubiquitination chain forms when the C-terminus of another ubiquitin is linked to the first methionine or one of the seven lysine residues of the ubiquitin coupled to substrate serine. Mono-ubiquitination affects cellular processes such as DNA repair, histone regulation, gene expression, and

endocytosis (Huang and D'Andrea, 2006). Polyubiquitination is diversified by the way of ubiquitin assembly, in which one of these seven Lys residues is linked to the former ubiquitin. To date, all forms of chain linked with these 7 lysines have been found in eukaryotic cells, especially K48 and K63-linked chains are most studied. Polyubiquitinations linked by K63 play critical roles in inflammation, polyubiquitination of K48 is highly related to protein proteasomal degradation (Ikeda and Dikic, 2008; Meierhofer et al., 2008; Peng et al., 2003). In addition, linear polyubiquitin chain linked with the N-terminal methionine (M1) of Ub by the linear Ub chain assembly complex (LUBAC), is critical for activation of the NF-κB signaling (Gerlach et al., 2011; Tokunaga et al., 2009) (Fig. 2). Taken together, these different ubiquitination forms regulate broad aspects of key cellular processes. The detailed functions of ubiquitination will be discussed as below.

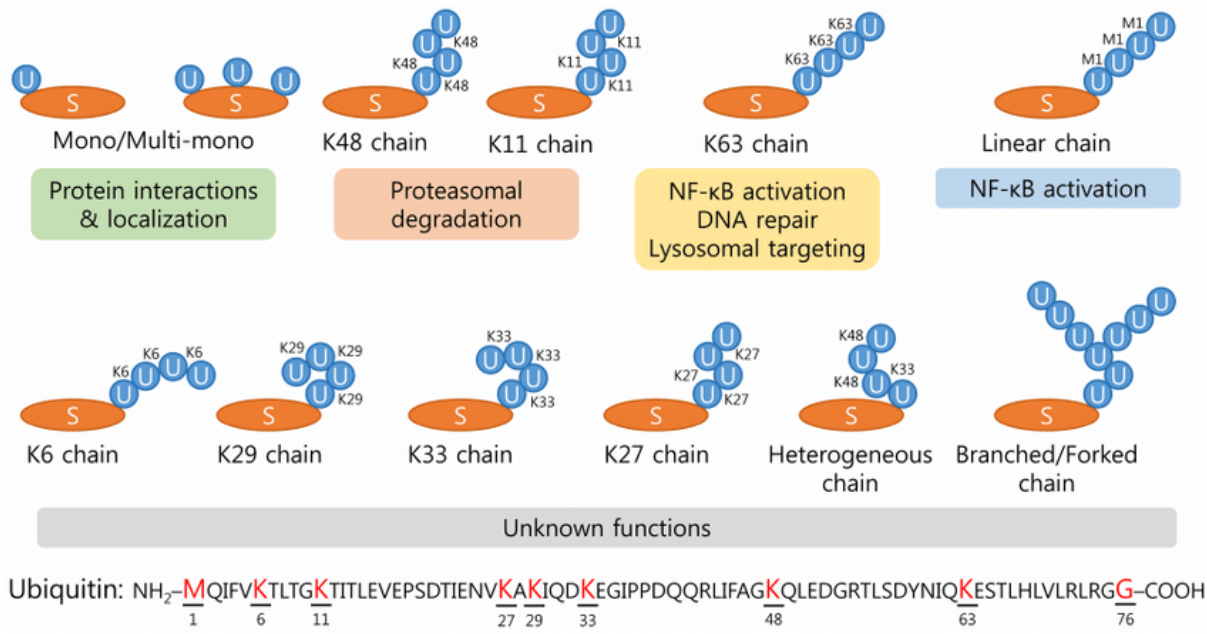


Figure 2. Functions of various types of ubiquitination.

The substrate proteins can be modified with monoubiquitin, multi-monoubiquitin, or polyubiquitin with different types of chain linkages. Different ubiquitination forms confer the proteins different fates. (Adapted from *Park et al. 2014*)

4.1.2 Ubiquitination regulates protein proteasomal degradation

Proteolysis plays important roles in protein quality control and self-renewal of cells, which contribute to maintain a healthy proteome. In eukaryotes from yeast to mammalian cells, precise complex machineries have been evolved for the regulation

of protein degradation, including two major degradative systems: the ubiquitin-proteasome system (UPS) and the autophagy-lysosome system. Many diseases including cancer and neurodegenerative disorders such as Alzheimer's disease, Parkinson's diseases, are characterized as caused by dysfunctional degradation and linked to mutations in many UPS and lysosomal genes (Rubinsztein, 2006). Pharmacological methods have been under study for the treatment of diseases by targeting ubiquitin-proteasome system and the autophagy-lysosome system (Thibaudeau and Smith, 2019). Given the importance of ubiquitin-proteasome and autophagy-lysosome mediated degradation systems, contributions to the discoveries of both of these two systems have been awarded with Nobel Prizes in 2004 and 2016. Of note, although both of these two processes require ubiquitin, they handle different tasks in different mechanisms. Mostly, membrane proteins, protein aggregates, endocytosed proteins and damaged organelles can be recognized and removed by the autophagy-lysosome system (Luzio et al., 2007). Whereas the intracellular soluble, shorted-lived, and misfolded proteins are specifically targeted by E3 ligases and degraded by the ubiquitin-proteasome system (Hershko et al., 2000).

It has been well studied that K48-linked polyubiquitinated protein is recognized and degraded to short peptides by 26S proteasome, a large ATP-dependent proteolytic complex. For example, p53 is regulated by the ubiquitin-proteasome system. p53 is a short-lived transcription factor that functions as a tumor suppressor through regulating the expression of numerous genes including P21. Even through p53 mutations have been identified in a number of human cancers, normally it is tightly regulated and maintained at a low level in cells that is hardly detected, to allow healthy cell growth and division. When cells are exposed to stresses like DNA damage, p53 will be stabilized and regulate cell growth. Clear evidence suggests that ubiquitin-proteasome system-mediated degradation plays a important role in the control of stability of p53 protein. Several E3 ligases have been found to be involved in negative regulation of p53, among these E3 ligases, RING finger ubiquitin ligase MDM2 is the key regulator that modified p53 with K48-linked ubiquitin chain and targets it for degradation, loss of Mdm2 leads to embryonic lethality in mice, demonstrating the role of MDM2 mediated p53 repression in normal development (Hock and Vousden, 2014; Kubbutat et al., 1997).

Another example for explaining the role of ubiquitination in protein degradation is cereblon (CRBN). CRBN is the binding target of the immunomodulatory drug

lenalidomide that used for the treatment of multiple myeloma (Ito et al., 2010). CRBN forms a cullin4-RING E3 ligase complex (CRL4-CRBN), with the DNA binding protein 1 (DDB1), Cullin-4A (CUL4A) and regulator of cullins1 (ROC1). In this E3 ligase complex, CRBN functions as a specific substrate receptor, thus targeting a number of substrates for ubiquitination and proteasomal degradation. A nonsense mutation of CRBN missing the C-terminal region is related to mental retardation due to dysregulation of its natural substrate during development (Higgins et al., 2004; Xu et al., 2013). On the other hand, as the target of lenalidomide, CRBN mediates the ubiquitination of IKZF1, IKZF3 and CK1α in the presence of lenalidomide as bridging compound, thus enhancing their degradation and suppressing cancer (Krönke et al., 2014). Under normal conditions, these proteins are not substrates of CRL4-CRBN, lenalidomide provides the binding interface for them to interact with CRBN, recruiting them for ubiquitination (Petzold et al., 2016). This compound-introduced high binding affinity between E3 ligase and cellular protein provides more opportunities for next-generation drug development.

To realize the goal of recruiting a target protein to E3 ligase for selective degradation, many efforts have been made on the development of proteolysis targeting chimera (PROTAC) technology. PROTAC consists of two linked molecules: one targets to a given protein meant for ubiquitination and removal by the proteasome, the other one binds to a E3 ligase. Since PROTAC itself can not be degraded, it hijacks E3 ligase and catalytically brings protein of interest for degradation for multiple rounds, thus exhibiting obvious advantage over traditional chemical inhibitors. So far several E3 ligases have been employed for PROTACs, including MDM2, CRBN, VHL, and Keap1 (Paiva and Crews, 2019).

As a digestive complex, the 26S proteasome comprises of a 20S core particle and 19S regulatory particle that recognizes ubiquitinated substrates. Due to the essential roles of proteasome in the degradation of cellular proteins, it has became a potential therapy target for many diseases. It has been proved that abundance and degradative capacity of the proteasome are critical for protein degradation rate, and that inhibition of proteasome induces cell apoptosis through stabilizing pro-apoptotic proteins. Based on this observation, several proteasome inhibitors including Ixazomib have been approved for the treatment of cancers (Moreau et al., 2016).

4.1.3 Ubiquitination is critical for autophagy

In addition to targeting proteins for proteasomal degradation, ubiquitination is also involved in autophagy-mediated degradation. Autophagy is a term for the clearance processes involving lysosomal degradation of unneeded or toxic cytoplasmic components including long-lived proteins, damaged organelles, or invading pathogens. Nutrient starvation, which regulates mTOR function, is one of the typical triggers of autophagy. Once autophagy is induced, the phagophore engulfs the cytoplasmic components that need to be degraded and forms a double membrane known as autophagosome. The autophagosome then transports the cargos to a lysosome, and the two organelles fuse. Eventually, the contents of the autophagosome are degraded by acidic lysosomal hydrolases in the lysosome.

Extensive efforts have been focused on understanding the initiation of autophagy and how autophagosome forms. As an essential post-translational modification for the regulation of numerous cellular pathways, it is not surprising that ubiquitination is also involved in the modulation of autophagy. The autophagy system contains a ubiquitin-like conjugation machinery, which is critical for autophagosome formation and substrate degradation (Grumati and Dikic, 2018; Nakatogawa et al., 2007). In yeast, over 30 autophagy-related (ATG) proteins have been identified. Among these ATG proteins, the ubiquitin-like modifier, ATG8 is activated and then modified by phosphatidylethanolamine (PE) in a ubiquitination-like way before it is docked on autophagosomal membranes. Firstly, ATG8 is cleaved by a protease ATG4, this results in a C-terminal glycine exposed ATG8 protein, which can be activated by the E1 enzyme ATG7. Activated ATG8 is then recognized by the E2 enzyme ATG3, followed by linkage to phosphatidylethanolamine by the E3 complex ATG5-ATG12-ATG16L. The lipid-modified ATG8 is then resided on autophagosomal membranes and plays important roles in autophagosome expansion and selection of autophagy substrates (Nakatogawa et al., 2007).

In addition to ubiquitination-like processes, ubiquitination modification of autophagy-related proteins is needed for the induction of autophagy. For example, when mammalian cells are suffered from starvation, mTORC1 activity is inhibited, which results in de-phosphorylation of AMBRA1 protein. The free AMBRA1 then binds to E3 ligase TRAF6 and bring TRAF6 to ULK1, which results in K63-linked polyubiquitination of ULK1. ULK1 is an important kinase complex contains Ulk1/2, the homologue of ATG1 of yeast, which is necessary for autophagosome biogenesis. Ubiquitin chain

activates ULK1, thus inducing phosphorylation of downstream targets and initiation of autophagy (Grumati and Dikic, 2018). In addition to the modification of core autophagy machinery proteins, ubiquitination is also essential to selective autophagy of substrates. Selective autophagy refers to autophagosome mediated specific degradation of protein aggregates, invading bacteria, or dysfunctional organelles without clearance of other healthy cytosolic components. In the case of ubiquitin-dependent selective autophagy, autophagy receptors that bind to ATG8 on the autophagosomal membranes recognize and bind ubiquitinated substrates through its ubiquitin-binding domain, thus recruiting ubiquitinated substrates for degradation. So far a number of receptors have been identified to be essential for substrate selective autophagy, such as p62 (also known as SQSTM1), optineurin (OPTN), NDP52 (also known as CALCOCO2) (Khaminets et al., 2016).

Cellular autophagic machinery also plays important roles in immune response including antibacterial and antiviral defense, in which ubiquitination is needed. One example for explaining the role of ubiquitination mediated autophagy in anti-infection is xenophagy. Xenophagy is a type of selective autophagy occurs in host cells for removing invading pathogens. Intracellular pathogens, such as *Mycobacterium tuberculosis*, *Salmonella*, *Legionella*, are targeted for degradation by the same cellular machinery mechanism that targets mitochondria for degradation. For example, during infection of the intracellular pathogen *Salmonella typhimurium,* bacteria replicate in a modified phagosome called *Salmonella*-containing vacuole, smaller percentage of bacteria exposed to the cytosol will be decorated with ubiquitination (Birmingham et al., 2006). The decoration of cytosolic *Salmonella* bacteria by ubiquitination can be recognized by autophagy adaptors like p62, NDP52, thus initiating the xenophagy and clearance of bacteria (Von Muhlinen et al., 2010; Zheng et al., 2009).

Mitophagy refers to the selective degradation of mitochondria by autophagy pathway. It often occurs to defective mitochondria caused by damage or cellular stress. So that mitophagy prevents the accumulation of dysfunctional mitochondria, which can lead to cellular degeneration, thus promoting the renewal of this organelle. It has been well characterized that mitophagy is regulated by PINK1 and E3 ligase Parkin. Briefly, in unhealthy mitochondria, PINK accumulates in the outer mitochondria membrane and then recruits the E3 ligase Parkin. Once Parkin is recruited, it will be phosphorylated by PINK. The phosphorylation modification activates Parkin, which results in the ubiquitination of proteins on the mitochondria surface. Ubiquitination chains decorated

on mitochondria is related to initiation of autophagy via recruiting autophagy adaptor p62 (Youle and Narendra, 2011). Taken together, ubiquitination pathway plays essential roles in the regulation of selective autophagy by affecting several stages of the autophagy process.

4.1.4 Ubiquitination plays important role in immune response

Besides the direct clearance of bacteria through xenophagy mentioned above, ubiquitination also plays essential role in innate immunity via signaling transduction. The nuclear factor κ enhancer binding protein (NF-κB) pathway is essential for the innate immune response to pathogens. NF-κB is a transcription factor comprises five Rel family proteins including RelA (also known as p65), RelB, c-Rel, NF-κB1 and NF-κB2. Normally, the NF-κB proteins are sequestered in the cytosol by inhibitor proteins of NF-κB (IκBs), and activated and translocated into the nucleus upon stimulation. Ubiquitination plays an essential role in the regulation of NF-κB pathway. Upon infection of pathogens, transcription factors NF-κB or IFN regulatory factor are activated to induce innate immune response. Stimulation of cells with inflammatory cytokines leads to activation of E3 ligase TRAF6 or recruitment of the linear ubiquitin chain assembly complex (LUBAC) followed by synthesis of polyubiquitination chains, leading to the activation of TAK1 and then activation of IKK. Activated IKK phosphorylates the inhibitor protein IκBα, resulting in K48-linked ubiquitination and then proteasomal degradation of IκBα followed by nuclear translocation of NF-κB (Chen and Chen, 2013).

In addition to the regulation of host innate immune response by NF-κB activation, ubiquitination mediated proteasomal degradation of pathogenic proteins is also a strategy for host defense. The core protein of Hepatitis C virus (HCV), is essential for the formation of HCV virion and regulation of host signaling pathways. Studies have revealed that HCV core protein is regulated by ubiquitin-dependent degradation and that N-terminal lysine residues of core protein are ubiquitinated in infected cells (Suzuki et al., 2009). Of note, ubiquitination also plays an opposing role in viral infection. There is emerging evidence suggesting that ubiquitination of viral protein is a strategy for tight regulation of viral proteins and better viral infection. A recent study showed that a RING-E3 ligase MARCH8 induce K63-linked poly-ubiquitination of HCV nonstructural 2 protein, a virus protein playing a critical role in viral assembly and maturation, in cells infected by virus (Kumar et al., 2019). Notably, instead of contributing to anti-viral

defense, the E3 ligase activity of MARCH8 is required for HCV infection (Kumar et al., 2019). Thus ubiquitination is not only involved in host anti-virus defense but also utilized by virus for effective infection, this makes ubiquitination system as potential target for anti-virus strategy.

4.1.5 Regulation of ubiquitination through deubiquitinase

Post-translational modifications, including the ubiquitination, can be reversed by enzymes harboring opposite functions. The process that cleaves ubiquitin molecules from ubiquitin-modified substrates by deubiquitinating enzymes (DUB, also known as deubiquitinase) is called deubiquitination (Wilkinson, 2000). Similar to E3 ligase, DUBs are functionally specific, only a few of substrates for each DUB, so DUBs comprise a large family of proteases. The human genome encodes more than 100 proteins predicted to be deubiquitinases according to their conserved sequences, based on which the DUBs can be further divided into different families. Of note, over 50 proteases containing conserved domain compose the largest ubiquitin-specific processing proteases (USP) DUB family. These cysteine proteases contains short conserved motif including catalytic triad residues needed for removal of ubiquitin from substrate proteins (Amerik and Hochstrasser, 2004).

DUBs function in various aspects including processing ubiquitin precursors, editing ubiquitin chains, reverse of protein ubiquitination and recycling of the free ubiquitin. All of these activities of DUB contribute to the maintenance ubiquitin pool homeostasis. For example, ubiquitin can be transcribed from different genes and translated as a single ubiquitin fused to ribosomal proteins or a linear fusion of polyubiquitin precursor proteins (Kimura and Tanaka, 2010; Redman and Rechsteiner, 1989), so that DUB are required to generate free ubiquitin molecules. In addition to generating free ubiquitin from polyubiquitin precursor, most DUBs function to hydrolyze the isopeptide bond between a Lys ε-amino group and a carboxyl group of the C-terminus of ubiquitin, which is dependent on a catalytic cysteine residue.

Since ubiquitination is involved in both proteasomal and lysosomal degradation of proteins, DUBs can remove ubiquitin molecules from modified proteins. Deubiquitination of a substrate protein targeted to proteasomal degradation is important for the regulation of protein stability (Komander et al., 2009). For example, as mentioned above, tumor suppressor protein p53 is a short-lived protein that can be ubiquitinated by Mdm2 and then degraded by the proteasome, dysfunctional p53 has

been detected in many cancer patients (Kubbutat et al., 1997). USP7 and USP10 have been identified as a p53 interacting proteins and can deubiquitinate p53 ubiquitination, then increase p53 stability and strongly inhibit cancer cell growth (Li et al., 2002; Yuan et al., 2010).

Besides its function in regulating protein stability, DUBs also remove ubiquitin or ubiquitin chains from the non-degradative signal (Elliott and Komander, 2016). For example, deubiquitination also plays critical roles in signal transduction. Ovarian tumor (OTU) family deubiquitinase OTULIN, which targets M1-linked linear-ubiquitin chain, is described to be essential for balancing immune homeostasis. Overexpression of OTULIN extensively reduced the M1-Ub chain on RIPK2 and NEMO recruitment, thus resulting in the inhibition of NF-κB activation (Fiil et al., 2013). On the contrary, OTULIN deficiency in mouse models leads to autoimmunity because of the accumulation of M1-Ub chain (Damgaard et al., 2016).

Given the significant roles of ubiquitination pathway in numerous cellular events, DUBs are important for maintaining cellular homeostasis via regulating key ubiquitination processes, can be new therapeutic targets in the future.

4.1.6 Ubiquitin-like proteins

Ubiquitin is the first small polypeptide described to be involved in covalent modification of other proteins in eukaryotic cells. After the discovery of ubiquitin, other proteins that share a similar structure with ubiquitin, like ISG15, ATG8, NEDD8, and SUMO, were also found to be conjugated to proteins to regulate activities of their targets or to lipid and play important roles in autophagy. Similar to ubiquitin, these ubiquitin-like proteins (Ubl) are also conjugated to targets through a E1-E2-E3 three enzyme-cascade.

ISG15, the first identified ubiquitin-like protein, is activated by interferon stimulation upon viral infection. ISG15 is encoded by the *ISG15* gene and expressed as a precursor protein that can be cleaved at the C-terminal region. This cleavage leads to the generation of an exposed ubiquitin-like RGG C-terminal tail that mediates the conjugation of ISG15 to target proteins. Even though ISG15 is conjugated to substrate proteins via a similar three-enzyme cascade as ubiquitination, there are not so many E3 ligases for ISG15 as ubiquitination pathway has. Moreover, single knockdown of the ISG15 E3 ligase HERC5 leads to almost completely abolishment of ISGylation, suggesting this E3 plays a predominant role in ISG15 mediated protein modification (Wong et al., 2006). ISG15 is important for the host to anti-virus as the ISG15-deficient

mice is more susceptible to Sindbis virus than wild-type mice (Lenschow et al., 2007). Studies revealed that ISG15 functions as an anti-viral factor though modifying viral proteins. NS1 protein of influenza A virus is essential for viral replication, ISG15 conjugation of NS1 catalyzed by HERC5 inhibits its nuclear translocation by blocking its interaction with the transport receptor importin α (Woods et al., 2014). In addition to conjugation to viral proteins, many host proteins have been identified as substrates of ISG15 upon IFN stimulation. For example, IRF3 is an interferon regulatory transcription factor that plays an important role in the antiviral innate immune response. It has been reported that IRF3 can be modified with ISG15, which inhibits ubiquitination and degradation of IRF3, thus resulting in stronger antiviral response (Morales and Lenschow, 2013).

NEDD8 is a small ubiquitin-like protein encoded by the *NEDD8* gene in humans. Similar to ubiquitin, NEDD8 can be conjugated to substrate proteins through its C-terminus in a ubiquitination-like manner. In the presence of NEDD8 E3 ligase, activated NEDD8 processed by NEDD activating E1 and UbcH12 E2 is conjugated to specific substrates such as Cullin family proteins, which are the well-defined major substrates of NEDD8. Neddlylation of Cullins has been characterized to activate ubiquitination functions of CRL ubiquitin E3 ligases via conformational changes. Moreover, emerging evidence suggests that NEDD8 conjugations play important roles in embryonic development and cell viability (Pan et al., 2004). These findings have led to the development of NEDD8-activating enzyme inhibitor, which specifically inhibits protein neddylation and can induce apoptosis of lymphoma cells, and now is under study for the treatment of lymphoma (Wang et al., 2015).

As mentioned above, ubiquitin-like protein ATG8 is the key regulatory factor, among all the identified autophagy-related proteins (ATG) in yeast. Other eukaryotes including humans possess different homologous proteins of ATG8. ATG8 family proteins contain not only a ubiquitin-like core, but also two N-terminal α helices. According to the sequence similarities, ATG8 family proteins can be classified into 3 different subfamilies: microtubule-associated protein 1 light chain 3 (LC3), γ-aminobutyric acid receptor-associated protein (GABARAP) and Golgi-associated ATPase enhancer of 16 kDa (GATE-16) (Shpilka et al., 2011). Upon autophagy initiation, LC3/GRABARAP family proteins target membranes via conjugation to phosphatidylethanolamine after ubiquitination-like lipidation. These ubiquitin-like proteins localize to autophagosomal

membranes and recruit different cellular proteins containing LC3 interacting regions (LIRs) for degradation (Stolz et al., 2014).

4.2 Bacteria hijack cellular pathways including ubiquitination

For efficient interactions with host cells, many intracellular pathogens have evolved specialized protein secretion systems, such as the well-elucidated type III or IV secretion systems (T3SS or T4SS), to deliver bacterial virulence proteins into the host cells. Both of these two secretion machines are composed of the protein complexes that cross the entire bacterial envelope to provide a channel for protein delivery. Extensive efforts have been made to reveal the biochemical functions of many bacterial effectors and contributed significantly to a better understanding of the biology of infection (Mattoo et al., 2007). Effectors injected into host cells can facilitate bacteria to escape from the immune response of the host cells or benefit bacterial intracellular proliferation via modulating a variety of host signaling pathways including cytoskeletal dynamics, membrane trafficking, transcription, cell cycle progression, and signal transduction (Galán, 2009; Hicks and Galán, 2013).

For example, *Salmonella*'s type III secretion system injects effectors structurally and functionally mimic G protein signaling components. Rho family of GTPases including RhoA, Rac, and Cdc42 are well-studied GTPases that play important roles in cellular events such as control of the actin cytoskeleton, cell cycle regulation, intracellular vesicular transport (Chimini and Chavrier, 2000). SopE, SopE2 and SptP injected by *Salmonella* type III secretion system mimic RhoGTPase-interacting proteins to modulate RhoGTPase signaling. SopE and SopE2 are homologous proteins that function as GEFs that can bind and then activate Cdc42 and Rac-1, thereby resulting in membrane ruffling and facilitating *Salmonella* entry into the host cell (Hardt et al., 1998). Oppositely, SptP acts as a GAP protein that counteracts SopE or SopE2 caused activation of RhoGTPase to restore cytoskeletal architecture once invasion succeeds and the pathogen is entered (Fu and Galán, 1999; Stebbins and Galán, 2000).

As mentioned above, xenophagy is a powerful strategy for the clearance of infectious pathogens and protecting the host cells. On the other hand, this explains why many bacterial pathogens have evolved weapons that target autophagy components and block the activation and maturation of autophagosomes, to benefit their intracellular survival and replication within host cells. For example, *Legionella* can interfere with autophagy by using several bacteria effectors. *Legionella* deletions screening

identified that effector RavZ is needed for autophagy inhibition. Further *in vitro* assay revealed that RavZ functions as cysteine protease and cleaves lipidated LC3 from autophagosome membranes (Choy et al., 2012). In addition to the reverse of LC3 lipidation, it is reported that another *Legionella* effector Lpg1137 is secreted into host cells and functions as a serine protease to cleave syntaxin 17 that mediates the fusion between autophagosome and lysosome, thereby resulting into inhibition of autophagy (Arasaki et al., 2017).

Another example is *Shigella flexneri*. IcsA, a *Shigella* effector that localizes on the bacterial surface, is required for bacterial adhesion to host cells during infection, thus playing a critical role in *Shigella* pathogenesis. Once the pathogen is inside the host cells, IcsA can be recognized by ATG5, thereby triggering xenophagy. This problem is solved by the other effector IcsB, which shares the motif required for binding to IcsA with ATG5. IcsB completes with ATG5 to bind IcsA and thus protects bacteria from being eliminated by host cells (Ogawa et al., 2005).

Moreover, pathogens have developed strategies interfering with host innate immune signaling via dampening NF-κB activation in different ways. For example, it was reported that *Yersinia* T3SS effector YopJ inhibits the activation of MAPK and NF-κB by acetylating upstream kinases MKK6 and IKK, thereby resulting in irreversibly blocking of their ability to phosphorylate and activate downstream proteins (Mukherjee et al., 2006; Orth et al., 2000). In addition to bacterial effectors counteracting host defense mechanisms by interfering with upstream pathways that control NF-κB activation, bacteria can also target NF-κB directly. It was shown that *Chlamydia* secretes an effector protein CT441 that directly cleaves the subunit of NF-κB p65, which significantly delays the expression of inflammatory cytokines in response to infection (Lad et al., 2007).

Not surprisingly, the significance of ubiquitination in cellular processes of eukaryotes makes it an ideal target for infectious pathogens. Increasing evidence suggests that bacterial pathogens utilize the ubiquitin pathway of host cells to facilitate bacterial infection. Although bacteria don't harbor the genes encoding ubiquitin, they have evolved effectors to hijack the host ubiquitination pathway by mimicking the activities of components of the ubiquitination pathway of host cells. Moreover, some pathogens manipulate host ubiquitination signaling via effectors with novel enzymatic activities that have not identified before in eukaryotic systems. Here we mainly summarize

regulation of ubiquitination by the bacterial effectors (Table 1, adapted from *Zhou and Zhu, 2015*).

Table 1. Bacterial effectors involved in host ubiquitination

Bacterial effectors	Pathogens	Host targets	Enzymatic activities	Biological functions	References
CHBP	*Burkholderia pseudomallei*	NEDD8, Ub	Glutamine deamidase	Cell cycle control	(Cui et al., 2010)
Cif	EPEC	NEDD8	Glutamine deamidase	Cell cycle control	(Cui et al., 2010)
OspI	*Shigella flexneri*	Ubc13	Glutamine deamidase	Inhibition the inflammatory response	(Sanada et al., 2012)
OspG	*Shigella flexneri*	Ubiquitin-conjugated E2	Half-kinase	Inhibition of NF-κB activation	(Kim et al., 2005)
SopA	*Salmonella Typhimurium*	TRIM56, TRIM65	HECT-type E3 ligase	Regulation of host inflammation	(Diao et al., 2008; Fiskin et al., 2017; Zhang et al., 2006)
NleL	EHEC/EPEC	JNKs	HECT-type E3 ligase	Promotion of EHEC-induced A/E lesions and bacterial infection	(Piscatelli et al., 2011; Sheng et al., 2017)
NleG	EPEC, EHEC	MED15, Hexokinase-2, SNAP29	U-box E3 ligase	Degradation of host proteins	(Tobe et al., 2006; Wu et al., 2010)
IpaH9.8	*Shigella flexneri*	U2AF35, NEMO	IpaH-type E3 ligase	Suppression of immune responses; NF-κB inactivation	(Ashida et al., 2010; Okuda et al., 2005)
IpaH7.8	*S. flexneri*	Glomulin	IpaH-type E3	Activation of inflammasomes	(Suzuki et al., 2018)
IpaH0722	*S. flexneri*	TRAF2	IpaH-type E3	Inhibition of NF-κB activation	(Ashida et al., 2013)
IpaH1.4	*S. flexneri*	HOIP	IpaH-type E3	Inhibition of NF-κB activation	(De Jong et al., 2016)
IpaH2.5	*S. flexneri*	HOIP	IpaH-type E3	Inhibition of NF-κB activation	(De Jong et al., 2016)
IpaH4.5	*S. flexneri*	p65, TBK1	IpaH-type E3	Inhibition of NF-κB activation	(Zheng et al., 2016)
SspH1	*Salmonella Typhimurium*	PKN1	IpaH-type E3	Inhibition of NF-κB activation	(Haraga and Miller, 2006)
SspH2	*S. Typhimurium*	Nod1, SGT1	IpaH-type E3	Promotion of Nod1-regulated inflammatory response	(Bhavsar et al., 2013)
SlrP	*S. Typhimurium*	Thioredoxin, ERdj3	IpaH-type E3	Promotion of the host cell death	(Bernal-Bayard et al., 2010)

SseL	*S. Typhimurium*	Ubiquitinated aggregates, RPS3	SENP1-like DUB	Inhibition of autophagic defence, inhibition of NF-κB signaling	(Mesquita et al., 2012; Wu et al., 2018)
ChlaDub1	*Chlamydia trachomatis*	IκBα	SENP1-like DUB	Inhibition of NF-κB activation	(Le et al., 2008; Misaghi et al., 2006)
ChlaDub2	*C. trachomatis*	Unknown	SENP1-like DUB	Unknown	(Misaghi et al., 2006)
ChlaOTU	*C. pneumonia*	Polyubiquitin chains, NDP52	OTU-like DUB	Remove ubiquitin accumulation at the bacterial entry site	(Furtado et al., 2013)

4.2.1 Bacteria hijack ubiquitin and ubiquitin-like protein

As the core machinery of the ubiquitination pathway, ubiquitin has been identified as the target of bacterial effectors. For example, Cif is a bacterial effector secreted by type III secretion system TTSS of *Enteropathogenic Escherichia coli* (EPEC), studies revealed that Cif and Cif homologue in *Burkholderia pseudomallei* (CHBP) are involved in inhibition of cell cycle process (Marchès et al., 2003). Ubiquitin protein treated with CHBP exhibited migration on the PAGE gel, compared with ubiquitin treated with CHBP mutants, mass spectrometry analyses revealed that CHBP catalyzes deamidation of Gln40 of ubiquitin. This modification does not affect Ub activation by E1 and Ub conjugation by E2. However, it inhibits ubiquitin transfer from E2 to the acceptor ubiquitin, thus blocking ubiquitin chain synthesis (Cui et al., 2010). Since ubiquitin-like proteins share a similar structure with ubiquitin, researchers also studied the effect of CHBP on these small proteins. Although Gln40 is conserved in NEDD8, SUMO, and LC3, only NEDD8 is deamidated, might because NEDD8 shares a much more similar primary sequence with ubiquitin. Actually, during bacterial infection, more NEDD8 were deamidated than ubiquitin, suggesting that Cif and CHBP harbor a preference for NEDD8. As mentioned above, NEDD8 is involved in the activation of Cullin-RING ubiquitin ligase by neddylating cullins. In cells infected by Cif catalytically defective mutant strain of EPEC, or cells expressing NEDD8 Q40E mutant, substrate proteins of CRLs E3 ligases, such as Nrf2 and p27, were markedly accumulated. Taken together, these effects on ubiquitin and ubiquitin-like protein explain how bacteria regulate cell cycle and exhibit virulence to host cells (Cui et al., 2010).

More recently, one study reported that *Chromobacterium violaceum* infection blocks the ubiquitination pathway of host cells during infection, in a manner that is dependent on the type III effector CteC. The biochemical study revealed that CteC can specifically

recognize ubiquitin and catalyze ADP-ribosylation of ubiquitin T66 by acting as an ADP-ribosyltransferase. *In vitro* reaction data suggested that CteC catalyzes ADP-ribosylation of not only mono-ubiquitin, but also ubiquitin of K48, K63 and M1-liked polyubiquitin chains without substrate preference. This modification inhibits the E1 to E2 transfer of mono-ubiquitin, thus preventing ubiquitination and synthesis of the polyubiquitin chain. In addition, polyubiquitin chains modified by CteC could not be cleaved by deubiquitinase, thus preventing ubiquitin recycle mediated by deubiquitinase. Moreover, modified polyubiquitin chains could not be recognized by protein harboring Ub-binding domains, such as TUBE and NEMO, resulting in dysfunction of ubiquitination in protein quality control and immune response (Yan et al., 2020).

4.2.2 Bacteria manipulate host E3 ligases

In addition to targeting ubiquitin, studies have found that a number of bacterial effectors mimic the activities of E3 ubiquitin ligases of the eukaryotic host cells. Among the identified bacterial effectors, NleG family effectors are secreted via the T3SS system of pathogenic *E. coli* strain (Tobe et al., 2006). Sequence analysis suggested that all NleG proteins contain a highly conserved region in the C-terminus, which has little similarity to any known eukaryotic protein. Interestingly, the NMR structure of NleG2-3, a member of the NleG family effectors, revealed that this conserved region possesses a RING-like structure, indicating that NleG family effectors may function as RING-type E3 ligase (Wu et al., 2010). *In vitro* ubiquitination assay confirmed that NleG2-3, as well as other NleG family effectors including NleG5-1, NleG6-2 and NleG9, harbors E3 ligase activity in the generation of ubiquitin chains. This provides evidence that NleG effectors mimic the RING/U-Box E3 ligase of the host. However, the substrates and detailed functions of this family of effector in host cells are still poorly understood.

Among the effectors that *Salmonella* secrets into the host via its T3SS system, there are also some enzymes targeting the ubiquitination pathway. For example, the *Salmonella* effector SopA is an E3-like enzyme. Despite the lack of similarity in the sequence with any known E3 ligase, SopA structurally resembles HECT E3 ligase with its L-shaped lopes comprise of an N-terminal putative substrates binding domain and a C-terminal HECT-like domain. Sequence comparison between the active site of SopA and other HECT-E3 ligases revealed a conserved catalytic cysteine of SopA.

Mutation of this Cys residue reduced the E3 ubiquitin ligase activity (Diao et al., 2008). SopA plays an important role in Salmonella pathogenicity, as it was shown that *sopA* deletion mutant strain induced a remarkable decrease in polymorphonuclear neutrophil transepithelial migration during infection, which has been used as a marker of *Salmonella* infection (Wood et al., 2000). A recent study of our group demonstrated that SopA recognizes and ubiquitinates TRIM56 and TRIM65, and leads them to proteasomal degradation during infection, resulting in inhibition of TRIM56/65 mediated ubiquitination (Fiskin et al., 2017).

Besides these bacterial effectors similar to eukaryotic E3 ligase, recent evidence has revealed that bacterial pathogens have evolved effectors that possess novel activities to modulate the ubiquitination pathway of host cells. IpaH effectors comprise a large novel E3 ligase family of many bacterial pathogens including *Shigella* and *Salmonella* (Rohde et al., 2007; Zhu et al., 2008). Despite the lack of similarity in sequence to any known E3 ubiquitin ligase of eukaryotes cells, this IpaH family effectors contain the essential elements needed for functioning as an E3 ligase: the E2-interacting surface residues that can hijack host E2 and receive ubiquitin; a conserved C-terminal E3 ligase domain containing catalytic cysteine residue for forming a thioester bond with ubiquitin followed by ubiquitin transfer; the hydrophobic leucine-rich repeats for protein interaction and substrate recognition. *Shigella* effector IpaH9.8 and *Salmonella* SspH1 are the first identified novel E3 ligases (Okuda et al., 2005; Rohde et al., 2007), now there are a number of IpaH effectors have been found in *Shigella* and other pathogens, revealing diverse functions of this family members. IpaH9.8 was found to interact with NEMO and a ubiquitin-binding adaptor protein ABIN-1, facilitating the polyubiquitylation of NEMO. Consequently, ubiquitination of NEMO brings it to proteasomal degradation, which impairs NF-κB activation (Ashida et al., 2010). This was confirmed by *in vivo* study showing that mice infected with *Shigella* strain expressing IpaH9.8 catalytic cysteine mutant exhibited severer inflammatory response with less bacterial replication, compared with mice infected with wild-type *Shigella* (Ashida et al., 2010).

Further studies revealed that another two *Shigella* effectors IpaH4.5 and IpaH0722 also inhibit NF-κB signaling. A Yeast two-hybrid screening identified that IpaH4.5 specifically interacts with the p65 subunit of NF-κB, resulting in ubiquitination of p65 and inhibition of the NF-κB signaling pathway. During infection, the *Shigella* mutant strain lacking IpaH4.5 caused more severe inflammatory responses with remarkable

higher induced pro-inflammatory cytokine levels and decreased bacterial replication than that in the wild type-infected mice (Wang et al., 2013). In the case of IpaH0722, it was shown that IpaH0722 blocks the inflammatory response by dominantly inhibiting the PKC-induced activation of NF-κB via catalyzing ubiquitination and proteasome-dependent degradation of TRAF2 (Ashida et al., 2013).

In addition to these *Shigella* effectors mentioned above, some *Salmonella* novel E3-like effectors including SspH1, SspH2 and SlrP, have also been identified as important for bacterial virulence during infection of animal models (Tsolis et al., 1999). For example, it was found that SspH1 localizes to the nucleus during infection and inhibits NF-κB signaling by interacting with PKN1, resulting in inhibition of inflammatory response the host with decreased proinflammatory cytokine levels (Haraga and Miller, 2006).

4.2.3 Bacteria hijack DUBs

As mentioned above, DUBs compose a large family of proteases that reverse ubiquitination by specifically hydrolyzing the isopeptide bonds between ubiquitin and lysines of substrates. Increasing studies have demonstrated that bacterial pathogens modulate cellular pathways via DUB-like effectors. For example, sequence analysis suggested that *Salmonella Typhimurium* effector SseL is similar to SENP1 cysteine protease. *In vitro* assay confirmed that purified SseL cleaves both K48-linked and K63-linked ubiquitin chains, with a preference for K63-linked ubiquitin chains. Cells infected with *Salmonella* that lacks SseL exhibited accumulated ubiquitinated proteins, confirming SseL ubiquitinates host proteins during bacterial infection (Rytkönen et al., 2007). Moreover, in the absence of SseL activity, these ubiquitinated substrates around SCV were recognized by p62, an autophagy receptor that recruits LC3 and targets them for autophagic degradation, thereby resulting in facilitation of intracellular bacterial replication. This indicates that SseL is involved in protecting bacteria from autophagic clearance. In addition to the resistance of anti-infection autophagy, SseL is also reported to be involved in the inhibition of NF-κB activation by deubiquitinating ribosomal protein S3 (RPS3), which is a important host transcriptional co-factor. Deubiquitination inhibits RPS3 nuclear translocation and subsequently innate immune response to bacterial infection (Wu et al., 2018).

In addition to this *Salmonella* DUB, there are two SENP1-like effectors ChlaDub1 and ChlaDub2 from *Chlamydia trachomatis*, and an otubain (OTU)-like effector ChlaOTU from *Chlamydia pneumonia* have been identified (Furtado et al., 2013; Misaghi et al.,

2006). ChlaDub1 and ChlaDub2 are the first enzymes that harbor both deubiquitinating and deneddylating activities (Misaghi et al., 2006). During infection, once inside the host cells, ChlaDub1 inhibits NF-κB activation by binding the NF-κB inhibitory subunit IκBα, interfering with its ubiquitination and proteasomal degradation (Le Negrate et al., 2008). *Chlamydia pneumonia* OTU-like effector ChlaOTU was demonstrated to be able to cleave K48-linked and K63-linked ubiquitin chains *in vitro*. During infection, injected ChlaOTU binds to ubiquitin and NDP52 to eliminate ubiquitin at the invasion sites and protects bacteria from autophagic clearance (Furtado et al., 2013). More studies are needed to identify other DUBs from the infectious pathogens to increase our understanding of bacterial pathogenesis.

4.3 Legionella regulates host ubiquitination pathway

4.3.1 Legionella pneumonia

Legionella pneumonia is a Gram-negative bacterial pathogen that causes severe Legionnaires' disease, a form of acute pneumonia in humans. It was first isolated and described after a lethal pneumonia outbreak among the participants of an American legion convention in 1976, which caused 34 deaths (Rowbotham, 1980). Since then *Legionella* has became a serious threat to public health. So far 65 *Legionella* species have been identified (Gomez-Valero et al., 2019), among which serogroup 1 of *Legionella pneumophila* is the primary agent that causes Legionnaires' disease. At present, antibiotics including macrolides and fluoroquinolones are most widely used for the treatment of Legionnaires' disease (Diederen, 2008). However, cases of antibiotic-resistant *Legionella*-caused pneumonia have been reported (Jia et al., 2019), so further studies on the pathogenesis of *Legionella* is needed for better treatment of the Legionnaires' disease.

Legionella is quite ubiquitous in many environments, especially freshwater and soil. It resides in biofilm and proliferates in the natural host amoeba, which phagocytizes bacteria as food source. Using the effectors secreted by the T4SS system, *Legionella* can establish a niche and survive inside (Fig. 3).

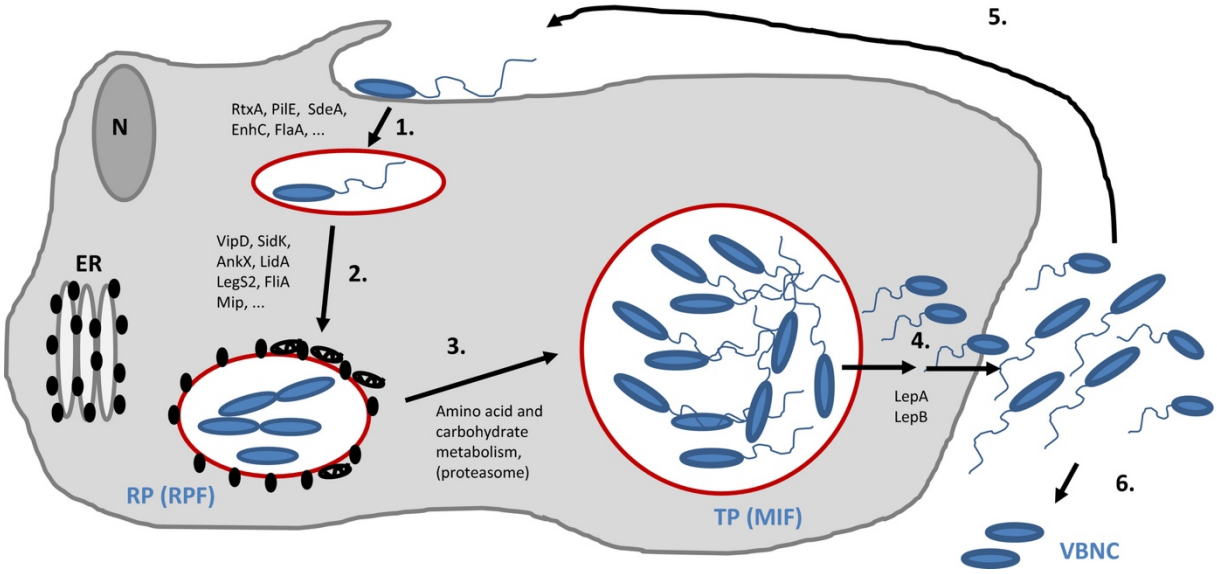


Figure 3. Intracellular life-cycle of *Legionella pneumophila.*

1. *Legionella* with high motility attaches to the natural host amoeba and is taken up by the host cell through phagocytosis, which results in the phagosome containing bacteria. 2. *Legionella* secrets effector proteins through the T4SS system and establishes the *Legionella* containing vacuole (LCV) by recruiting ER membranes and transfer into replicative phase form (RPF). 3. The LCV expands and the pathogen replicates inside. Bacteria grow into the flagellated transmissive form (MIF) when the nutrients become limited. 4-6. Host cell breaks and the bacteria released out of the host cell can infect other new cells, some develop into viable-but-not culturable (VBNC) form in which the pathogen can stay for a long time in the environment (Adapted from *Eisenreich and Heuner, 2016*).

In humans, the mechanism of *Legionella pneumophila* infection is similar to that in amoeba. When aerosol containing *Legionella* is inhaled by humans, *Legionella* invades alveolar macrophages and replicates within the host cells, and can eventually cause the lung disease (Horwitz and Silverstein, 1980). Therefore the development of water-cooled air conditioning system introduces higher possibility of *Legionella* infection in people. Once inside the host cell, the phagosome containing bacteria undergoes a phosphoinositide decoration conversion from PI(3)P to PI(4)P, and recruits membranes from ER and other organelles such as mitochondria and ribosomes (Weber et al., 2018). *Legionella* quickly establishes a specialized compartment called *Legionella*-containing vacuole (LCV), which has the features of ER (Xu and Luo, 2013). Formation and maturation of the LCV prevent the invading bacteria from fusion with the lysosome and allow it to proliferate within the host cell, thereby are crucial for *Legionella* pathogenesis (Isberg et al., 2009).

4.3.2 Legionella hijacks cellular pathways of the host

Given the essential role of the Dot/Icm T4SS secretion system in the formation of *Legionella* containing vacuole and bacterial replication inside human macrophages, studies have been conducted to identify substrates of the T4SS system (Burstein et al., 2009; Zhu et al., 2011), these efforts have led to identification of more than 300 *Legionella* effectors and revealed the largest identified arsenal of effectors, compared with other bacterial pathogens (Ensminger, 2016). These effectors facilitate *Legionella* infection and growth through many-sided manipulations of the host cells. However the traditional genetic approach to study the function of a *Legionella* effector though deleting the corresponding gene is not effective, due to the high functional redundancy of the effectors. So far only one effector IroT (also known as DimB or MavN) has been found to be essential for bacteria intracellular growth in host cells (O'Connor et al., 2011). Although the functions of most of these effectors are still unknown, considerable efforts have been made and revealed that various approaches are utilized by *Legionella* to regulate host cells to its advantage, which has improved significantly our understanding of *Legionella* infection. Like other pathogenic bacteria have been described above, *Legionella* also manipulates host cellular pathways with diverse mechanisms via its effectors (Hubber and Roy, 2010; Qiu and Luo, 2017a).

4.3.2.1 Legionella escapes from the host endo-lysosomal pathway

Upon reaching to the host cell plasma membrane, *Legionella* can be taken up immediately by macrophages through phagocytosis, resulting in the *Legionella* residing in a phagosome where the bacteria replicates. Eukaryotic cells possess an organized endosomal system for membrane trafficking and cargo sorting. This system also leads pathogens to lysosome for degradation (Gruenberg and Van Der Goot, 2006). To avoid being captured by the endo-lysosomal pathway and clearance, *Legionella* converts the phagosome and eventually establishes a safe compartment called *Legionella*-containing vacuole, which is dependent on the effectors. LegC7/YlfA has been identified as involved in the prevention of the LCV from the endo-lysosomal degradation through disrupting the endosomal systems. Expression of LegC7/YlfA in yeast results in apparent vesicular accumulation and eventually cell death. Deletion of a gene *VSP27*, which is one of the genes for the endosome sorting complex, reduces the toxicity of LegC7 (O'Brien et al., 2015). Another effector VipD was reported to act as a phospholipase and change the lipid decoration on the endosomal membrane

(Gaspar and Machner, 2014). It is well-known that the phospholipid PI(3)P is necessary for early endosomal antigen EEA1 and other fusion factors mediated membrane fusion between early endosomes (Mills et al., 2001). VipD possesses an N-terminal phospholipase, once secreted into the host cells, VipD localizes to endosome. Binding to Rab5 and Rab22 through the C-terminal domain activates the phospholipase activity of VipD, which leads to the PI(3)P elimination from endosomal membranes, thereby inhibiting the fusion of endosome with the early LCV (Gaspar and Machner, 2014). Of note, to avoid the risk of getting trapped in the endosomal pathway, *Legionella* effectors not only modulate the endosomal vesicles, but also function on the LCV. Lipid decoration of LCV membrane also affects protein recruitment and membrane fusion with endosome. It has been identified that, once the bacteria enters host cells, phosphatidylinositol phosphates (PIPs) of LCV is rapidly converted from PI(3,4,5)P_3 to PI(3)P, which is then removed and replaced with PI(4)P as the main PIP species (Weber and Hilbi, 2014). So far 2 effectors have been reported to be related to LCV PIP subversion. Effector SidP is a phosphatidylinositol polyphosphate 3-phosphatase that recognizes PI(3)P and PI(3,5)P_2, and specifically hydrolyses them *in vitro* (Toulabi et al., 2013). In addition to SidP, SidF is the other identified phosphatase and specifically targets PI(3,4)P_2 and PI(3,4,5)P_3 *in vitro*. SidF facilitates the recruitment of proteins to the LCV, less SidC, an effector containing PI(4)P binding domain, was detected on the LCV without SidF (Hsu et al., 2012). Thus manipulation of the PIP decoration enables the LCV to escape from the endo-lysosome pathway, on the other hand, it also allows the LCV to recruit host proteins for the maturation of the LCV and facilitates the consequent intracellular growth.

4.3.2.2 Legionella escapes from the host autophagy pathway

In addition to being captured by the endo-lysosome pathway, invading pathogens can be trapped in autophagy system and get removed in host cells. Effector protein RavZ is indispensable for inhibiting autophagy during *Legionella* infection (Choy et al., 2012). RavZ acts a cysteine protease and directly cleaves ATG8 proteins from membranes. Moreover, it hydrolyzes the bond between C-terminal glycine conjugated to the phosphatidylethanolamine on autophagosome membranes and the adjacent tyrosine residue of ATG8 proteins, thus inhibiting autophagy by irreversibly blocking ATG8 proteins (Choy et al., 2012). Besides RavZ, there are other effectors have been found to be involved in the anti-autophagy response. It was showed that effector Lpg1137 functions as a serine protease that targets and cleaves STX17 (syntaxin 17).

Degradation of STX17 caused by Lpg 1137 leads to the inhibition of autophagy through blocking the fusion of autophagosome with lysosome (Arasaki et al., 2017). Moreover, it was reported that *Legionella* strain lacking SdhA (*ΔsdhA*) showed defective intracellular growth (Laguna et al., 2006). It was revealed that *ΔsdhA* bacteria were more colocalized with LC3 during infection, compared to the wild-type *Legionella* strain, suggesting that SdhA may also contribute to the prevention of LCV from autophagy degradation (Creasey and Isberg, 2012). Further studies are necessary to identify the targets of SdhA for better understanding of its functions.

Interestingly, unlike the effectors that function to block the interaction of the LCV with autophagic degradation system, one effector protein LegA9 was shown to facilitate the recognition of the LCV by autophagy (Khweek et al., 2013). Colony forming units (CFUs) assay revealed that intracellular replication of *Legionella legA9* mutant in mice bone marrow derived macrophages was remarkably facilitated compared to the wild-type strain. The LCV of *legA9* mutant acquired less ubiquitin labeling and p62 recruitment, therefore resulting in the inhibition of the targeting by autophagy (Khweek et al., 2013). It will be interesting to study function of this effector in different hosts in future.

4.3.2.3 Legionella hijacks organelles for establishing the LCV

Once the bacteria is taken up by host cell, the LCV is then modified to establish a safe compartment for efficient infection and bacterial replication. In the process of maturation, LCV recruits ER-derived vesicles before the fusion with ER (Derré and Isberg, 2004). Effector SidC was reported to be essential for the recruitment of ER protein calnexin to the LCV. SidC localizes to the LCV through binding to PI(4)P via its C-terminal PI(4)P binding domain. *Legionella* lacking SidC and its homologue protein SdcA is defective for recruitment of GFP-tagged calnexin to LCV, suggesting SidC plays an important role in ER-derived vesicles acquisition by LCV (Ragaz et al., 2008). In addition to the initial recruitment of ER to LCV, in *Dictyostelium discoideum* amoebae, the LCV moves quickly along the microtubules and is eventually transported to the ER, where the LCV expands and matures into a ER-associated compartment (Lu and Clarke, 2005). It was reported that this movement of LCV is dependent on the effector LegG1. Secreted LegG1 localizes to the LCV, where it activates the small GTPase Ran through its GEF domain. LegG1 stabilizes microtubules in cells infected with *Legionella* and promotes LCV motility in host. *Legionella* strain lacking *legG1* exhibited compromised intracellular replication, compared to the wild-type strain

(Rothmeier et al., 2013). When the LCV reaches to ER, it attaches and fuses with ER to get more membrane material required for the maturation of the LCV, in a manner dependent on atlastin3 (Steiner et al., 2017). Atlastin3 was identified from the LCVs purified from the infected cells. Atlastin3 is not necessary for the earlier ER recruitment to LCV, but facilitates the ER remodeling and fusion with LCV, which promotes to the LCV maturation and therefore the intracellular bacterial replication (Steiner et al., 2017). More studies will be needed to identify the effectors involved in the fusion of LCV with ER.

4.3.2.4 Legionella effectors regulate host proteome

Legionella can also manipulate host cellular pathways by directly regulating the mRNA transcription and protein translation of host cells. Effector RomA/LegAS4 was found to localize to cell nucleus and methylate K14 of histone H3 through its methyltransferase activity during *Legionella* infection, which decreases histone H3 acetylation and gene expression of host cells, including the genes related to the innate immune response (Rolando et al., 2013). In line with the role of RomA in regulation of host gene expression, RomA is needed for efficient intracellular replication of *Legionella* during infection. Bacterial growth of *Legionella* strain lacking RomA was remarkably decreased compared with the wild-type strain (Rolando et al., 2013).

In addition to the regulation of gene expression by DNA methylation, it has been reported that *Legionella* effectors induce transcription of anti-apoptotic genes by activating NF-κB. In a *Legionella* effectors screening study, LegK1 was identified as a a enzyme harboring NF-κB activating activity (Ge et al., 2009). LegK1 mimics the host IKK and phosphorylates the IκB family inhibitors, therefore resulting in the activation of NF-κB pathway. Deletion of LegK1 in *Legionella* does not introduce remarkable effect to intracellular bacterial growth, which is similar to other known effectors. However, cells infected with *Legionella* lacking LegK1 are more sensitive to staurosporine for apoptosis induction (Ge et al., 2009). This suggested that LegK1 is involved in the prevention of apoptosis in host cells, probably through regulating the gene expression. These findings help us to better understand how bacteria responds to the host anti-infection defense. It will be interesting to identify other effectors that manipulate host transcriptome and proteome.

4.3.3 Legionella hijacks ubiquitination pathway of the host

Among the *Legionella* effectors with reported functions, a number of the proteins have been revealed to hijack components of the host ubiquitination system, including hijacking E2, mimicking DUBs and E3 ligases. Here we summarize the effectors involved in the regulation of host ubiquitination pathway (Table 2, adapted from Qiu and Luo, 2017).

Table 2. Legionella effectors targeting host ubiquitination pathway

Gene ID	Effector	Target	Enzymatic activity	Function	References
Lpg0171	legU1	BAT3	F-Box containing protein, form an E3 complex with SKP1 and Cullin1	Unknown	(Ensminger and Isberg, 2010)
Lpg1408	licA	SKP1	F-Box containing protein, form an E3 complex with SKP1 and Cullin1	Unknown	(Ensminger and Isberg, 2010)
Lpg2144	ankB	Parvin B	F-Box containing protein, form an E3 complex with SKP1 and Cullin1	Bacterial intracellular replication; Recruitment of polyubiquitinated species to LCV	(Ensminger and Isberg, 2010; Price et al., 2009)
Lpg2224	PpgA	Unknown	F-Box protein containing protein, E3 ubiquitin ligase	Unknown	(Ensminger and Isberg, 2010)
Lpg2455	GobX	Unknown	U-Box E3 ubiquitin ligase	Unknown	(Lin et al., 2015)
Lpg2830	LubX	Clk1, SidH	U-Box protein, E4	Inavtivation of Clk1; Degradation of effector SidH	(Kubori et al., 2008)
Lpg1111	RavN	Unknown	U-Box E3 ubiquitin ligase	Unknown	(Lin et al., 2018)
Lpg2577	MavM	Unknown	RING E3 ligase	Unknown	(Lin et al., 2018)
Lpg2498	MavJ	Unknown	HECT E3 ligase	Unknown	(Lin et al., 2018)
Lpg2510	SdcA	Unknown	E3 ubiquitin ligase	Recruitment of ER vesicles and polyubiquitinated species to LCV	(Hsu et al., 2014)
Lpg2452	SdcB	Unknown	E3 ubiquitin ligase	Recruitment of ER vesicles and polyubiquitinated species to LCV	(Lin et al., 2018)
Lpg2511	SidC	Unknown	E3 ubiquitin ligase	Recruitment of ER vesicles and polyubiquitinated species to LCV	(Hsu et al., 2014)

Lpg2153	SdeC	Rab33b, Rtn4	Novel ubiquitin ligase	Bacterial intracellular replication; ER recruitment to LCV	(Kotewicz et al., 2017; Qiu et al., 2016)
Lpg2156	SdeB	Rab33b, Rtn4	Novel ubiquitin ligase	Bacterial intracellular replication; ER recruitment to LCV	(Kotewicz et al., 2017; Qiu et al., 2016)
Lpg2157	SdeA	Rab33b, Rtn4	Novel ubiquitin ligase	Bacterial intracellular replication; ER recruitment to LCV	(Kotewicz et al., 2017; Qiu et al., 2016)
Lpg1621	Ceg23	K63-linked ubiquitination chain	Deubiquitinase	Regulation of Lys-63-linked ubiquitin signaling on the LCV	(Ma et al., 2020)
Lpg2248	LotA	Unknown	Deubiquitinase	Regulation of ubiquitin signaling on the LCV	(Kubori et al., 2018)
Lpg2149	MavC	Ubiquitin, UBE2N	Transglutaminase	Ubiquitination UBE2N; Inhibition of NF-κB signaling	(Gan et al., 2019a)(Mu et al., 2020)
Lpg2148	MvcA	ubiquitinated UBE2N	Deamidase	Deubiquitination of UBE2N; temporal regulation of the activity of UBE2N	(Gan et al., 2020)

4.3.3.1 Legionella effectors manipulate host E2

As shown in the Table 2, a number of the identified *Legionella* effectors have been revealed to hijack components of the host ubiquitination system, including hijacking E2, mimicking DUBs and E3 ligases. Studies have revealed that E2 enzymes play important roles in the formation of specific ubiquitin chains (Stewart et al., 2016). For example, UBE2N is a main E2 enzyme that mediates the synthesis of K63-linked ubiquitin chains, which is important for cellular signaling transduction including NF-κB activation (Hodge et al., 2016). Recently, a study showed that UBE2N is conjugated to ubiquitin at Gln40 during infection, which is catalyzed by *Legionella* effector, a transglutaminase, MavC. Ubiquitination of UBE2N caused by MavC abolishes its E2 activity, thereby dampening NF-κB signaling and inflammatory response of the host against the pathogen (Gan et al., 2019). Moreover, MavC activity is temporally regulated during infection, another effector Lpg2149 encoded by an adjacent gene counteracts its effects by binding to MavC and inhibiting its activity (Valleau et al., 2018).

4.3.3.2 Legionella DUBs

Like other bacterial pathogens mentioned above, *Legionella* also secrets DUBs to host cells and modulate host signaling pathways. For example, crystal structure

demonstrated that *Legionella* effector protein LupA (Lpg1148) possesses a typical ubiquitin protease domain, which was confirmed by the deubiquitinase activity of LupA *in vitro*. In addition, LupA only recognizes ubiquitin, but not other ubiquitin-like proteins such as NEDD8 or SUMO, indicating that LupA probably functions in cells specifically (Urbanus et al., 2016). LupA rescues the toxicity of *Legionella* effector LegC3 expression to yeast. When HEK293T cells are co-transfected with LegC3 and active LupA, polyubiquitination of LegC3 is diminished (Urbanus et al., 2016). As another example, a recent study has revealed that Ceg23 harbors a catalytic motif resembling that of OUT family DUBs, despite the amino acid sequences have very little similarity (Ma et al., 2020). *In vitro* assay demonstrated that Ceg23 specifically cleaves K63-linked ubiquitination. In addition, it was shown that Ceg23 down-regulates the K63-linked ubiquitination on the LCV (Ma et al., 2020). Further studies remain to be done to address how this DUB affects *Legionella* infection and intracellular proliferation.

4.3.3.3 Legionella effectors mimic host E3 ligases

To date, the majority of ubiquitin E3 ligases identified in eukaryotic cells are RING-type E3 ligases that contain a conserved RING domain. E3 ligases containing RING domain can function as a single-subunit protein to bind to E2 and substrates and directly catalyze the ubiquitin transfer to substrates. U-box domain is highly similar to RING domain at the level of structure, and U-box E3 ligases share a similar ubiquitin transfer mechanism with RING-type E3 ligases, therefore, E3 ligases containing U-box domain are classified as RING-E3 ligases. Moreover, RING-type domains can also function as subunit of Cullin-RING E3 ligase complexes (CRLs), among which SCF (Skp1-Cul1-F-box protein) family is the most studied one. SCF ligase contains the RING-domain protein Rbx1 for E2 binding, scaffold protein Cullin 1, and Skp1 that binds an F-box domain, which is able to specifically recognize substrates via its leucine-rich repeat motif (Hatakeyama et al., 2001). To date, most of the identified ubiquitination involving pathogenic effectors are E3 ligase-like proteins (Maculins et al., 2016). In the case of *Legionella* carrying the largest effector armory, it is not surprising that there are a number of proteins have been identified as E3 ligase like enzymes.

To date, all sequenced *Legionella* strains possess the genes encoding putative F-box domain-containing proteins. Studies focusing on screening for Legionella proteins that possess typical features of eukaryotic proteins have identified a number of F-box domain containing proteins including LegU1, LicA, AnkB, PpgA and two U-box domain-containing proteins LubX and GobX (Hubber et al., 2013). For example, AnkB is

a *Legionella* effector essential for intracellular proliferation within both macrophages and protozoan hosts. This effector harbors an N-terminal F-box interacting with host Skp1 and a C-terminal domain for recognizing substrates, thus functions to the assembly of an SCF-E3 ligase (Price et al., 2009). Immunostaining assay demonstrated that AnkB plays an essential role in the acquisition of polyubiquitinated proteins by the LCV. In addition, the expression of AnkB F-box domain mutant results in bacterial intracellular proliferation defect during infection (Price et al., 2009). Furthermore, it has been revealed that AnkB causes K48-linked polyubiquitination and followed degradation of proteins on LCV, which provides amino acids required for bacterial intracellular proliferation (Price et al., 2011).

Another example of *Legionella* effector containing a functional F-box domain is LegU1, which forms a SCF E3 ligase complex through interacting with Skp1, Cullin1. A study reveals that exogenously expressed LegU1 in cells specifically recognizes and ubiquitinates the host chaperone protein BAT3 (Ensminger A.W. and Isberg R.R., 2010). However, no obvious growth defect for *Legionella* LegU1 mutant is observed during infection, suggesting that other redundant effectors might alternatively function to modulate BAT3 activity (Ensminger A.W. and Isberg R.R., 2010). More studies remain to be done to decipher the effect of this F-box-containing E3 ligase in bacterial infection.

Moreover, using catalytically dead mutant as bait, Kubori *et al*. found that LubX has strong interaction with host Clk1 (Cdc2-like kinase 1). Either *in vitro* ubiquitination assay or *in vivo* experiments confirmed that Clk1 is a substrate of LubX. Further biochemical assays suggested that U-box 1 of LubX functions in interacting with E2 whereas U-box 2 plays an unconventional role and serves as the Clk1 binding site (Kubori et al., 2008). In addition to manipulating host cellular events by directly hijacking the host machineries as many other effector proteins, LubX has been found to recognize and ubiquitinate secreted effector. SidH, the protein product of a gene proximal to the gene encoding LubX, is secreted into the host cell cytosol during *Legionella* infection, and recognized by LubX, resulting in the proteasomal degradation of SidH (Kubori et al., 2010). SidH is likely needed only at the early stage of infection, as the protein level in the bacteria is high when infection is initiated, until the first hours upon infection, then intracellular SidH is ubiquitinated and decreased over time. On the contrary, protein level of injected LubX increases gradually upon host cell infection. U-box-type E3 ligase LubX contains two U-box domains, one of which serves as an E2

binding site, the other one recognizes substrate SidH. Therefore, studies demonstrated that E3 ligase LubX temporally regulates the stability of SidH to shutdown unnecessary *Legionella* effector protein at the later stage of infection (Kubori et al., 2010).

Another example of *Legionella* effector that contains the U-box domain is GobX. Although GobX does not present high sequence similarity to any known protein, the central region has been predicted to be a U-box-like domain due to the conserved hydrophobic residues involved in E2 binding. *In vitro* ubiquitination assay demonstrated that GobX has E3 ubiquitin ligase activity when incubated with the E2 ubiquitin-conjugating enzymes UbcH5a, 5b, 5c, or UbcH6v (Lin et al., 2015). Expression of GFP-GobX extensively localizes to the Golgi via *S*-palmitoylation at Cys175, however, it does not significantly impact Golgi function. Furthermore, expression of GobX in *S. cerevisiae* does not exhibit any toxicity in yeast (Lin et al., 2015). Future study on the identification of the host substrates of GobX is needed for understanding how ubiquitination activity of GobX benefit *Legionella* infection and bacterial proliferation.

Once inside the host cell, surface phosphoinositide lipid decoration of the LCV where *Legionella* resides gradually converts to PI(4)P from PI(3)P, which is beneficial to recruit proteins to LCV and avoid lysosomal clearance of bacteria. SidC and its paralogue SdcA are *Legionella* effectors that harbor a PI(4)P binding domain at the C-terminal regions, which ensures to anchor them to LCV during infection (Ragaz et al., 2008). This makes SidC an excellent marker of LCV in many studies using the antibody against SidC. *Legionella* mutant strain lacking SidC/SdcA recruits less ER and ER-derived vesicles for building a replication-permissive vacuole in host cells, compared with wild type *Legionella* (Ragaz et al., 2008). Despite that studies of the structure did not find SidC/SdcA have structural homology to any known protein, sequence analysis revealed that Cys46, His444, and Asp446 on the surface of SidC form a potential canonical catalytic triad. Further study demonstrated that SidC and SdcA exhibit E3 ligase activity and catalyze the polyubiquitination chain recruitment to LCV (Hsu et al., 2014). Recently, a genome-wide CRISPR-Cas9 screen has been performed to systematically identify host factors hijacked by *Legionella*. This screen revealed that the host Rab10 is required for ER recruitment to LCV and bacterial proliferation. Furthermore, it was shown that Rab10 is recruited to LCV and modified with ubiquitin by SidC (Jeng et al., 2019). More studies remain to be done to identify other substrates

modified by SidC and SdcA, which will definitely address how these E3 ligases benefit *Legionella* infection and intracellular bacterial replication.

4.3.4 Legionella SidE family effectors mediate a novel phosphoribosyl-linked serine ubiquitination

Extensive efforts have been made to identify other substrates of the T4SS system of *Legionella*, one study revealed that SidE family proteins are secreted through binding to IcmS of Dot/Icm system by incubating IcmS fused to Glutathione S-transferase (GST) with *Legionella* lysate (Bardill et al., 2005). For *Legionella*, the absence of an effector usually does not lead to significant intracellular growth defect, because of the extensive functional redundancy among the Dot/Icm substrates. One case in point is this SidE effector family that consists of 4 proteins: SidE, SdeC, SdeB, and SdeA, one of which shares sequence homology with others. Notably, genes encoding SdeC, SdeB and SdeA are structured in the same locus with the gene encoding another *Legionella* effector SidJ, whereas gene encoding SidE locates separately (Bardill et al., 2005). Expression of SidE effector family increases with bacterial growth and reaches to the peak in early stationary phase, which is similar to other known substrates that secreted by T4SS system early in the formation of the LCV, indicating that effectors of SidE family effectors might play important roles during infection. Indeed, SidE family deletion *Legionella* strain exhibited approximately 10-fold less growth than wild type strain inside protozoan *Acanthamoeba castellanii,* the natural host of *Legionella*. Moreover, complement of SdeA alone rescues bacteria from growth defect. This suggests that SidE family effectors are required for bacterial full virulence of *Legionella* (Bardill et al., 2005). Moreover, an assay using yeast genetic system revealed that expression of SdeA is very toxic to yeast, of note, this toxicity can be suppressed by low amount expression of SidJ (Jeong et al., 2015). Even though SidE family effectors play a critical role in *Legionella* pathogenesis, their function had previously been unknown. In 2016, a study led by Qiu reported that SdeA possesses a mono-ADP-ribosyltransferase (mART) motif: R766-S820-E860S861E862, according to the sequence alignment with known proteins. Mutations of glutamic acids inside the catalytic motif to alanines completely erased the toxicity of SdeA to yeast. Importantly, the mART domain is important for the function of SdeA in intracellular replication of *Legionella* during infection. The SidE family deletion *Legionella* mutant (*ΔsidEs*) showed attenuated virulence against the host *Dictyostelium discoideum*, replenishment of wild-type SdeA in a *ΔsidEs* strain almost completely restored its ability to grow within the host, whereas

supplement of SdeA EE/AA mutant did not rescue bacteria. Given the ADP-ribosyltransferase domain in SdeA, researchers investigated the effect of SdeA on mammalian cell proteins, however, no ADP-ribosylation activity was detected. Further biochemical and mass spectra studies revealed that SdeA catalyzes the ADP-ribosylation of ubiquitin on Arg42, then eventually ubiquitinates Rab small GTPases including Rab1 and Rab33b utilizing NAD^+, without the need of E1 and E2 (Qiu et al., 2016).

Taken together, unlike the conventional ubiquitination involving a three-enzyme cascade mediated formation of isopeptide bonds between the ubiquitin C-terminus and lysines in substrate proteins by consuming ATP, SdeA-mediated ADP-ribosylation of ubiquitin and consequent substrate ubiquitination expands our knowledge about this post-translational modification and function regulation by ubiquitin molecule.

5 Objectives

As described above, The finding of this all in one ubiquitin E3 ligase from bacteria constitutes a novel ubiquitination entirely distinct from the canonical enzymatic cascade characterized in eukaryotic cells. However the detailed mechanism of SdeA-mediated ubiquitination has been poorly understood. The regulation of SdeA E3 ligase activity by other effectors needs to be elucidated. Moreover, identification of other potential substrates of SdeA is necessary for explaining the toxicity of SdeA expression in eukaryotic cells and subsequent biological functions of SdeA-catalyzed ubiquitination in host cells.

In this thesis, the main objectives have been prosed:

I: To study the effect of phosphoribosylation of ubiquitin caused by SdeA mART domain on ubiquitin activity and conventional cellular ubiquitination pathways and the mechanism of phosphoribosyl-linked serine ubiquitination.

II: To investigate the mechanism of the regulation of SdeA-catalyzed phosphoribosyl-linked serine ubiquitination by other *Legionella* effectors.

III: To identify the substrates of SdeA and dissect the biological functions of the SdeA-catalyzed ubiquitination in host cells.

6 Materials and Methods

6.1 Antibodies and reagents

All reagents were from Sigma, Roche or Roth. The following antibodies were used: antibodies against HA (C29F4), GFP (sc-9996), GRASP65 (sc-374423) from Santa Cruz; ubiquitin (P4BD) and ubiquitin (ab7254) from Cell signaling and Abcam respectively; antibodies against mCherry (ab125096), Tubulin (ab6046), Calnexin (ab22595), Legionella (ab20943) from Abcam; antibodies against GM130 (D6B1), GAPDH (D16H11) GST (91G1) from Cell signaling; GM130 (610823) from BD for IF; GRASP55 (10598-1-AP) from Proteintech, TGN46 (AHP500) from Biorad. His_6 (31358000) from Roche; Anti-HA-Agarose mouse antibody (HA-7) from Sigma. Anti-Myc-Agarose antibody (9E10) from Santa Cruz.

6.2 Cloning and mutagenesis

For protein expression in mammalian cells, GFP or mCherry tagged DupA, wild type EGFP-SdeA and catalytically defective mutants SdeA H277A and SdeA EEAA were generated as described previously (Bhogaraju et al., 2016; Shin et al., 2020). SdeA plasmids were digested with BamHI and XhoI, then inserted into mCherry-C1 vectors digested with BamHI and XhoI to generate N terminally mCherry tagged wild type and mutated SdeA. Truncated deletions SdeA 1-907, 1-1096, 1-1233 and 1234-C were amplified from the full-length SdeA cDNA and digested with BamHI and XhoI. The digested DNA fragments were inserted into pEGFP-C1 vectors digested with BamHI and XhoI. GFP or HA-tagged GRASP55 and GRASP65-GFP were generated by PCR from GRASP55 or GRASP65 cDNA and digested with XhoI and BamHI or HindIII and KpnI respectively, then inserted into the pEGFP-N1 or pHA-N1 vectors. Serine to Alanine mutations were generated by site-directed mutagenesis. For protein expression in E. coli, SdeA was amplified from SdeA cDNA and digested with BamHI and XhoI. The digested DNA fragments were inserted into pGEX-6p-1 vectors digested with BamHI and XhoI. GRASP55 and GRASP65 cDNA were amplified from mammalian vector and digested with NdeI and BamHI and cloned into pET15b and pGEX-6p-1 vector respectively.

6.3 PCR and Quickchange site-directed mutagenesis

A 50 µL PCR reaction components mixture was assembled as described below. All reagents were thawed on ice and added in the following order: water, 10 µL of 5X Phusion HF buffer, 1 µL of 10 mM dNTPs, 1.5 µL of Forward and Reverse primer respectively, 200 ng template DNA, 1.5 µL DMSO. 0.5 µL Phusion DNA Polymerase was added in the end to prevent any primer degradation caused by the exonuclease activity., All components liquid were mix by tapping tube and collected to the bottom of the tube by a quick spin with a mini-centrifuge. For a PCR program, thermocycling conditions were programmed as follows: Step1: Preheat samples to 98 °C for 3 min. Step 2: Denature sample at 98 °C for 15 seconds, anneal primers at 58 °C for 30 seconds, 72 °C for the extension for several minutes according to the length of the DNA. 30 cycles for amplification. Step 3: Final extension for 10 min. For site-directed mutagenesis PCR, primers were designed with Agilent QuikChange Primer Design tool (https://www.agilent.com/store/primerDesignProgram.jsp). 20 units of DpnI (New England Biolabs) was used to digested methylated template DNA at 37 °C for 2 hrs.

6.4 DNA Transformation

DNA transformation was performed as the protocol provided by New England Biolabs. Thaw 50 µL NEB® Turbo Competent *E. coli* (High Efficiency) in 1.5 mL Eppendorf tube on ice. Add approximately 1 µg (2 µL) of plasmid, or 5 µL ligation product, mix gently by pipetting up and down or flicking the tube to mix the cells and DNA. Do not vortex. Place the mixture on ice for 30 min without mixing. Heat shock at 42 °C thermomixer (Eppendorf) for 30 seconds without mixing. Add 800 µL of room temperature LB medium to the tube. Shake tube at 37 °C vigorously (800 rpm) for 1 hour. Warm antibiotic selection plate to 37 °C. Spread 50–100 µL of the cells onto the plate with an inoculation loop and incubate at 37 °C incubator overnight. As for the transformation of *E. coli* expression vector, T7 Express Competent *E. coli* (High Efficiency) was used.

6.5 Plasmid constructs

The constructs used in this thesis were generated according to standard cloning.

Table1: Information of plasmid constructs used for mammalian expression

Construct name	Vector	Insert	Species	Cloning site	Antibotic resistance
HA-FLAG-SdeA WT	pHA C1	SdeA	Legionella	XhoI/BamHI	Kanamycin
HA-FLAG-SdeA H277A	pHA C1	SdeA	Legionella	XhoI/BamHI	Kanamycin
HA-FLAG-SdeA E860E862/AA	pHA C1	SdeA	Legionella	XhoI/BamHI	Kanamycin
GFP-FLAG-SdeA WT	pEGFP C1	SdeA	Legionella	XhoI/BamHI	Kanamycin
GFP-FLAG-SdeA H277A	pEGFP C1	SdeA	Legionella	XhoI/BamHI	Kanamycin
GFP-FLAG-SdeA E860E862/AA	pEGFP C1	SdeA	Legionella	XhoI/BamHI	Kanamycin
mcherry-SdeA WT	pmcherry C1	SdeA	Legionella	KpnI/BamHI	Kanamycin
mcherry-SdeA H277A	pmcherry C1	SdeA	Legionella	KpnI/BamHI	Kanamycin
mcherry-SdeA E860E862/AA	pmcherryC1	SdeA	Legionella	KpnI/BamHI	Kanamycin
pEGFPN1-GRASP55	pEGFP N1	GRASP55	human	XhoI/BamHI	Kanamycin
GRASP55-Myc	pcDNA3.1	GRASP55 Myc	human	BamHI/XhoI	Ampicilin
GRASP55-FLAG	pcDNA3.1	GRASP55 FLAG	human	BamHI/XhoI	Ampicilin
Myc-GRASP55	pCMV3	Myc GRASP55	human	KpnI/XbaI	Kanamycin
pEGFPN1-GRASP65	pEGFP N1	GRASP65	human	HindIII/KpnI	Kanamycin
ACBD3-Myc	pCMV3	ACBD3 myc	human	KpnI/XbaI	Kanamycin
Myc-Rab33b	pHA C1	Rab33b	human	ECoRI/BamHI	Kanamycin
GFP-SidJ	pEGFP C1	SidJ	Legionella	KpnI/BamHI	Kanamycin
mCherry-SidJ	pmCherry C1	SidJ	Legionella	KpnI/BamHI	Kanamycin
GFP-DupA	pEGFP C1	DupA	Legionella	XhoI/BamHI	Kanamycin
mCherry-DupA	pmCherry C1	DupA	Legionella	XhoI/BamHI	Kanamycin
GFP-DupB	pEGFP C1	DupB	Legionella	XhoI/BamHI	Kanamycin
mCherry-DupB	pmCherry C1	DupB	Legionella	XhoI/BamHI	Kanamycin

GFP-SdeA 1-909	pEGFP C1	SdeA 1-909	Legionella	XhoI/BamHI	Kanamycin
GFP-SdeA 1-972	pEGFP C1	SdeA 1-972	Legionella	XhoI/BamHI	Kanamycin
GFP-SdeA 1-998	pEGFP C1	SdeA 1-998	Legionella	XhoI/BamHI	Kanamycin
GFP-SdeA 1-1233	pEGFP C1	SdeA 1-1233	Legionella	XhoI/BamHI	Kanamycin
GFP-SdeA 1-1350	pEGFP C1	SdeA 1-1350	Legionella	XhoI/BamHI	Kanamycin
GFP-SdeA 1-1443	pEGFP C1	SdeA 1-1443	Legionella	XhoI/BamHI	Kanamycin

Table2: Information of plasmid constructs used for *E. coli* expression

Construct name	Vector	Insert	Species	Cloning site	Antibotic resistance
His-GRASP55	pET15b	GRASP55	human	NdeI/BamHI	Ampicilin
GST-GRASP55	pGEX6p1	GRASP55	human	BamHI/XhoI	Ampicilin
GST-GRASP65	pGEX6p1	GRASP65	human	EcoRI/NotI	Ampicilin
His-SdeA 909-C	pET15b	SdeA 909-C	Legionella	BamHI/XhoI	Ampicilin
His-SdeA 1158-C	pET15b	SdeA 1158-C	Legionella	BamHI/XhoI	Ampicilin
SdeA GST	pGEX6p1	SdeA	Legionella	BamHI/XhoI	Ampicilin
His-sfGFP-SdeA	His-sfGFP	SdeA	Legionella	SfiI/XhoI	Kanamycin
GST-DupA	pGEX6p1	DupA	Legionella	BamHI/XhoI	Ampicilin
GST-DupB	pGEX6p1	DupB	Legionella	BamHI/XhoI	Ampicilin
His-Rab33b	pET21a	Rab33b	human	NotI/BamHI	Ampicilin

Table 3: Primers information of plasmid constructs

Construct	Vector	Primer	Sequence (5’ to 3’)
SdeA	pEGFP-C1	Forward	GCGCCTCGAGCTGATTACAAGGATGACGACG ATAAGGGCAGCGGCAGCATGAGTTTGGGAGA AGCCATAATGC
		Reverse	GCGCGCGGATCCTTAAAATCCTATAGTTTTTT TATTGGATTCATCTTCTTCAACCATGAC
SdeA 1-972	pEGFP-C1	Reverse	GCGCGCGGATCCTTAAGGACCAACGGTGTTT TTTAGAAAATTTTTGTAACC

SdeA 1-1089	pEGFP-C1	Reverse	GCGCGCGGATCCTTAAGGGGTAACAACAGCACGCTG
SdeA 1-1233	pEGFP-C1	Reverse	GCGCGCGGATCCTTATGGATCTTTCGATGAACCGTGTTTTTTG
SdeA 1234-C	pEGFP-C1	Forward	GCGGGGTACCATGCTGGATCTGTCTGATTTGGATAAATTAAGCGG
		Reverse	GCGCGCGGATCCTTAAAATCCTATAGTTTTTTTATTGGATTCATCTTCTTCAACCATGAC
GRASP55-GFP	pEGFP-N1	Forward	GCGCGCCTCGAGATGGGCTCCTCGCAAAGC
		Reverse	GCGCGCGGATCCCGAGGTGACTCAGAAGCATTGGC
GRASP65-GFP	pEGFP-N1	Forward	GCGCGCAAGCTTATGGGCCTGGGCGTC
		Reverse	GCGCGCGGTACCGTTTCTGTGGTAGAGATCTGGGCC
GRASP55-HA	pEGFP-N1	Forward	GCGCGCCTCGAGATGGGCTCCTCGCAAAGC
		Reverse	GCGCGCGGATCCTTAAGCGTAGTCTGGGACGTCGTATGGGTAGCCGCCAGGTGACTCAGAAGCATTGGC
His-GRASP55	pet15b	Forward	GCGCGCCATATGATGGGCTCCTCGCAAAGC
		Reverse	GCGCGCGGATCCTTAAGGTGACTCAGAAGCATTGGC
GST-GRASP65	pGEX6p1	Forward	GCGCGCGAATTCATGGGCCTGGGCGTC
		Reverse	GCGCGCGCGGCCGCTTATTCTGTGGTAGAGATCTGGGCC
GST-DupA	pGEX6p1	Forward	GCGCGCGGATCCATGCCCATAATTTTAGATCCAGAAGTATTAAAAGTAGCAGAG
		Reverse	GCGCGCCTCGAGTCA TAGTTGTGCTTTTTTTTCCAAGACCTCCG
GFP-DupA	pEGFP-C1	Forward	GCGCGCCTCGAGCTATGCCCATAATTTTAGATCCAGAAGTATTAAAAGTAGCAGAG
		Reverse	GCGCGCGGATCCTCA TAGTTGTGCTTTTTTTTCCAAGACCTCCG
GST-DupB	pGEX6p1	Forward	GCGCGCGGATCCATG CCTATTATTCTAGATTCTGATGTGTTGGAAGTGG
		Reverse	GCGCGCCTCGAGCTA AGGTTTATTAGTCTTCTCCGTTGTTGGTTG
GFP-DupB	pEGFP-C1	Forward	GCGCGCCTCGAGCTATG GTGCCTATTATTCTAGATTCTGATGTGTTG
		Reverse	GCGCGCGGATCCCTA AGGTTTATTAGTCTTCTCCGTTGTTGGTTG

Table 4: Primers information of mutation

Gene	Mutation	Primer	Sequence (5' to 3')

SdeA	H277A	Forward	GGCGGTCGAATGGTGTACAGACAACATGCGGGACTTTCCCATACTTTACGGACCATGGC
		Reverse	GCCATGGTCCGTAAAGTATGGGAAAGTCCCGCATGTTGTCTGTACACCATTCGACCGCC
SdeA	E860A	Forward	CCATGGCGAAGGCACCGCAAGTGAATTCTCCGTTTATTTGCC
		Reverse	GGCAAATAAACGGAGAATTCACTTGCGGTGCCTTCGCCATGG
SdeA	E860E862AA	Forward	GGCGAAGGTACCGCAAGTGCATTCTCCGTTTATTTGCCG
		Reverse	CGGCAAATAAACGGAGAATGCACTTGCGGTACCTTCGCC
DupA	H67A	Forward	CAGAGACTTCATCGGCCCATTGCTGGTTTGGCTCATACCATGCG
		Reverse	CGCATGGTATGAGCCAAACCAGCAATGGGCCGATGAAGTCTCTG
DupA	E126A	Forward	GTGGCTGGACGAGAAAGCGCGGCCTCTTATGGTGATGCTTATCACC
		Reverse	GGTGATAAGCATCACCATAAGAGGCCGCGCTTTCTCGTCCAGCCAC
DupA	H189A	Forward	CGCTGAAGGCTATCTCATTCACTTATCGGCCATGATCGATTTAATGCGCTGTAAAAGCC
		Reverse	GGCTTTTACAGCGCATTAAATCGATCATGGCCGATAAGTGAATGAGATAGCCTTCAGCG
DupB	H67A	Forward	GCAAGCAGCTTCATCGCCCTATCGCTGGGTTAGCTCATACGATGCGC
		Reverse	GCGCATCGTATGAGCTAACCCAGCGATAGGGCGATGAAGCTGCTTGC
DupB	E126A	Forward	CGGGCGCGAAAGCGCGGCTTCTTATGGTGATGCTTATCATCG
		Reverse	CGATGATAAGCATCACCATAAGAAGCCGCGCTTTCGCGCCCG
DupB	H189A	Forward	GATGGTACCCCTGAAGGTTATATAATTCACTTGTCAGCCATGATCGATTTGATGCGTTGCAAAAG
		Reverse	CTTTTGCAACGCATCAAATCGATCATGGCTGACAAGTGAATTATATAACCTTCAGGGGTACCATC
GRASP55	S408S409AA	Forward	CAAAGGCAGACGCTGCCGCCGCACTCACTGTGGATGTGACGCC
		Reverse	GGCGTCACATCCACAGTGAGTGCGGCGGCAGCGTCTGCCTTTG
GRASP55	SS34TT	Forward	CGGCAGCCATATGATGGGCACCACGCAAAGCGTCGAGATCCCGG
		Reverse	CCGGGATCTCGACGCTTTGCGTGGTGCCCATCATATGGCTGCCG

GRASP55	S441A	Forward	CAGTCAGCGAGAAGCCTGTTGCTGCGGCTGTGGATGCC
		Reverse	GGCATCCACAGCCGCAGCAACAGGCTTCTCGCTGACTG

6.6 Plasmid mini-preparation

Plasmids of high-quality and yield for transformation or transfection were isolated as the protocol provided with NucleoSpin Plasmid kit (MACHEREY-NAGEL). Pellet overnight cultured 10 mL of bacterial culture by centrifugation at 16,000 x g for 30 seconds, discard the supernatant. Resuspend pellet with 250 µL Plasmid Resuspension Buffer contains RNase A (Buffer A). Vortex or pipet up and down to ensure cells are completely resuspended without visible clumps. Lyse cells by adding 250 µl Plasmid Lysis Buffer (Buffer B), invert tube immediately and gently 5-6 times until color changes to blue, and the solution is clear and viscous. Neutralize the lysate by adding 300 µL of Plasmid Neutralization Buffer (B3). Gently invert tube until the color is uniformly white. Centrifuge the lysate for 10 minutes at 16,000 x g. Carefully transfer the supernatant to a spin column and centrifuge for 30 seconds, discard flow-through. Re-insert column in the collection tube and add 600 µL of Plasmid Wash Buffer that removes RNA, protein and endotoxin. Centrifuge for 30 seconds, discard the flow-through. Re-insert column in the collection tube and add 600 µL of 70% ethanol, centrifuge for 30 seconds. Discard flow-through. Further centrifuge the column for 2 minutes at 16,000 x g to remove remaining ethanol. Put column in a new Eppendorf tube, add 40 uL Elution buffer (25 mM Tris-HCl, pH 8.0) or H_2O to dissolve DNA. Centrifuge for 10 minutes at 16,000 x g to collect plasmid. DNA concentration was measured with nanodrop (Thermo).

6.7 Cell lines culture and transfection

HEK293T, A549, COS7, HeLa cells were purchased from ATCC, GRASP55/65 knockout HeLa cells were kind gift from Dr. Yanzhuang Wang at Michigan University. Cells were cultured in high glucose Dulbecco's Modified Eagles Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/mL Penicilin and 100 mg/mL streptomycin at 37 °C, 5% CO_2 in a humidified incubator. For transient protein expression in cells, transfection was performed using polyethyleneimine (PEI) reagent. HEK293T cells were seeded at $3x10^6$ cells per well in 6-well plate with DMEM complete medium and incubated in the 37 °C incubator for 24 hrs. 2 µg of DNA was diluted into

200 µL of Opti-MEM medium (Gibco) and 6 µL of PEI was gently added (1 µg DNA: 3 µL PEI) dropwise into 200 µL DNA dilution. The diluted DNA and PEI were mixed in the tube and incubated for 15-20 min at room temperature. The transfection mix was added to the HEK293T cells and the cells were cultured for further 24-48 h to express the proteins.

6.8 Legionella culture and infection

Wild-type *Legionella* strain, SidE family effectors deletion mutant, DupA/B deletion mutants were obtained from Dr. Zhao-Qing Luo lab (Purdue University, USA). Bacterial cells were streaked with inoculation loop under a laminar flow hood and then cultured at 37°C on N-(2-acetamido)-2-aminoethanesulfonic acid (ACES)-buffered charcoal-yeast extract (BCYE) agar plates for 3 days, followed by colony inoculation and growth in 3 mL CYE liquid media for 20 h at 37 °C with shaking at 225 rpm/min. During the growth, optical density at 600 nm (OD600) was determined by an Ultrospec Cell Density Meter. HEK293T cells were transfected with FCγRII and GRASP55-GFP or GRASP65-GFP for 24 hrs. Post-exponential *Legionella* with OD_{600} between 3.6-3.8 were used to infect A549 or HEK293T cells. Indicated *Legionella* strains were opsonized with antibody against *Legionella* (1:500) at 37 °C for 30 min before infection. The HEK293T cells were infected with different *Legionella* strains at an MOI of 2 (for confocal imaging), or 10 (for Western blot) for the indicated time. After 2 hrs infection, cells were washed 3 times with PBS to remove bacteria outside cells. Cells were cultured with fresh complete DMEM medium for further several hours as indicated.

6.9 DNA sequencing

Sequencing samples (10 uL plasmid DNA and enclosed sequencing primer or standard primers provided) were prepared for sequencing (Microsynth Seqlab, Germany). Sequencing alignment was done with SnapGene Viewer.

6.10 Protein extraction from mammalian cells

Cell culture medium was aspirated, cells were washed once with pre-cold PBS and detached by pipetting up and down with cold PBS and gently transferred into a pre-cooled Eppendorf microcentrifuge tube. Cells were collected by centrifugation in a microcentrifuge at 4 °C for 3 min at 1000 x g and lysed with 1 mL ice-cold RIPA lysis buffer (150 mM NaCl, 50 mM Tris-HCl, pH 7.5, 1% Triton X-100, 0.1% SDS, 5 mM

EDTA, protease inhibitor cocktail). For Immunoprecipitation, cells were lysed with mild lysis buffer containing 150 mM NaCl, 50 mM Tris-HCl, pH 7.5, 0.5 % NP-40, 5 mM EDTA, protease inhibitor cocktail. Protein lysate supernatant was gently transferred to a new pre-cold Eppendorf tube, protein concentration of the cell lysate sample was determined with bicinchoninic acid (BCA) protein assay kit. Once the concentration of each sample was measured, samples were frozen at -80 °C or denatured by adding 5X Laemmli loading buffer (5% β-Mercaptoethanol, 0.02% Bromophenol blue, 30% Glycerol, 10% SDS, 250 mM Tris-HCl, pH 6.8), boiled samples were used for loading onto an SDS-PAGE gel, followed by Coomassie staining or immunoblotting.

6.11 SdeA mediated PR-ubiquitination reaction

SdeA mediated PR-Serine ubiquitination *in vitro* reaction was done as previously described (Kalayil et al., 2018). Briefly, 5 μM GRASP proteins were incubated with 1 μM of SdeA and 25 μM ubiquitin in the presence of 200 μM of NAD^+ in 40 μL of reaction buffer (50 mM NaCl and 50 mM Tris, pH 7.5) for 1 hour at 37°C. Deubiquitination assay was performed by incubating PR-ubiquitinated proteins with 1 μg of GST-DupA at 37 °C for 1 h in reaction buffer (150 mM NaCl, 50 mM Tris-HCl pH 7.5). The reaction products were analyzed by SDS-PAGE with Coomassie staining or western blotting using antibodies against GST (cell signaling technology), His (Roche), GRASP55 (Proteintech), GRASP65 (Sino biotech.), Ub (Abcam/Cell signaling technology). To assay the PR-ubiquitination of GRASP55 and GRASP65 in cells, plasmids expressing GRASP-GFP, GFP-SdeA or mCherry-SdeA, were co-transfected into HEK293T cells, then culture cells at 37 °C for 24 hrs. The whole cell lysates were subjected to immunoprecipitation with GFP-trap beads and the products or the rest whole cell lysates were separated with SDS-PAGE and blotted with antibodies against GFP or GRASP.

6.12 *In vitro* ubiquitination reactions using Legionella lysates

Different *Legionella* strains were grown in 3 mL of AYE broth until they reached the early stationary phase (OD_{600}=3). Same amount of cells of different strains were collected by normalizing the OD_{600} followed by centrifugation. Cells were lysed with lysis buffer for bacteria (50 mM HEPES pH7.5, 200 mM NaCl, 0.1% Triton X-100, 1 mM EDTA, 1 mM PMSF and 0.1 mg/ml lysozyme) by incubating cell pellets at room temperature. After 20 min, lysates were centrifuged, supernatants were used for *in*

vitro reactions. 5 µg of ubiquitin and 2 mM NAD^+ were added to each *Legionella* lysate and incubated at 37 °C. The mixed were denatured with SDS loading dye and probed with CS-Ub and abcam-Ub antibodies.

6.13 Western blotting and immunoprecipitation

Cell lysates or immunoprecipitated proteins were mixed with SDS sample buffer, heated at 95°C for 5 min, centrifuged, and separated by Tris-Glycine SDS-PAGE, and transferred to PVDF membrane (Millipore) in the cold room. Blots were blocked with 5% nonfat milk for 1 hour at room temperature and incubated with primary antibodies overnight at cold room or 2 hours at room temperature and washed with TBST (0.1% Tween 20 in TBS) three times. The blots were further incubated with secondary antibodies for 1 h at room temperature and washed 3 times with TBST. The blots were incubated with ECL reagents (advansta), and chemiluminescence was acquired with the Bio-Rad ChemiDoc system. For immunoprecipitation, HEK293T cells expressing GFP or HA-tagged proteins were lysed with mild immunoprecipitation buffer containing 150 mM NaCl, 50 mM Tris-HCl, pH 7.5, 0.5% NP40, 1 mM PMSF, protease inhibitor cocktail (Sigma Aldrich), mixed with 10 µL GFP-trap or HA antibody-conjugated agarose, and incubated for 4 h in the cold room with end to end rotation. Beads were washed 3 times in IP buffer containing 500 mM NaCl. Proteins were eluted by resuspending with 2 x SDS sample buffer followed by boiling for 5 min at 95°C. Samples were then submitted to western blotting analysis.

6.14 Protein expression and purification

GRASP55 and GRASP65 cDNA were cloned into p15b and pGEX-6p-1 vector respectively. Full-length SdeA was cloned into pGEX-6P-1 vector. *E. coli* competent cells (NEB T7 express) were transformed with plasmid, colonies were inoculated and cultured overnight in LB medium at 37 °C, Next day 5 mL culture was transferred to 1 L flask for further culture at 37 °C until the OD_{600} reached to 0.6-0.8. Protein expression was induced by adding 0.5 mM IPTG and cells were further cultured overnight at 18 °C. Cells were harvested and the cell pellet was resuspended in lysis buffer (300 mM NaCl, 50 mM Tris-HCl pH 7.5) followed by sonication and centrifuged at 13,000 rpm to clarify the supernatant.

For His_6-tagged GRASP55, the supernatant was incubated for 1 hour with TALON beads pre-equilibrated with lysis buffer containing 10 mM imidazole and then washed

with lysis buffer for 3 times. His_6-tagged GRASP55 proteins were eluted with elution buffer containing 200 mM imidazole in 300 mM NaCl, 50 mM Tris-HCl pH 7.5.
For GST-tagged proteins, the supernatant was incubated for 1 hour with glutathione-S-Sepharose pre-equilibrated with washing buffer containing 500 mM NaCl, 50 mM Tris-HCl pH 7.5, and washed for 3 times. GST-tagged proteins were eluted with elution buffer containing 50 mM NaCl, 15 mM glutathione, 50 mM Tris-HCl pH 7.5. For GRASP65 without tag, glutathione beads were incubated with PreScission 3C protease overnight at 4 °C. Proteins were further concentrated with filters and then purified by anion exchange chromatography on HitrapQ (GE Healthcare) and collected fractions were further loaded onto a size exclusion column (Superdex 75 16/60, GE Healthcare). Proteins were concentrated and used for *in vitro* reactions.

6.15 Identification of PR ubiquitination serine sites on GRASP55 and GRASP65

His-GRASP55 and GRASP65 were purified from *E. coli* and PR-ubiquitinated SdeA *in vitro*. Urea buffer containing 8 M urea, 0.1 M Tris, pH 7.5 was added to the reaction mixture to a final volume of 200 µl, then the reactions were transferred to 30 kDa filter (Amicon Ultra, 0.5 mL, Merck) and washed 3 times with 200 µL urea buffer by centrifugation to remove free ubiquitin. Then the proteins were washed 2 times with 50 mM ABC and digested with trypsin in 50 mM ABC pH 7.5 at trypsin to protein ratio 1: 50 for 6 hrs and subsequently desalted by C18. Digestion product was analyzed by LC-MS/MS to obtain initial high-resolution HCD spectra for all peptide species. Possible bridged peptides were subjected to targeted CID and ETD fragmentation to yield high-quality spectra to determine bridge structure and localization, respectively. Targeted ETD spectra for bridged peptides were searched and annotated using StavroX 3.6.

6.16 Data quantification

Western blot band intensity and Golgi area quantifications were performed with ImageJ (https://imagej.nih.gov/ij/). At least 3 independent experiments were performed, p values were determined using Student's t test and figures were generated with GraphPad Prism (https://www.graphpad.com/scientific-software/prism/). Data are shown as means ± SEM of more than 60 cells taken from three independent experiments. ***P<0.001, **P<0.01, *P<0.05.

6.17 VSVG trafficking assay

HEK293T or A549 cells were co-transfected with VSV-G-GFP and CD32 or transfected with VSVG-GFP respectively and cultured at 37 °C for 24 hours to express the proteins before transferred to 40 °C. After 16 hours incubation at 40 °C, cycloheximide was added into medium to inhibit further protein synthesis, after 2 hours treatment cells were infected with *Legionella* for another 2 hours then washed 3 times with PBS and cultured with fresh medium at 32 °C to remove the bacteria outside host cells, and then moved to 32 °C for 0 min,15min, 30min,60min, 90min,120min to release VSVG from ER. A549 cells were fixed and VSVG trafficking was acquired with confocal microscopy after immunofluorescence staining. HEK293T cells were lysed with lysis buffer containing 1% SDS, 50mM Tris, pH 8.0, DNAse benzonase was added to reduce the viscosity caused by released DNA. Cell lysates were mixed with denaturing buffer then boiled for 10 min, 95 °C, denatured proteins were incubated with EndoH for 3 h at 37 °C to cleave the EndoH sensitive form of glycosylation, final products were separated with SDS-PAGE and the EndoH caused band shift was analyzed by blotting GFP.

6.18 ELISA

To investigate the effect of PR-ubiquitination on secretion pathway, we analyzed the secretion of pro-inflammatory cytokines in PMA-treated THP-1 cells upon infection with *Legionella*. Cytokine secretion analyses were performed with the ELISA kits ordered from R&D system according to the manufacturer's protocol: 1. Reconstitute IL-1β Capture Antibody in a vial with PBS, then dilute it in PBS without carrier protein to the working concentration. Transfer 100 µL of the diluted Capture Antibody with a multichannel pipettor to each well of a 96-well microplate and incubate overnight at room temperature to coat the plate. 2. Wash the plate 3 times with washing buffer containing 0.05% Tween 20 in PBS, complete remove liquid after wash by inverting the plate and flapping it against clean paper towels. 3. Block plates by adding 300 µL of blocking buffer containing 1% BSA in PBS to each well and incubating at room temperature for 1 hour. 4. Repeat the wash then add 100 µL of the samples or IL-1β standard in blocking buffer. Seal with an adhesive cover and incubate 2 hours at room temperature. 5. Repeat the wash then add 100 µL of the IL-1β Detection Antibody diluted in blocking buffer to each well. Seal with a new adhesive cover and incubate 2 hours at room temperature. 6. Repeat the wash then add 100 µL of the diluted

Streptavidin-HRP to each well then incubate for 20 minutes followed with wash and addition of 100 µL of 1: 1 mixed Substrate Solution A and B to each well. 7. After 20 minutes incubation, add 50 µL of Stop Solution 2 N H_2SO_4 to each well and mix gently. 8. Determine the optical density of each well immediately at 450 nm, with wavelength correction at 540 nm. Cytokine concentrations of samples were calculated according to the four parameter logistic (4-PL) standard curve.

6.19 Staining of WGA

For immunofluorescence analysis of protein glycosylation with lectin, HeLa cells transfected with plasmid encoding SdeA or A549 cell infected with *Legionella* on cover slides were fixed in 4% paraformaldehyde (PFA) for 5 min. After blocking with 1% BSA (in PBS), surface glycosylated proteins were labeled with dye-conjugated lectin WGA (EY laboratories) that binds with sialic acid (SA), a terminal capping monosaccharide residue on glycoprotein, in the dark for 30 min at 4 °C. After being washed with blocking buffer, the cells were permeabilized with 0.05% saponin and processed for immunofluorescence with the indicated antibodies.

6.20 Preparation of Legionella medium

For CYE-Agar plate: dissolve 10 g ACES and 10 g Yeast extract in 900 mL deionized H_2O, adjust pH to 6.9 with 10 M KOH, then add H_2O to reach 1 L in a beaker. Weigh out 2 g activated charcoal and 15 g agar, place in a flask, transfer the liquid to a flask, autoclave for 15 min at 121 °C. Cool autoclaved medium to 60 °C, then add filter-sterilized 200X Cysteine stock solution (final concentration: 3.3 mM) and 5 mL of 200X Ferric Nitrate stock solution (final concentration: 0.6 mM) while stirring, pipet 20 mL to each 10 cm plate, dry the plates under a fuming cupboard for overnight, then store them at 4 °C fridge.

For AYE liquid medium, dissolve 10 g ACES and 10 g Yeast extract in 900 mL deionized H_2O, adjust pH to 6.9 with 10 M KOH, then add H_2O to reach 1 L in a beaker. Add 5 mL of 200X Cysteine (final concentration: 3.3 mM) and 5 mL of 200X Ferric Nitrate (final concentration: 0.6 mM) slowly while stirring. Then filter medium with 1 L filter several times using GE glass fiber until the medium gets clear, finally sterilize medium with 0.22 µM filter under a fuming cupboard in the cell culture room. Store medium at 4 °C fridge.

6.21 Immunofluorescence

HEK293T, COS7 or A549 cells were seeded on a coverslip in 12-well plates and cultured in a CO_2 incubator. Next day cells were transfected with plasmids encoding SdeA. 24 h after transfection, cells were washed once with PBS, pH 7.4, and fixed with 4% paraformaldehyde (PFA) in PBS for 10 min at room temperature. Cells were washed again with PBS for two times, then permeabilized with 0.1% saponin in PBS for 10 min, and blocked with blocking buffer containing 0.1% saponin and 2% BSA in PBS for 1 h at room temperature. Cells were stained with antibodies diluted in blocking buffer overnight at 4 °C and washed with PBS three times the next day. Cells were further incubated with Alexa Flour dyes-conjugated secondary antibodies for 1 h at room temperature in the dark and washed with PBS and incubated with DAPI in PBS, followed with further 2 times washing with PBS. Confocal imaging was performed using the Zeiss LSM780 microscope system. Images were analyzed with Fiji software. Over 60 cells taken from three independent experiments were used for quantification analysis.

6.22 Yeast transformation

Inoculated the yeast strain from YPD plate, resuspended it into 3 mL of YPD medium. Cells were grown overnight at 30 °C. Next day, the culture was diluted (1: 10) and transferred to a new tube and cultured for another 4 hours. Cells were then pelleted at 1500 g for 5 min, and washed with 0.1 M LiAc. Transformation mixure was added to cell pellet: PEG 3350 (50%) 100 µL, LiAc (1.0 M) 15 µL, carrier DNA (2 mg/mL) 20 µL, pAG416GAL-SdeA plasmid, mixed thoroughly with vortex. The mixture was incubated at 30 °C for 30 min, followed by heat shock at 42 °C for 40 min. Cells were then pelleted at 1500 g for 10 min and washed twice with sterile water. Cells were resuspended with 20 µL of sterile water, plated on selective agar plates without Ura and incubated at 30 °C for 3 days.

7 Results

7.1 Function of SdeA-catalyzed unconventional ubiquitination

7.1.1 Identification of phosphodiesterase domain in SdeA

SdeA-mediated ubiquitination is entirely different from conventional ubiquitination involving a three-enzyme cascade, the mechanism of how ADPR-ubiquitin is transferred to substrate proteins remained unknown. Thus, in order to better understand the chemistry behind this novel protein ubiquitination, we attempted to identify other functional domains in SdeA by analyzing its sequence features.

Previous structural and biochemical studies have revealed that SdeA possesses an N-terminal deubiquitinase (DUB) domain (amino acid 1-200) and an mART domain spanning residues 500 to 1000 (Qiu et al., 2016; Sheedlo et al., 2015). Protein structural prediction analysis showed that the region between residues 200 and 500 of SdeA is similar to the predicted phosphodiesterase (PDE) domain in the *Legionella* effector lpg1496 (Bhogaraju et al., 2016; Wong et al., 2015). The putative SdeA PDE region showed 48% sequence similarity to the PDE domain of lpg1496 and 23% sequence similarity to the PDE domain in the well-characterized phosphodiesterase PA4781 from *Pseudomonas aeruginosa* (Bhogaraju et al., 2016; Rinaldo et al., 2015). Based on this observation, and in view of the fact that mART-modified ubiquitin is transferred to the the substrate, with the detection of AMP (Qiu et al., 2016), we hypothesized that SdeA has PDE activity and the predicted PDE domain in SdeA may cleave the pyrophosphate bond between two phosphates ADP in ADP-ribosylated ubiquitin, which results in the formation of phosphoribosylated Ub and release of AMP. To test this, PDE domain sequence of SdeA was aligned with the sequence of the PDE domain of lpg1496 (PDB code: 5BU1), and the predicted catalytic residues of the PDE domain and the mART domain in SdeA constructs spanning residues 200 to 1005 (ΔNC SdeA) were mutated (Bhogaraju et al., 2016) (Fig. 4A). Ubiquitin treated with SdeA WT was detected with phospho-specific Pro-Q Diamond stain due to the exposed phosphate of the phosphoribosyl moiety (Daniels et al., 2014), whereas Ub incubated with the PDE mutants did not show staining of Pro-Q Diamond (Fig. 4B). Together, these biochemical experiments confirmed the PDE domain that catalyzes the conversion of ADP-ribosylated ubiquitin to phosphoribosyl-ubiquitin and releases AMP.

Because of the lack of a specific antibody for detecting PR-modified ubiquitin induced by SdeA, it was challenging to directly indicate the effect of SdeA in vivo. In order to facilitate the detection of the SdeA-mediated Ub modification, we developed a strategy to assay phosphoribosylation of Ub in complex mixtures such as bacterial and human cell lysates. Various Ub antibodies were screened to check whether the modification of ubiquitin caused by SdeA interferes recognition of ubiquitin by antibody. We noticed that the Ub antibody from Cell Signaling Technology (referred to as CS-Ub antibody) recognized both SdeA-modified and un-modified Ub with similar efficiency, whereas the antibody from Abcam (referred to as Abcam-Ub antibody) selectively recognized the unmodified wild-type Ub only and failed to recognize the modified Ub (Fig. 4C). So based on this observation, this Abcam antibody can be used for indicating Ub modified by SdeA.

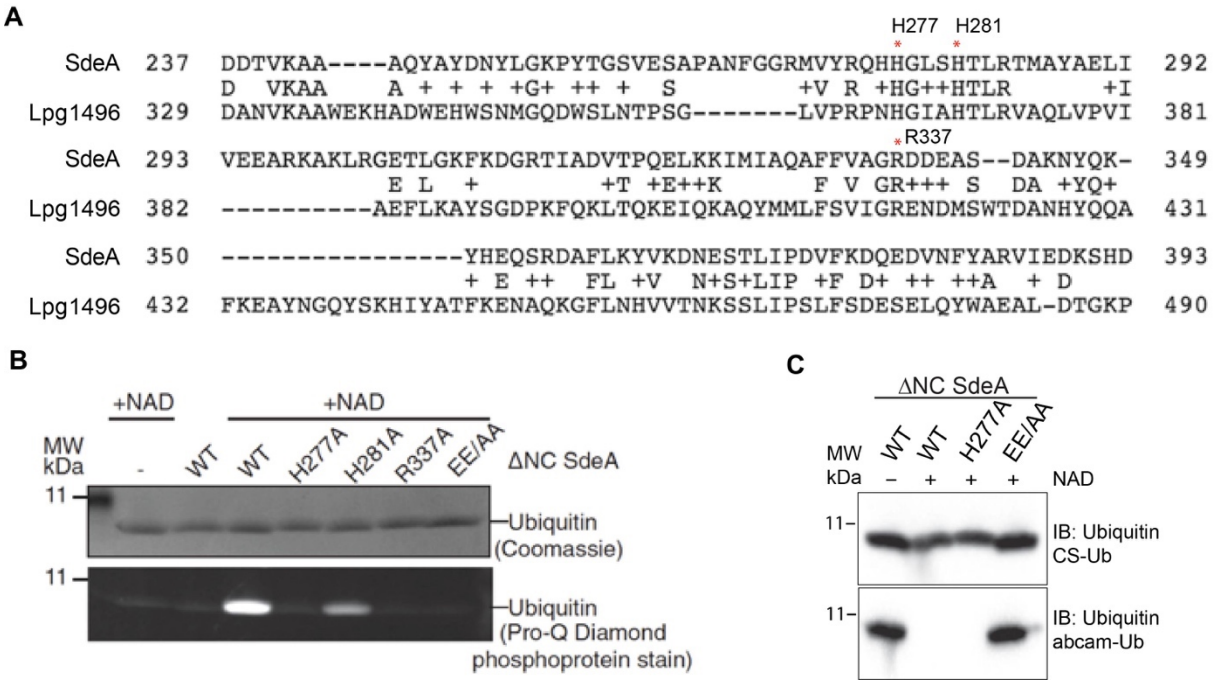


Figure 4. Identification of PDE domain in SdeA

(**A**) sequence alignment of SdeA and PDE domain of Lpg1496. Amino acids marked in red are predicted as conserved catalytic residues. (**B**) Pro-Q Diamond phosphor-protein staining of ubiquitin treated with SdeA WT and mutants. (**C**) Recognition of SdeA mediated ubiquitin modification by antibodies. Ubiquitin was modified by WT or the indicated mutants of SdeA *in vitro* in the presence of NAD^+. Reaction mixtures were separated by SDS-PAGE and blotted with the different antibodies against ubiquitin. Unmodified ubiquitin and SdeA-modified ubiquitin are differentially recognized by two commercial antibodies. Ubiquitin antibody from Cell signaling (CS-Ub) recognizes both modified and unmodified forms of

ubiquitin equally well, whereas the antibody from Abcam (abcam-Ub) selectively recognizes unmodified ubiquitin.

* (Experiments for Figure 4 were performed by Sagar Bhogaraju)

7.1.2 SdeA catalyzes modification of ubiquitin *in vitro* or in cells

We then checked whether endogenous SdeA extracted from *Legionella* bacteria possesses Ub modification activity similar to that of recombinant SdeA protein expressed and purified from *E. coli*. *Legionella* WT strain and various mutants were grown in AYE broth liquid media overnight until they reached to the early stationary phase. Cells were collected and lysed with lysis buffer. *In vitro* reactions were performed in S2 lab to study the activity of *Legionella* lysate on ubiquitin. Western blotting indicates lysate of the WT *Legionella* but not the *ΔsidEs* strain were able to modify ubiquitin, as the ubiquitin incubated with WT *Legionella* lysate could not be detected by Abcam Ub antibody, by contrary, ubiquitin treated with *ΔsidEs Legionella* lysate was recognized by either CS or Abcam antibody (Figure 5A). Notably, replenishing the *ΔsidEs* mutant strain with WT SdeA but not mART mutant SdeA EE/AA restored modification of ubiquitin, indicating that the endogenous *Legionella* protein SdeA and other SidE family members modify Ub in an mART domain dependent manner.

In view of that SdeA is translocated to host cells upon *Legionella* infection, to mimic this physiological condition without bacteria, we then checked whether ectopically expressed SdeA modifies the cellular endogenous free ubiquitin pool and affect ubiquitination pathway in cells. HEK293T cells were grown and transfected with plasmids encoding SdeA proteins. Western blotting indicates that expression of SdeA WT or H277A mutant led to the modification of ubiquitin in cells, as the detection of ubiquitin by Abcam antibody was interfered, whereas the mART mutant did not (Figure 5B). This is in agreement with the observation made on *in vitro* experiment described above.

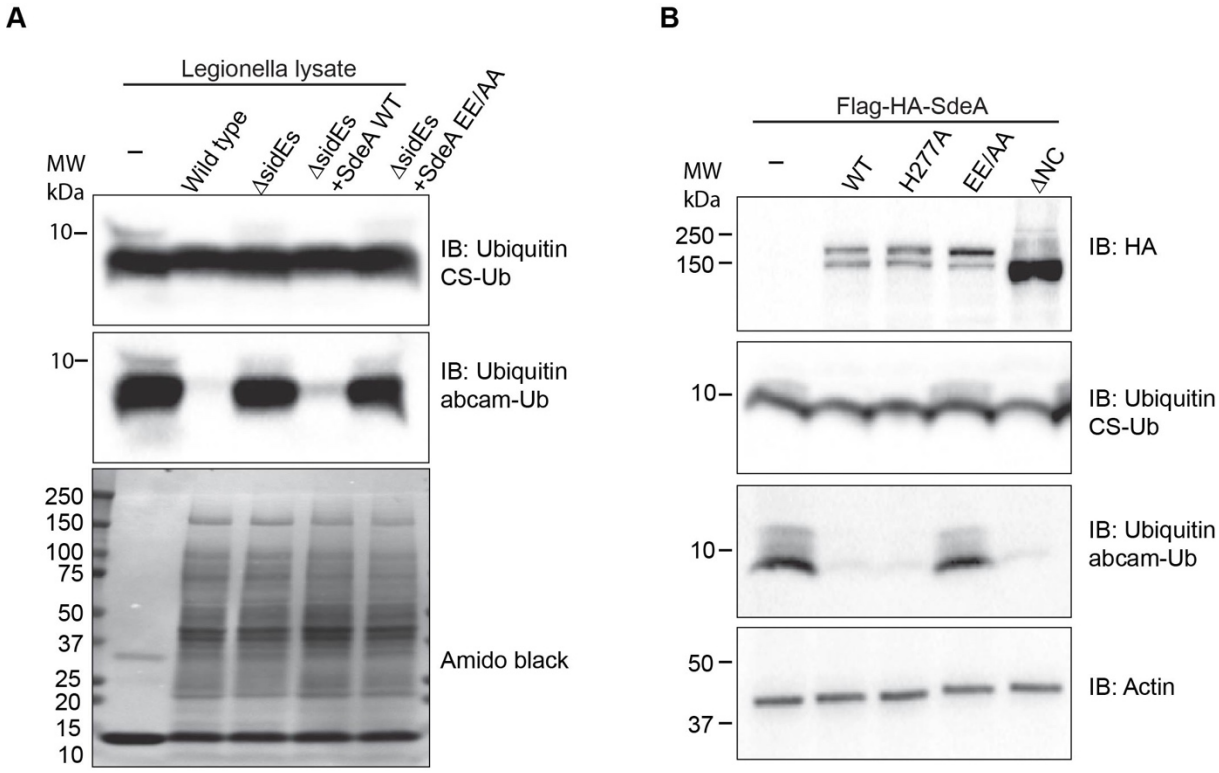


Figure 5. Modification of Ubiquitin by Legionella SidE family effector SdeA.

(**A**) WT (*lp02*) and mutant *Legionella* strains were grown until early stationary phase (OD_{600}=3), normalized, and lysed in non-denaturing conditions. Clarified lysate was used in a reaction mix with ubiquitin and NAD+. Ubiquitin was probed using CS-Ub and abcam-Ub antibodies. (**B**) HEK293T cells were transfected with WT and various mutants of Flag-HA-SdeA. Cell lysates were subjected to SDS-PAGE and probed with CS-Ub and abcam-Ub antibodies to monitor ubiquitin modification. Analysis of ubiquitin signal from both antibodies depicts the dependence of ubiquitin modification on the mART motif of SdeA.

7.1.3 Phosphoribosylation of ubiquitin impairs the conventional ubiquitination

7.1.3.1 SdeA reduces free active ubiquitin

Identification of the PDE domain of SdeA expands our understanding of the mechanism of how SdeA catalyzes the non-conventional ubiquitination of substrate proteins. On the other hand, it demonstrates that SdeA has at least two functions: this effector not only ubiquitinates substrate proteins such as Rab33b, but also ADP-ribosylates (mART domain) and phosphoribosylates (mART domain + PDE domain) ubiquitin. Given the reported toxic effect of SdeA expression in yeast (Jeong et al., 2015; Qiu et al., 2016), we attempted to figure out which function of SdeA leads to cell death. For this reason, we conducted yeast assay by expressing wild-type SdeA, SdeA mART EE/AA mutant or PDE H277A mutant in yeast under the control of Gal promoter.

Yeast cells transformed with SdeA plasmids were grown normally on the Glucose plate. Under the Galactose condition, protein expression was induced, expression of the ccdB carried by the empty vector or SdeA EE/AA mutant did not affect the growth of yeast cells, while Galactose-induced expression of wild-type SdeA and SdeA H277A were extremely toxic to yeast cells (Fig. 6). This data suggested that toxicity effect of SdeA expression on yeast is caused by ubiquitin modification, indicating that modification of ubiquitin by SdeA may lead to broader consequences in host cells.

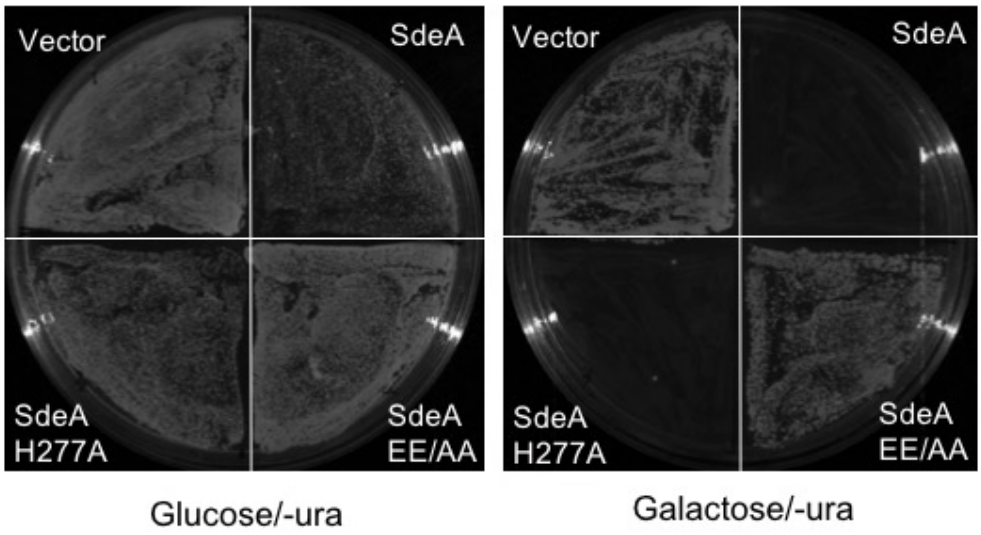


Figure 6. SdeA-catalyzed ubiquitin phosphoribosylation is toxic to yeast cells.
Yeast strain BY4743 cells were grown in 3 mL of YPD medium at 30 °C. Next day, the subcultured cells were transformed with pAG416GAL empty vector or plasmids inserted with SdeA cDNA. Transformants were plated on two different plates and grown for 3 days at 30 °C.

Crystal structure of phosphoribosylated Ub revealed that phosphoribosyl modification caused position changes of Arg42 and Arg72, which are important for ubiquitin activation by E1, thereby making ubiquitin unable to be utilized for ubiquitination reaction (Bhogaraju et al., 2016). Given the fact that polyubiquitin chains undergo constant recycle due to proteasomal degradation and DUB enzymes including SdeA itself, phosphoribosylation of ubiquitin may eventually lead to accumulation of modified free mono-ubiquitin, thereby affecting the conventional ubiquitination pathway. To verify changes in the levels of mono-ubiquitin caused by SdeA expression, we transfected HEK293T cells with plasmids encoding wild-type SdeA and SdeA mutant, then performed fluorescence-activated cell sorting (FACS) of GFP-SdeA transfected cells followed by immunoblotting to compare mono-ubiquitin level in the cells

expressing GFP-SdeA WT with that in cells expressing GFP-SdeA H277A or non-transfected cells. Western blotting result indicates that expression of GFP-SdeA H277A but not SdeA WT led to an increase of free ubiquitin (Fig. 7A). A possibility is that WT SdeA utilizes ubiquitin to modify substrates, thus resulting in the reduction of free ubiquitin.

It is elucidated that proteotoxic stress caused by inhibition of proteasomal degradation can decrease the level of ubiquitinated histone H2A as many cellular processes compete for the limited amount of free Ub (Dantuma et al., 2006). We hypothesized that free ubiquitin decrease caused by SdeA protein may trigger the ubiquitin recycle from ubiquitinated proteins. To further confirm that SdeA expression affects the cellular ubiquitination pathway, we transfected cells with SdeA plasmids and analyzed the cell lysates to verify whether exogenously expressed SdeA leads to reverse of H2A ubiquitination. Western blotting result shows that similar to the effect of proteasomal inhibition, SdeA expression led to a decrease in H2A ubiquitination (Fig. 7B). This data indicates that the hijack of ubiquitin by SdeA results in a shortage of free ubiquitin and subsequently boosts the recycle of ubiquitin from ubiquitinated proteins.

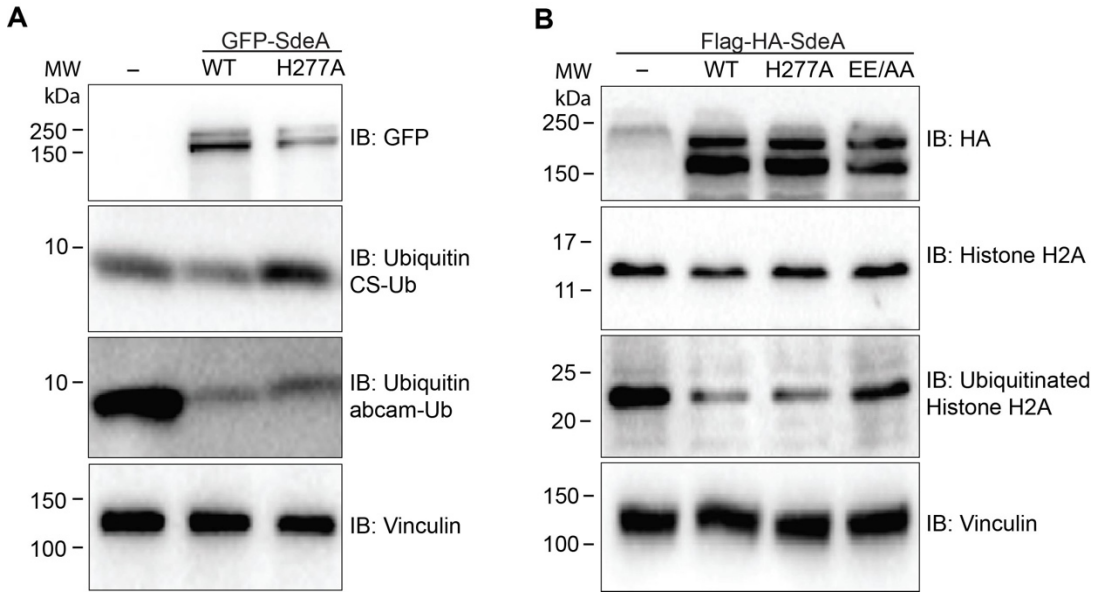


Figure 7. SdeA-catalyzed ubiquitin phosphoribosylation reduces free ubiquitin.

(**A**) GFP-SdeA WT and H277A mutant expressing HE293T cells were FACS sorted and the mono-ubiquitin in the cells was monitored using CS-Ub and Abcam-Ub antibodies. (**B**) Levels of ubiquitinated-H2A were assayed in total cell lysates of HEK293T cells transiently expressing SdeA WT, H277A, and EE/AA mutants.

Based on these observations, we next sought to check whether this modification of ubiquitin in cells affects total ubiquitination in cells. For this reason, HEK293T cells were transfected with SdeA plasmids, whole cell lysates of cells ectopically expressing SdeA WT or H277A mutant or EE/AA mutant were blotted for total ubiquitination using FK2 antibody against ubiquitinated proteins (Fig. 8A). Western blotting result suggests that ubiquitination levels decreased in samples expressing either SdeA WT or SdeA H277A, compared with that in control cells or cells expressing SdeA EE/AA mutant. This demonstrates that phosphoribosylation or ADP-ribosylation modification of ubiquitin by SdeA severely interferes the conventional ubiquitination of cellular proteins. Given different linkage types of polyubiquitin chains target substrates to different fates, we further sought to decode the details of the interference of SdeA on the conventional ubiquitination. To this end, HEK293T cells were transfected with SdeA plasmids, whole cell lysates of cells ectopically expressing SdeA WT or H277A mutant or EE/AA mutant were blotted for K63-Ub chains, and K48-Ub chains using the respective antibodies (Fig. 8B). Interestingly, the blotting results showed that SdeA expression reduced either K48-linked or K63-linked ubiquitination, moreover, SdeA seems to have preference on K63 chains than on K48 chains, which is in agreement with the previous report showing the SdeA DUB domain specifically targets K63 chains (Sheedlo et al., 2015).

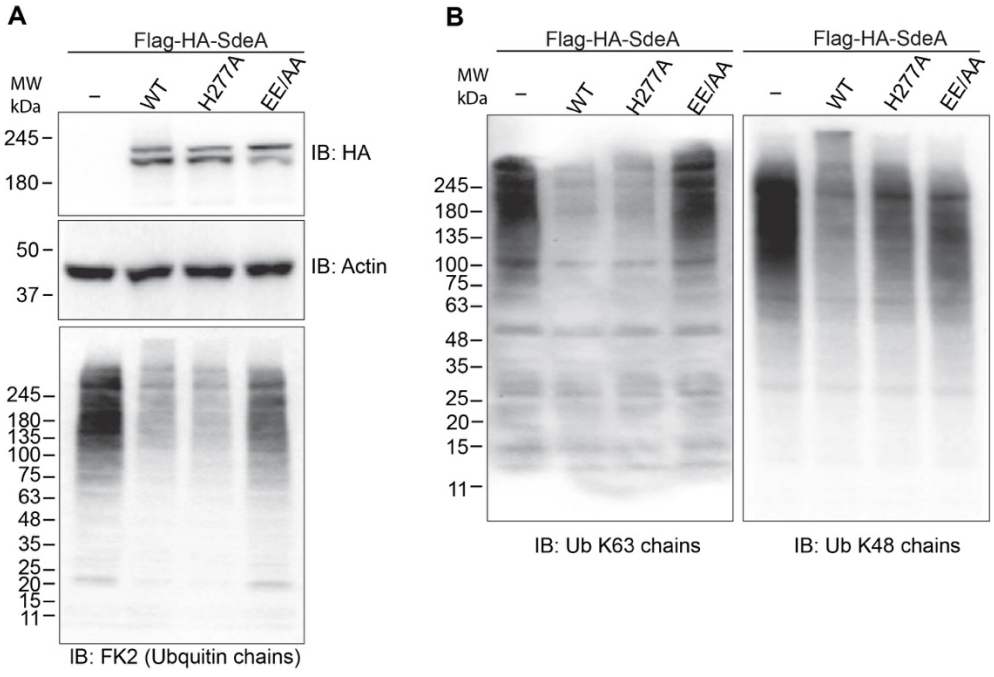


Figure 8. Phosphoribosyl-linked ubiquitination blocks conventional K64, K48 linked ubiquitination in cells

(**A**) GFP-SdeA WT and H284A mutant expressing HE293T cells were FACS sorted and the mono-ubiquitin in the cells was monitored using CS-Ub and Abcam-Ub antibodies. (**B**) Levels of K63 ubiquitination, and K48 ubiquitination were assayed in total cell lysates of HEK293T cells transiently expressing SdeA WT, H284A, and EE/AA mutants

7.1.3.2 SdeA impairs proteasomal degradation

As SdeA induces accumulation of the modified free ubiquitin, which enhances the recycle of ubiquitin, such as K48-linked ubiquitin from ubiquitinated proteins in cells, we hypothesized this will subsequently affect proteasomal degradation of proteins. To test this hypothesis, we attempted to check the protein stability of hypoxia-inducible factor 1-α (HIF1-α) in cells expressing SdeA. Under normal conditions, HIF1-α constantly undergoes ubiquitination catalyzed by the E3 ligase von Hippel-Lindau protein (pVHL) and subsequent degradation by the proteasome (Kim and Kaelin, 2003). Western blotting result shows that expression of SdeA WT or H277A mutant but not the mART mutant led to HIF1-α stabilization in HeLa cells (Fig. 9). This observation indicates that the SdeA-mediated modification of ubiquitin is a robust inhibitor of the cellular ubiquitin system and severely inhibits many ubiquitin-dependent cellular pathways. Our data demonstrates that SdeA has at least two functions: one is to ubiquitinate substrate proteins such as Rab33b, and the other is to phosphoribosylate ubiquitin, leading to inactivation of the ubiquitin system.

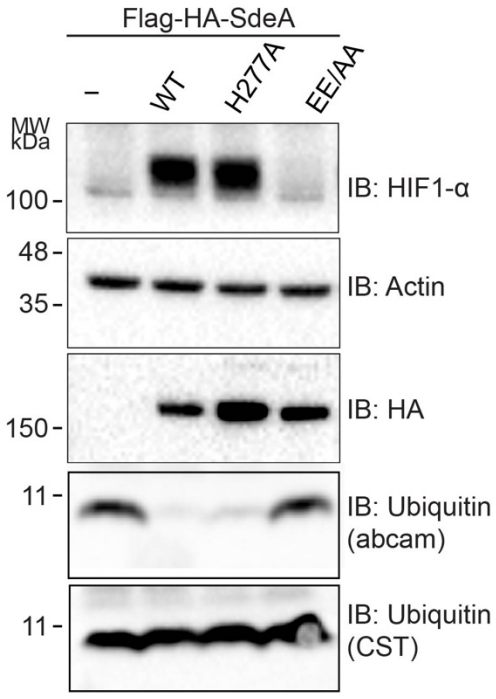


Figure 9. SdeA impairs HIF1-α proteasomal degradation by inhibiting ubiquitination.
Degradation of HIF1-α under normoxic conditions was probed in HeLa cells expressing WT and mutants of HA-Flag-SdeA.

7.1.3.3 SdeA impairs NF-κB signaling

In addition to regulating protein stability directly, ubiquitination also affects other cellular processes including autophagy and NF-κB signaling as mentioned above, we hypothesized that inhibition of ubiquitination caused by SdeA-catalyzed phosphoribosylation of ubiquitin regulates these cellular events. To test this hypothesis, we expressed SdeA in cells and checked the effects of SdeA expression on such broad ubiquitin involved processes in cells by western blotting and immunostaining. In the case of mitophagy, ubiquitination of the mitochondrial membrane proteins by the E3 Ub ligase Parkin is one of the markers of mitochondria autophagy, so we transfected plasmids encoding SdeA WT or mutants into HeLa cells expressing Parkin, then treated cells with CCCP to initiate mitophagy and induce ubiquitination on the outer mitochondrial membrane surface, which was indicated with ubiquitin staining. Immunostaining result shows that in HeLa cells without SdeA transfection, CCCP treatment effectively induced ubiquitination at mitochondria, whereas expression of WT SdeA or H277A mutant remarkably interfered ubiquitination at mitochondria (Fig. 10A). In addition, the Ub chains signal in the cytoplasm also decreased heavily in cells expressing either WT SdeA or the DNC construct of SdeA lacking the N-terminal DUB domain. On contrary, expression of the SdeA mART mutant EE/AA did not lead to a remarkable decrease in ubiquitination at the mitochondria, suggesting that SdeA's inhibitory effect on the mitochondria ubiquitination is the result of its mART domain activity for modification of ubiquitin (Fig. 10B, C).

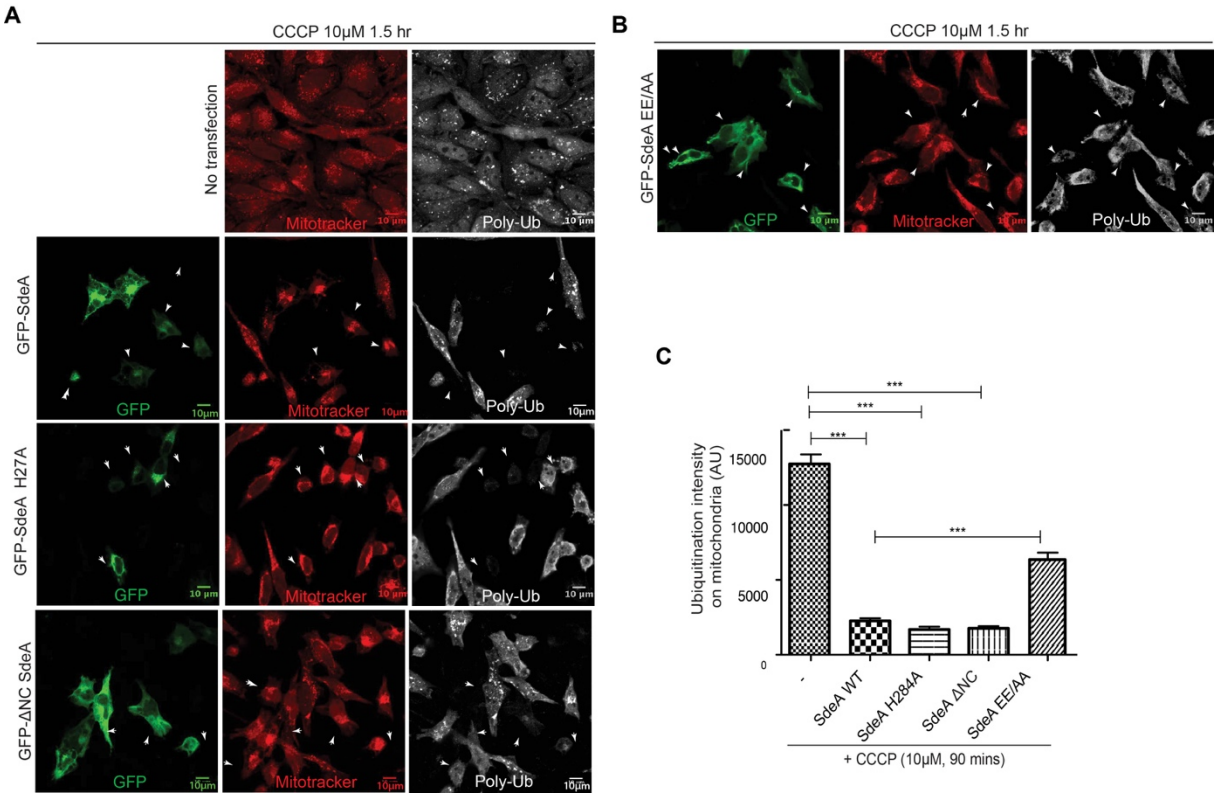


Figure 10. SdeA impairs CCCP induced mitochondria ubiquitination

(**A**) Images showing the polyubiquitination on mitochondria after mitophagy induction. HeLa cells stably expressing HA-Parkin were transfected with plasmids encoding WT or GFP-tagged SdeA H277A PDE mutant. 24 h after transfection, cells were treated with 10 mM CCCP for 90 min followed by staining with mitochondria marker MitoTracker and antibody against FK2 (poly-Ub). GFP-positive cells are marked with a white arrow. **(B**) Images showing the polyubiquitination on mitochondria after mitophagy induction in cells expressing SdeA EE/AA. (**C**) Quantitative analysis of CCCP-induced poly-ubiquitination at mitochondria of cells expressing SdeA WT or various SdeA mutants. Data are represented as mean ±SEM.

7.1.3.4 SdeA impairs TNF-α signaling

Ubiquitination of multiple proteins involved in different stages of TNF signaling transduction is critical for eventual activation and nuclear translocation of the transcription factor p65, which leads to the induction of the expression of its target genes (Chen and Chen, 2013). To verify whether SdeA expression also affected TNF-α-dependent activation of the NF-κB pathway, HeLa cells were transfected with plasmids encoding SdeA. After 24 h transfection, cells were fixed, p65 translocation was indicated with immunostaining followed by microscopy imaging. The result shows that expression of SdeA WT, the H277A mutant, or the DNC construct severely

inhibited p65 nuclear translocation, whereas the SdeA mART mutant EE/AA did not significantly affect activation of p65 (Fig. 11).

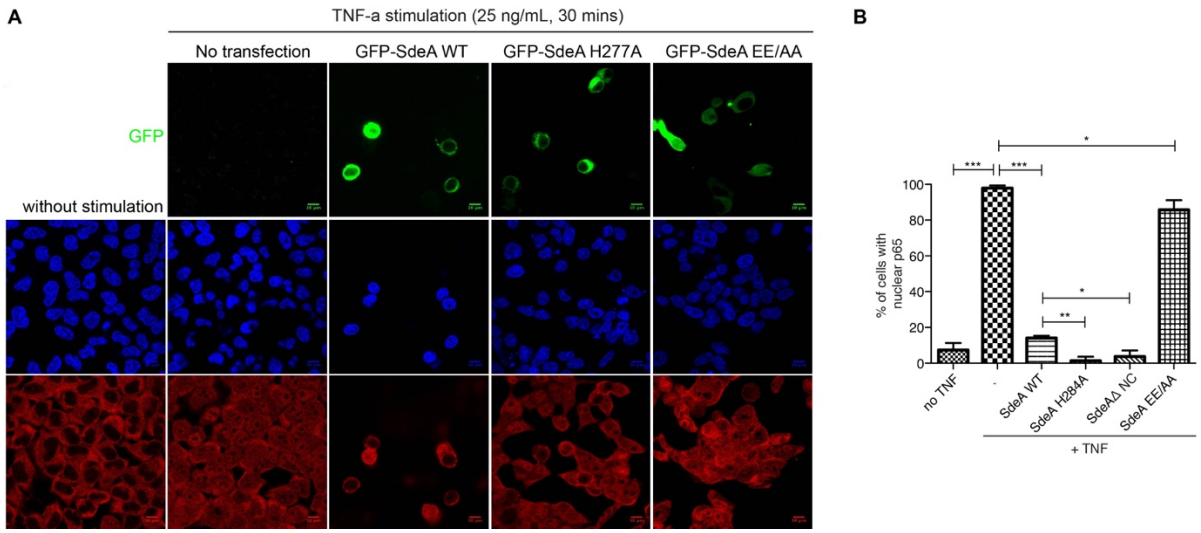


Figure 11. SdeA impairs NF-κB signaling by inhibiting ubiquitination.

(**A**) Monitoring SdeA effect on p65 translocation to nucleus. HeLa cells expressing WT and various mutants of SdeA were treated with TNF-α for 30 min followed by staining with anti-p65 and DAPI. Number of cells with p65 nuclear translocation was quantified in all the samples. (**B**) Quantitative analysis of effects of SdeA WT and its mutants on p65 nucleus translocation , data are represented as mean ± SEM.

7.1.4 SdeA does not modify ubiquitin-like proteins

Following the discovery of ubiquitin, multiple additional evolutionarily-related proteins of ubiquitin have been described, these ubiquitin-like proteins are involved in a wide array of cellular events with chemistry similar to ubiquitination. In particular, LC3 and GABARA proteins have been reported to be involved in xenophagy that protects host cells from pathogen invasion (Verlhac et al., 2015). Given that members of the UBL family are small proteins sharing a common structure with ubiquitin, we hypothesized that SdeA modifies these ubiquitin-like proteins as well by the same mechanism to facilitate bacterial infection and intracellular proliferation. In order to test this hypothesis, we purified LC3 and GABARA proteins from *E. coli* and then performed *in vitro* reactions by incubating them with purified SdeA H277A protein, in the presence of NAD^+. Ubiquitin was used as a positive control. Reaction products were separated with SDS-PAGE followed by immunoblotting with the antibody against ADP-ribose. Interestingly, the result showed that only ubiquitin treated with SdeA H277A was ADP-

ribosylated and detected by ADP-ribose antibody, other proteins were hardly detected as modified by SdeA H277A (Fig. 12). This data indicates that SdeA modifies only ubiquitin but not other UBL proteins.

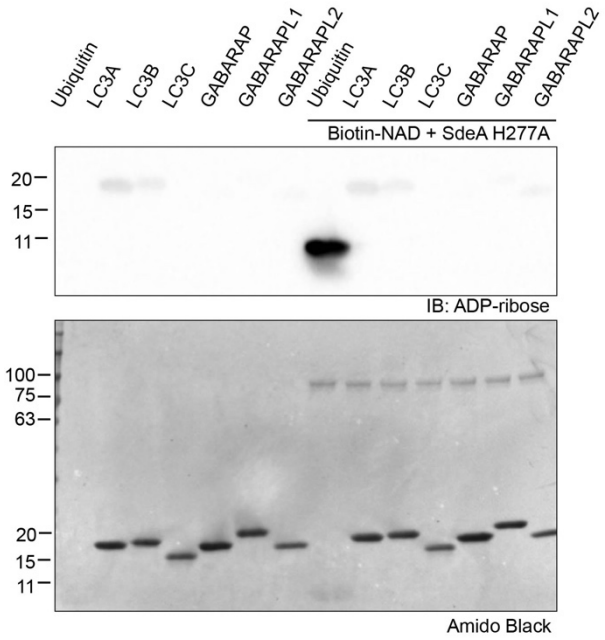
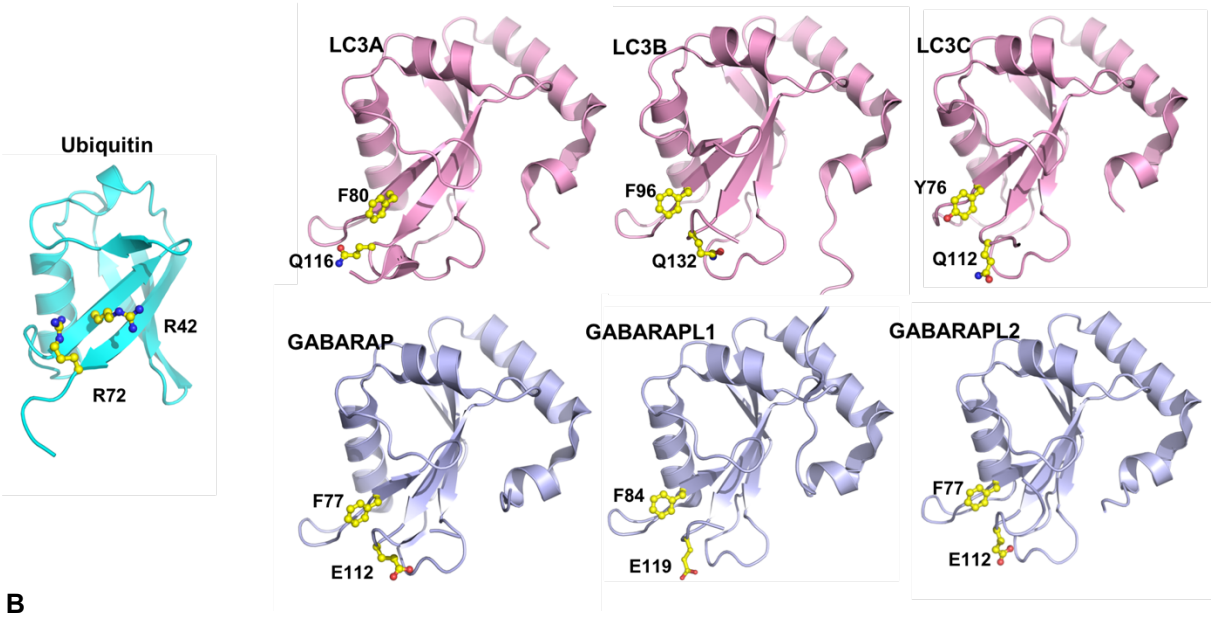


Figure 12. SdeA does not modify autophagy associated ubiquitin-like proteins.

Purified ubiquitin-like proteins LC3 A, B, C or GABARAP, L1, and L2 were incubated with SdeA ΔNC (200-1005) PDE mutant H277A in the presence of biotin labeled NAD^+. Reaction mixtures were separated with SDS-PAGE and stained with amido black dye or probed with a pan-ADP-ribose antibody.

According to the study by Qiu *et al*., SdeA ADP-ribosylates ubiquitin at R42 (Qiu et al., 2016). Recently, in studies of the SdeA structure, it was shown that Ub R72 and Ub R74 are bound in the negatively charged groove of SdeA mART, both of these two Arg in ubiquitin are essential for recognition by SdeA (Dong et al., 2018; Kalayil et al., 2018).To address the question why SdeA targets ubiquitin only, we analyzed the arginine residues on the surface of these proteins, and conducted a comparison of the ubiquitin-like modifiers to see how similar the structures are to ubiquitin. Interestingly, as shown, Arg42 is not conserved in other molecules LC3 and GABARA, instead, they have hydrophobic residues at R42 position and glutamine/glutamate (negatively charged) at Arg72 position of ubiquitin (Fig. 13). This structure comparison explains nicely the specificity of SdeA in targeting ubiquitin.

A



B

```
Ubiquitin         1 ----------------------------MQIFVKTL------TGKT--ITLEVEPSDTIEN 25
LC3A              3 -SDRPFKQRRSFADRCKEVQQIRDQHPSKIPVIIERYKGEKQLP-VLDKTKFLVPDHVNMSE 62
LC3B              2 PSEKTFKQRRTFEQRVEDVRLIREQHPTKIPVIIERYKGEKQLP-VLDKTKFLVPDHVNMSE 62
LC3C             11 ---RPFKQRKSLAIRQEEVAGIRAKFPNKIPVVVERYPRETFLP-PLDKTKFLVPQELTMTQ 68
GABARAP           1 -MKFVYKEEHPFEKRRSEGEKIRKKYPDRVPVIVEKAPKA-RIG-DLDKKKYLVPSDLTVGQ 59
GABARAPL1         1 -MKFQYKEDHPFEYRKKEGEKIRKKYPDRVPVIVEKAPKA-RVP-DLDKRKYLVPSDLTVGQ 59
GABARAPL2         1 -MKWMFKEDHSLEHRCVESAKIRAKYPDRVPVIVEKVSGS-QIV-DIDKRKYLVPSDITVAQ 59

                                    R42                                  R72
Ubiquitin        16 VKAKIQDKE--GIPPD-QQRLIFA-GKQL-EDGRTLSDY--NIQ---KESTLHLVLRLR-GG 76
LC3A             63 LVKIIRRRL--QLNPTQAFFLLVNQHSMV-SVSTPIADIYEQEKDEDGFLYMVYASQETFGF 121
LC3B             63 LIKIIRRRL--QLNANQAFFLLVN-GHSMVSVSTPISEVYESEKDEDGFLYMVYASQETF-- 119
LC3C             69 FLSIIRSRMVLRA-T-EAFYLLVNNKSLV-SMSATMAEIYRDYKDEDGFVYMTYASQETF-  125
GABARAP          60 FYFLIRKRI--HLRAEDALFFFVN-NVIP-PTSATMGQLYQEHHEEDFFLYIAYSDESVYG- 116
GABARAPL1        60 FYFLIRKRI--HLRPEDALFFFVN-NTIP-PTSATMGQLYEDNHEEDYFLYVAYSDESV--- 114
GABARAPL2        60 FMWIIRKRI--QLPSEKAIFLFVD-KTVP-QSSLTMGQLYEKEKDEDGFLYVAYSGENTFGF 117
```

Figure 13. Ubiquitin R42 and R72 are not conserved in ubiquitin-like ATG8 proteins

(**A**) The overall structures of ubiquitin and ATG8 proteins. R42 and R72 of ubiquitin were shown as yellow sticks. The figures were prepared using the program Pymol (pymol.org). (**B**) Structure-based sequence alignment of ubiquitin and ATG8 homologue proteins was performed with VMD program. R42, R72 of ubiquitin were marked in red. Amino acids of ATG8 proteins aligned with R42, R72 were shown in blue and green respectively.

7.2 Regulation of PR-ubiquitination by DupA/B

7.2.1 SidJ counteracts PR-ubiquitination in a non-deubiquitinase manner

Although the effectors of intracellular pathogens are important for bacterial infection and replication through modulating host cellular pathways, these secreted toxins need to be timely regulated once their tasks are completed, as pathogens require alive host cells to reside. The regulations of infectious bacteria effector activities by other

effectors have been previously documented. For example, during infection of *Salmonella enterica*, bacterial effectors SopE and SptP are injected into host cells by the type III secretion system. SopE serves as a guanine nucleotide exchange factor (GEF) and facilitates *Salmonella* infection through manipulating the host cytoskeleton. Excessive activation Rho GTPases by SopE is antagonized by SptP, which results in the protection of bacteria from host immune defense (Kubori and Galán, 2003). SidE family effectors are injected into host cells in the early stage of infection. To check whether these toxic effectors are controlled tightly, *Legionella* effectors were screened with deletion strains. It was shown that effector SidJ is able to specifically counteract the toxicity of SdeA and other members of the SidE family effectors (Jeong et al., 2015). Expression of low amounts of SidJ highly suppressed the toxicity caused by the transformation of SdeA plasmid in yeast (Jeong et al., 2015). In a study led by Qiu, a substrate of SdeA, Rab33b, was used as a monitor to test whether SidJ regulates the E3 ligase activity of SdeA during *Legionella* infection. Interestingly, infection of HEK293T cells expressing Rab33b with wild-type bacteria induced ubiquitination of Rab33b at short time (2 h) after bacterial uptake, however, at 4 h post infection, ubiquitination of Rab33b decreased in cells infected with *Legionella*, when the infection was extended to 6 h, ubiquitination of Rab33b could hardly be detected (Qiu et al., 2017).

In the study of Qiu *et al*., they argued that SidJ plays a critical role in *Legionella* intracellular growth during infection by functioning as a deubiquitinase for PR-ubiquitination, removing the ubiquitin from modified substrates of SdeA by cleaving the phosphodiester bond that links phosphoribosylated ubiquitin to the serines of protein substrates. Thus SidJ temporally regulates the effects of the SidE family effectors during bacterial infection by directly reversing SidE family effector mediated PR-ubiquitination (Qiu et al., 2017).

However, it is notable that the SidJ protein used in this study was purified from *Legionella* bacteria. We validated the SidJ protein expressed and purified from *E. coli*, but it did not work as DUB to cleave the ubiquitin from Ub-Rab33b. As full-length SidJ expressed in *E. coli* is not stable, which can be seen from the degraded bands of the protein on the Coomassie blue stained gel, we attempted to further confirm the activity of SidJ by expressing this protein in mammalian cells. In order to do this, we co-transfected Myc-Rab33b and N-terminally tagged mCherry-SidJ plasmids with GFP-tagged SdeA WT or SdeA H277A mutant into HEK293T cells. 24 h after transfection,

cells were lysed and the whole cell lysates were blotted with antibodies. As expected, expression of wild type SdeA ubiquitinated Myc-tagged Rab33b in cells, which is indicated by the shifted band of Myc-Rab33b, whereas SdeA H277A was not able to modify Rab33b, and ubiquitin was ADP-ribosylated but not phosphoribosylated due to the inactivation of PDE domain. Interestingly, expression of SidJ indeed diminished the ubiquitination of Rab33b catalyzed by SdeA in cells (Fig. 14A). This result in part supports the opinion that SidJ is a deubiquitinase that targets PR-ubiquitination, and is in agreement with a previous study showing that SidJ regulates the toxicity of SdeA by antagonizing the activity of this bacteria effector.

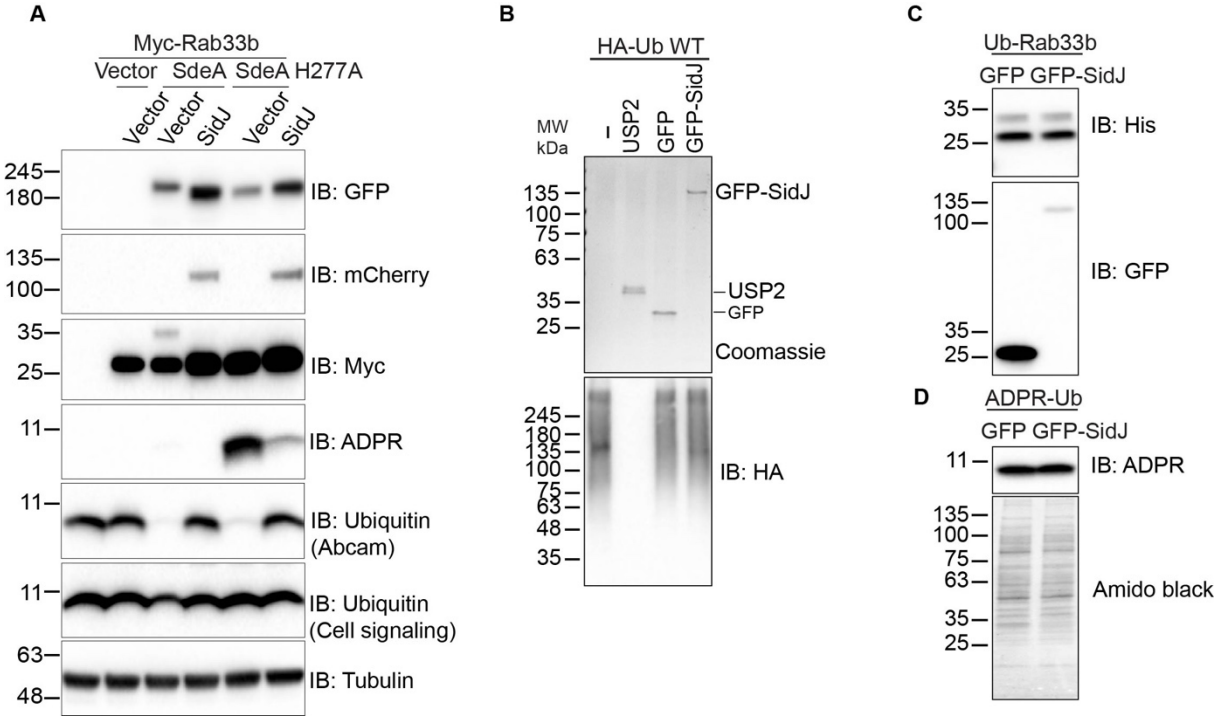


Figure 14. SidJ removes PR-ubiquitination in cells, but not *in vitro*

(**A**) HEK293T cells were co-transfected with plasmids encoding Myc-tagged Rab33b, SidJ with wild-type SdeA or PDE mutant SdeA H277A. Whole cell lysates were probed with antibodies against Myc or ADP-ribose to detect the ubiquitination of Rab33b or ADP-ribosylation of ubiquitin. (**B**) Conventionally ubiquitinated proteins were purified from cells expressing HA-tagged ubiquitin, then incubated with purified SidJ or deubiquitinase USP2 catalytic domain. Reaction mixtures were separated with SDS-PAGE and stained with Coomassie blue or probed with HA antibody. (**C**) SidJ purified from HEK293T cells transfected with SidJ plasmids was incubated with ubiquitinated His-tagged Rab33b for *in vitro* reaction. Reaction mixtures were separated with SDS-PAGE and blotted with antibodies against His_6 or GFP tag. (**D**) Cells expressing SidJ were lysed with lysis buffer without detergent using 30 G needle. Clarified lysates were incubated with ADP-ribosylated ubiquitin. Reaction mixtures were separated with SDS-PAGE and blotted with antibodies against ADP-ribose.

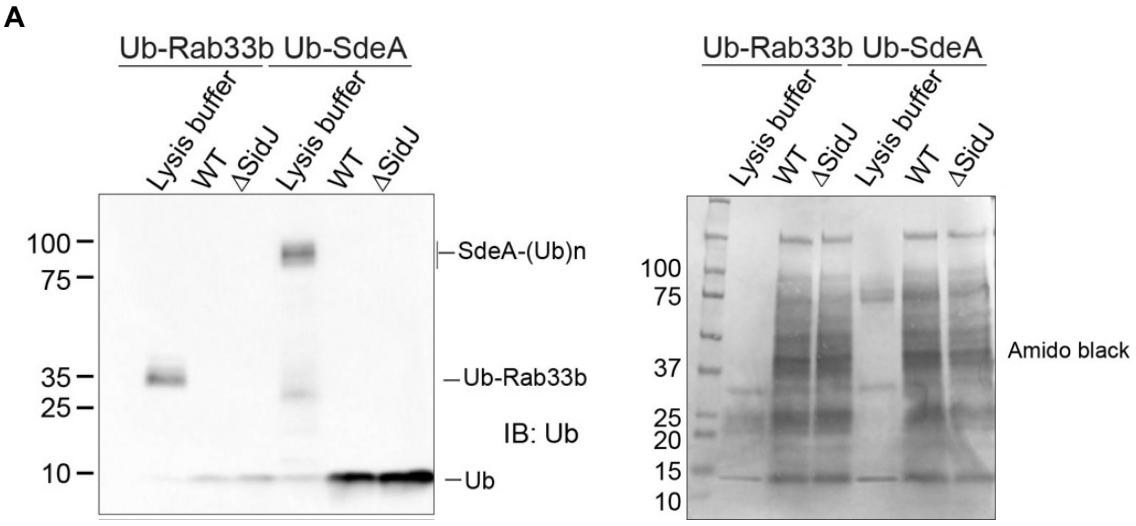
To further test the deubiquitinase activity of SidJ, we purified N-terminally tagged GFP-SidJ with GFP-trap beads, then did *in vitro* on beads-reaction by incubating pre-washed beads with purified ubiquitinated Rab33b at 37 °C for 1 h. Reaction products were separated with SDS-PAGE and stained with Coomassie blue, or blotted with the antibody against His_6 tag of Rab33b. The result shows that purified SidJ from cell lysate did not remove ubiquitin from PR-ubiquitinated proteins *in vitro*, as no decrease of the ubiquitination was detected, which is not consistent with the result of *in vivo* experiment. We then validated that whether exogenously expressed and purified SidJ from mammalian cells lysate displays classical deubiquitinase activity that showed in the previous study (Qiu et al., 2017). To test this, we transfected HA-tagged ubiquitin plasmid into HEK293T cells and then purified expressed HA-Ub with HA antibody to get classically ubiquitinated proteins. Purified ubiquitinated proteins were then incubated with purified GFP-tagged SidJ or USP2 catalytic domain at 37 °C for 1 h. The reaction products were separated with SDS-PAGE and stained with Coomassie blue or blotted with the antibody against HA tag. As expected, USP2 completely removed ubiquitin from the purified ubiquitinated proteins, whereas SidJ did show any deubiquitinase activity (Fig. 14B). One possibility is that SidJ catalyzes the deconjugation of ubiquitination in the presence of co-factor that might be washed off during the purification of GFP-SidJ from cells. To test this hypothesis, we attempted to use cell lysate containing GFP-SidJ to retain possible co-factor for treating the ubiquitinated substrates. We expressed GFP-SidJ in HEK293T cells and lysed cells with 30 G needle and 1 mL syringe by passing cells suspension through the needle 20 times. Cell lysates were incubated with purified Ub-Rab33b or ADP-ribosylated Ub at 37 °C for 1 h. The data showed that cell lysate containing GFP-SidJ did not cleave ubiquitin from either Ub-Rab33b or ADPR-Ub *in vitro* (Fig. 14C,D). So we questioned the possibility that SidJ inhibits SdeA activity as a deubiquitinase by directly reversing ubiquitination. Given that SidJ protein used in the published study was purified from *Legionella*, it is possible that other effector proteins that are able to erase ubiquitin from the substrates of SdeA were con-precipitated with SidJ. Thus, the *Legionella* effectors translocated to host cells capable of reversing SidEs-induced ubiquitination are also responsible for the decrease of ubiquitination of Rab33b in cells infected with *Legionella* for 6 hours.

7.2.2 Legionella effector DupA and DupB target PR-ubiquitination

7.2.2.1 Identification of SdeA PDE-like proteins from Legionella

To test whether there is another *Legionella* effector protein that possesses deubiquitinase activity, we cultured wild-type *Legionella* and also *Legionella* strain missing SidJ gene and extracted proteins from bacteria pellet for *in vitro* reaction. Ub-Rab33b proteins were incubated with protein lysates of different *Legionella* strains at 37 °C. Reaction products were separated with SDS-PAGE and stained with Coomassie blue or blotted with the antibody against ubiquitin. Interestingly, western blotting suggests that deletion of SidJ did not reverse the elimination of ubiquitination of Rab33b and SdeA, indicating the existence of deubiquitinase expressed by *Legionella*, which is not SidJ (Fig. 15A).

The processing of ADP-ribosylated Ub and linkage of PR-Ub to substrate serine residues by SdeA is catalyzed by its PDE domain (Bhogaraju et al., 2016; Kalayil et al., 2018). Moreover, ubiquitin cleaved from Rab33b by *Legionella* lysate was not detectable for the ubiquitin antibody from Abcam, indicating that cleaved ubiquitin might be still phosphoribosylated, and that the cleavage happens at the phosphodiester bond. As the PDE domain is capable of cleaving the phosphodiester bond in ADP-ribosylated Ub, we hypothesized that there are other *Legionella* effector proteins possessing PDE activity. To test this hypothesis, we sought out to do a screen of *Legionella* proteins that structurally similar to PDE of SdeA using sequence BLAST analysis. Based on sequence similarity, we identified 2 SidE family PDE like *Legionella* proteins: Lpg2154 (also named LaiE), and Lpg2509 (LaiF) (Fig. 15B).



B

```
SdeA-PDE      PQDKSVPVWNGFSLYTDDTVKAAAQYAYDNYLGKPYTGSVESA----------------- 263
LaiE          -----------MPIILDPEVLKVAEYVYQERLSKPYTEVGPEWEYNHKNPYATRATGTGH  49
LaiF          -----------MPIILDPDVLKVAEYVYQERLSKPYTEVGPEWEYNHKTPYATHATGTGH  49
                          : :  *  *  .*:*.*:: *.****    .

SdeA-PDE      ----PANFGGRMVYRQHHGLSHTLRTMAYAELIVEEARKAKLRGETLGKFKDGRTIADVT 319
LaiE          NLQRFITIDDQKLHRPIHGLAHTMRTLFYSQLMYEAAKRQ----PHPHRCADGRTIADLS 105
LaiF          NLQRFITINDQKLHRPIHGLAHTMRTLFYSQLMYEAAKRQ----PHPHRCADGRTIADLS 105
                    .:..: ::*  ***:**:**: *::*: * *::         :  *******::

SdeA-PDE      PQELKKIMIAQAFFVAGRDDEASDAKNYQKYHEQSRDAFLKYVKDNESTLIPDVFKDQED 379
LaiE          VEDLKKLNIAQLFFVAGRESEASYGDAYHRYHLYGAKQFEEYARKHLTHL----FS-EKE 160
LaiF          VQDLKKLNIAQLFFVAGRESEASYGDAYHRYHLYGAKQFEAYARKHLTHL----FS-EKE 160
               ::***: *** ******:.*** .. *::**  . . *  *.:.: : *    *. :::

SdeA-PDE      VNFYARVIEDKSH-DWESTPAHVLINQGHMVDLVRVKQPPESFLQRYFSS------MQRW 432
LaiE          IVLYSRCIEDRIGDRFDETAEGYLIHLSHMIDLMRCKSPVEVFIGHSRGVSGIVPTLIQL 220
LaiF          ITLYSRCIEDRIGDRFDETAEGYLIHLSHMIDLMRCKSPVEVFIGHSLGVSGIVPTLIQL 220
              : :*:* ***:    ::.*    **: .**:**:* *.* * *: :  .       : :
```

Figure 15. Legionella effector eliminates PR-ubiquitination caused by SdeA

(**A**) Verification of the effect of SidJ on PR-ubiquitination with *Legionella* lysates. Wild type *Legionella* and mutant strain lacking SidJ were grown to the early stationary phase and then lysed with lysis buffer for bacteria. PR-ubiquitinated Rab33b or SdeA were incubated Clarified lysates at 37 °C for 1 h. Reaction products were separated with SDS-PAGE and blotted with the antibody against ubiquitin. (**B**) Sequence alignment of SdeA PDE with *Legionella* effectors LaiE and LaiF generated with ClustalW. Essential residues in the PDE catalytic core were marked in red.

7.2.2.2 DupA (LaiE) and DupB (LaiF) specifically reverse PR-ubiquitination

To validate the functions of these two putative PDE effector proteins, we cloned Lpg2154 and Lpg2509 genes from *Legionella* cDNA of strain Philadelphia 1 (ATCC 33152), inserted them into *E. coli* expression vector for expressing and purifying recombinant proteins. We then attempted to check whether LaiE and LaiF cleave

PR-ubiquitinated SdeA or Rab33b at the phosphodiester bond by performing an *in vitro* reaction. Purified PR-ubiquitinated SdeA or PR-ubiquitinated Rab33b proteins were incubated with purified LaiE or LaiF at 37 °C for 1 h, the reaction products were separated with SDS-PAGE and stained with Coomassie blue or blotted with the antibody against ubiquitin, the result shows that ubiquitination bands of SdeA and Rab33b were removed, indicating that LaiE/LaiF indeed cleaved ubiquitin from these two modified substrates (Fig. 16A,B). Considering SdeA PDE functions to cleave ADP-ribosylated ubiquitin at the phosphodiester bond, we did the similar *in vitro* reaction experiment to check whether LaiE/LaiF also cleave ADPR-Ub, the result suggests that ADP-ribose signals diminished when the purified ADPR-Ub proteins were treated with LaiE or LaiF, indicating that LaiE and LaiF also cleave ADPR-Ub as SdeA PDE domain does (Fig. 16C).

To further confirm this observation and verify the activities of LaiE and LaiF, we co-expressed mCherry-tagged LaiE or LaiF with SdeA and its substrate Rab33b. 24 h after transfection, cells were lysed and the whole cell lysates were blotted with the antibody against ADP-ribose to check whether LaiE and LaiF remove the ADP ribosylation modification induced by SdeA PDE mutant in cells. Consistent with earlier observation, expression of SdeA WT induced ubiquitination of Rab33b, which is indicated by the shifted band with higher molecular weight. SdeA H277A ADP-ribosylated ubiquitin, but did not catalyze the ubiquitination of Rab33b due to the inactivation of PDE in SdeA. Similar to the result of *in vitro* reaction of recombinant proteins, both LaiE and LaiF expressed in cells removed the ADPR modification of ubiquitin catalyzed by mART activity of SdeA. In addition, LaiE and LaiF also eliminated the ubiquitination of Rab33b caused by wild type SdeA (Fig. 16D). Our data suggest that LaiE and LaiF not only remove the ADP-ribosylation of ubiquitin, but also reverse the PR-ubiquitination of host substrates caused by SdeA.

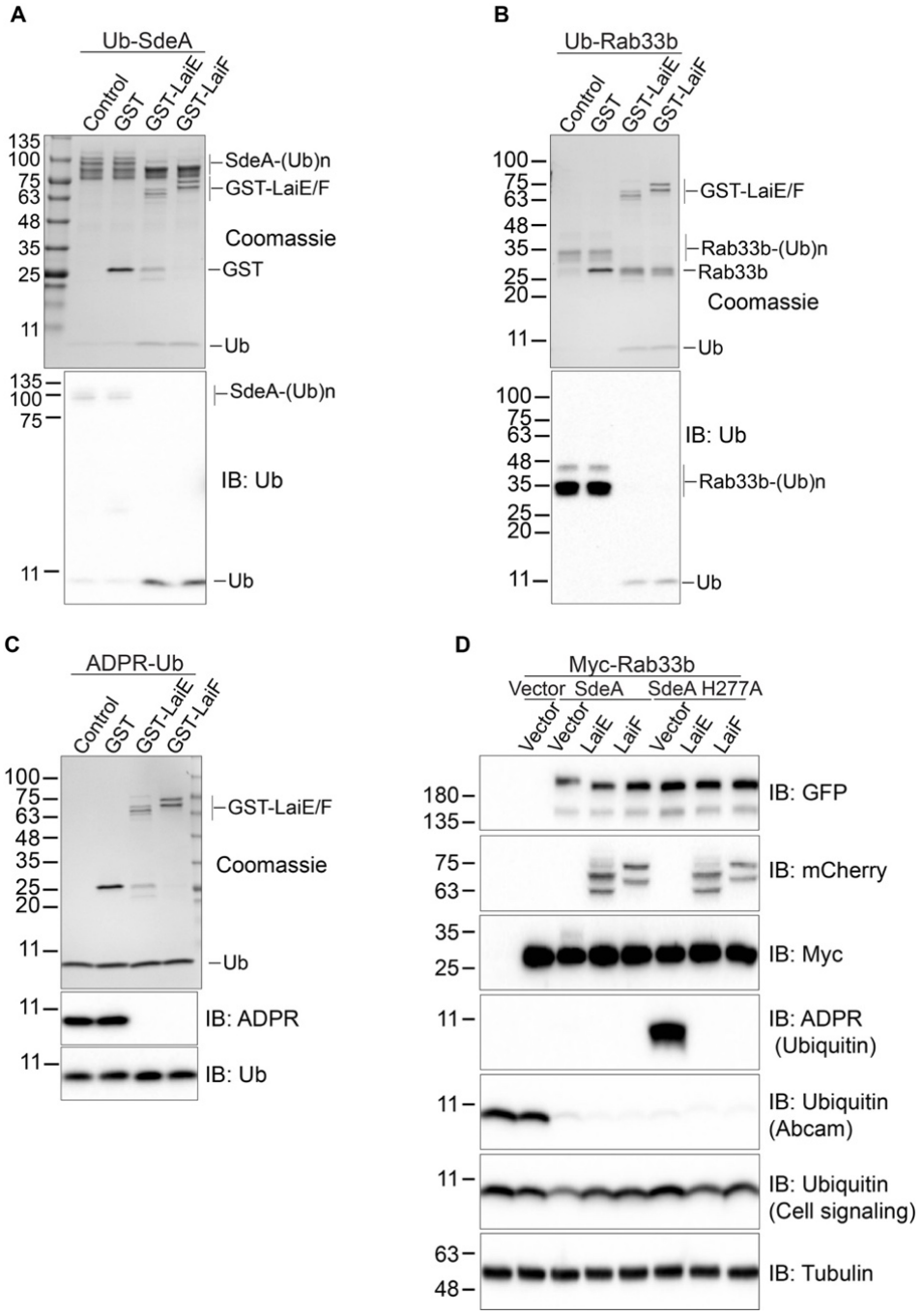


Figure 16. Legionella effectors LaiE/LaiF eliminate PR-ubiquitination caused by SdeA

(**A**, **B**) Putative *Legionella* PDE enzymes LaiE and LaiF were expressed and purified from *E. coli*, then incubated with ubiquitinated Rab33b or SdeA. Reaction mixtures were separated with SDS-PAGE and then stained with Coomassie blue or blotted with antibody against ubiquitin. (**C**) Purified LaiE/LaiF were incubated with ADP-ribosylated ubiquitin. Reaction mixtures were separated with SDS-PAGE and then stained with Coomassie blue or blotted with antibodies against ubiquitin or ADP-ribose. (**D**) LaiE or LaiF genes were cloned into a mammalian expression vector, then co-transfected into HEK293T cells with SdeA and Myc-Rab33b plasmids. Whole cell lysates were probed with the antibodies against Myc or ADP-ribose.

To understand the specificity of LaiE and LaiF on ubiquitination, we generated conventional ubiquitination of proteins by expressing HA-tagged ubiquitin in HEK293T cells, and novel PR-ubiquitination of proteins by co-expressing SdeA and HA-tagged Ub without C-terminal two glycine residues (ΔGG), which can only mediate PR-ubiquitination but not classical ubiquitination (Fig. 17A). Ubiquitinated proteins were then immunoprecipitated with HA antibody and incubated with LaiE or LaiF. Catalytic domain of the deubiquitinase USP2 was used as a positive control for reverse of conventional ubiquitination, it indeed completely cleaved the ubiquitin from the purified proteins, but did not alter the novel PR-ubiquitination. On contrary, LaiE and LaiF removed the PR-ubiquitination caused by SdeA, but did not change the normal ubiquitination at all (Fig. 17B). This data suggests that LaiE and LaiF specifically deconjugates the novel serine ubiquitination catalyzed by SdeA. Thus these two *Legionella* effectors were renamed as Deubiquitinases for PR-ubiquitination (DUPs): DupA/LaiE and DupB/LaiF.

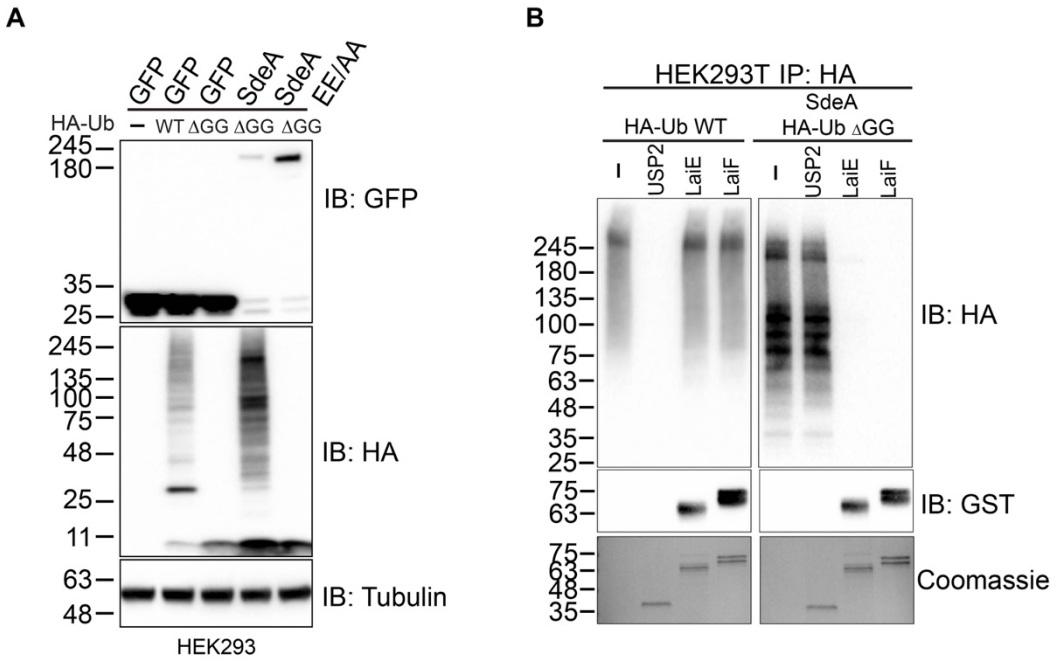


Figure 17. Legionella effectors LaiE/LaiF specifically target PR-ubiquitination caused by SdeA

(**A**) Generation of PR-serine ubiquitination by co-expression SdeA and HA-UbΔGG in HEK293T cells. Plasmids encoding SdeA or HA-tagged UbΔGG missing C-terminal Glycine were transfected into HEK293T cells, whole cell lysates were probed with antibodies against GFP or HA. (**B**) Conventionally ubiquitinated proteins or PR-serine ubiquitinated proteins were purified with HA antibody beads, then incubated with deubiquitinase USP2 catalytic domain or *Legionella* effectors LaiE/LaiF. Reaction mixtures were separated with SDS-PAGE and stained with Coomassie blue or blotted with the antibody against HA tag.

To examine whether there are other Legionella effectors that are capable of cleaving ubiquitin from PR-ubiquitinated proteins, *Legionella* mutant strain that lacks DupA and DupB was grown and the cell lysate was incubated with PR-ubiquitinated Rab33b at 37 °C for 1 h. Wild type *Legionella* and strain lacking SidJ were used as positive controls for cleaving. Mixtures were analyzed with immunoblotting using the antibody against ubiquitin. The result shows that lysate of DupA/B depletion *Legionella* strain failed to hydrolyze PR-ubiquitin from Rab33b (Fig. 18A), indicating that DupA/B might be the only two enzymes that cleave ubiquitin from the modified substrates of SdeA. We then asked whether this kind of deubiquitinating proteins specific for PR-ubiquitination exists in other bacteria that used in our lab. To test this, different *E. coli* strains, *Salmonella typhimurium*, *Shigella flexneri*, as well as *Legionella pneumophila* were grown in S2 lab, cells were collected and lysed for extraction of proteins. Clarified lysates were used for incubation with ubiquitinated Rab33b or SdeA. Reaction mixtures were probed with the antibody against ubiquitin to verify the effect of bacterial lysates on PR-ubiquitination. Western blotting result showed that only *Legionella* possessing DupA/B reversed PR-ubiquitination of substrates of SdeA, indicating that catalysis and reverse of PR-ubiquitination are likely specifically existed in *Legionella* (Fig. 18B).

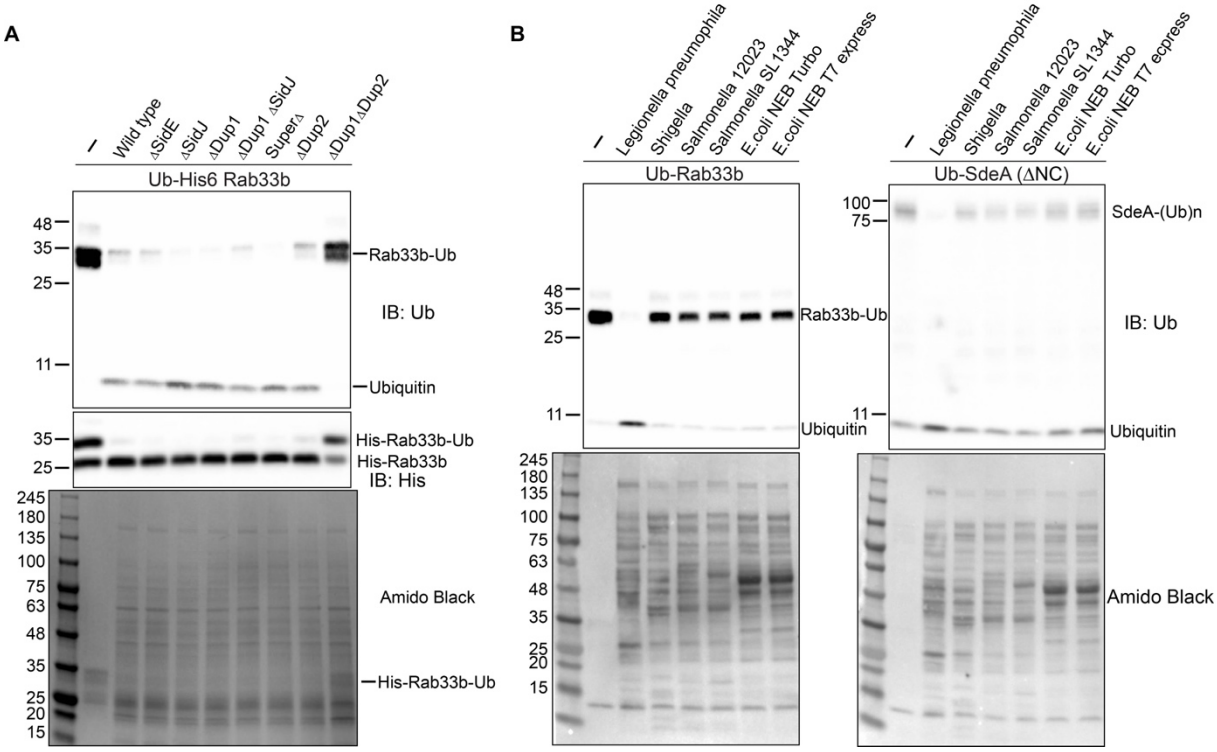


Figure 18. Legionella effectors DupA/B are the only two deubiquitinases that specifically target PR-ubiquitination caused by SdeA.

(**A**) Cells of WT *Legionella* and other mutation strains were grown and lysed. Clarified lysates were used to incubation with Ub-Rab33b at 37°C for 1 hour. Reaction products were separated with SDS-PAGE followed by Coomassie blue staining or probed with the antibody against ubiquitin. (**B**) Cells of *Legionella* and other bacterial strains were grown and lysed. Clarified lysates were used to incubation with Ub-Rab33b or Ub-SdeA at 37°C for 1 hour. Reaction products were separated with SDS-PAGE followed by Coomassie blue staining or probed with the antibody against ubiquitin.

7.2.2.3 DupA and DupB function as deubiquitinase during infection

Next, we sought to validate the activity of DupA/B during *Legionella* infection. We used wild-type *Legionella* or different mutant strains and checked the PR-ubiquitination of proteins in infected HEK293T cells. Infection with wild-type *Legionella* strain caused PR-ubiquitination of Rab33b at 2 h post-infection and subsequent reduction at 6 h post infection. Whereas cells infected with a strain lacking DupA/DupB maintained more PR-ubiquitinated Rab33b at 2 h, compared to cells infected with the wild-type *Legionella*, moreover, this accumulation of Ub-Rab33b was further increased at 6 h post infection (Fig. 19). This indicates that DupA and DupB are not only active as deubiquitinating enzymes *in vitro*, but also critical in the regulation of PR-ubiquitination during bacterial infection.

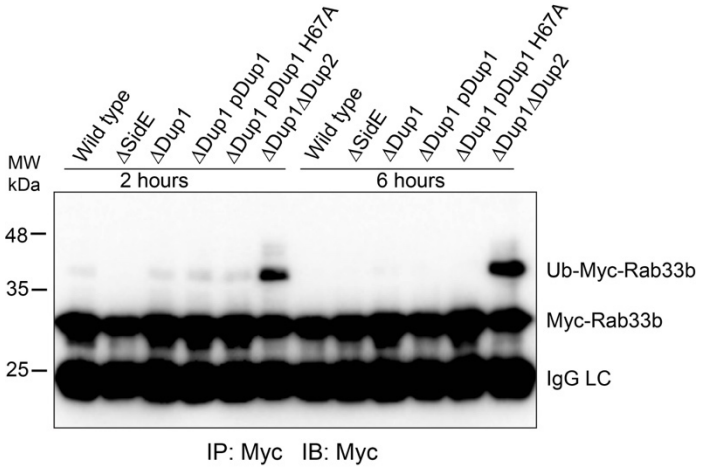


Figure 19. DupA and DupB regulate PR-ubiquitination caused by SdeA during infection.
HEK293T cells expressing Myc-Rab33b were infected with wild type *Legionella* or different mutant strains for 2 hours or 6 hours. Cells were lysed then the clarified lysates were used for Myc-IP. The isolated proteins were probed with the antibody against Myc tag.

7.2.3 DupA and DupB cleave the phosphodiereaster bond

7.2.3.1 PDE essential amino acids are conserved in DupA/B

Recently, studies of different groups determined structure of the catalytic core of SdeA, according to which the catalytic mechanism of serine PR-ubiquitin is uncovered. Three amino acids (H277, E340 and H407) of catalytic core in PDE domain are critical for the transfer of ubiquitin moiety to substrates (Akturk et al., 2018; Kalayil et al., 2018). Briefly, E340 functions in the formation of transient phosphohistidine intermediate by linking PR-ubiquitin and H277 of SdeA PDE, while H407 plays a role in the activation of substrate serine and final transfer of PR-ubiquitin to substrates (Kalayil et al., 2018). Sequence alignment revealed that the catalytic residues of the SdeA PDE domain are highly conserved in these 2 putative PDE *Legionella* proteins (Fig. 15 B). To determine whether DupA/DupB function as PDE enzyme by the same mechanism with the PDE domain of SdeA, 3 amino acids E126, H67, H189 in DupA or DupB predicted to be critical in the catalytic core were substituted with alanine. Purified mutant proteins from *E. coli* were incubated with Ub-Rab33b and ADPR-Ub. As expected, the result showed that wild-type DupA cleaved the modified Rab33b and ubiquitin. Interestingly, the mutants were almost completely inactive to remove ubiquitin from modified Rab33b or eliminate APR-ribose signal from ADPR-Ub (Fig. 20A, B). Consistently, mutating H67 of DupA or DupB to alanine inhibited the removal of PR-ubiquitination of Rab33b in cells expressing SdeA (Fig. 20C). These data suggest that DupA/B may share the same catalytic residues with the PDE domains of SidEs to mediate opposite reactions: removal of PR-Ub from substrates (Deubiquitination).

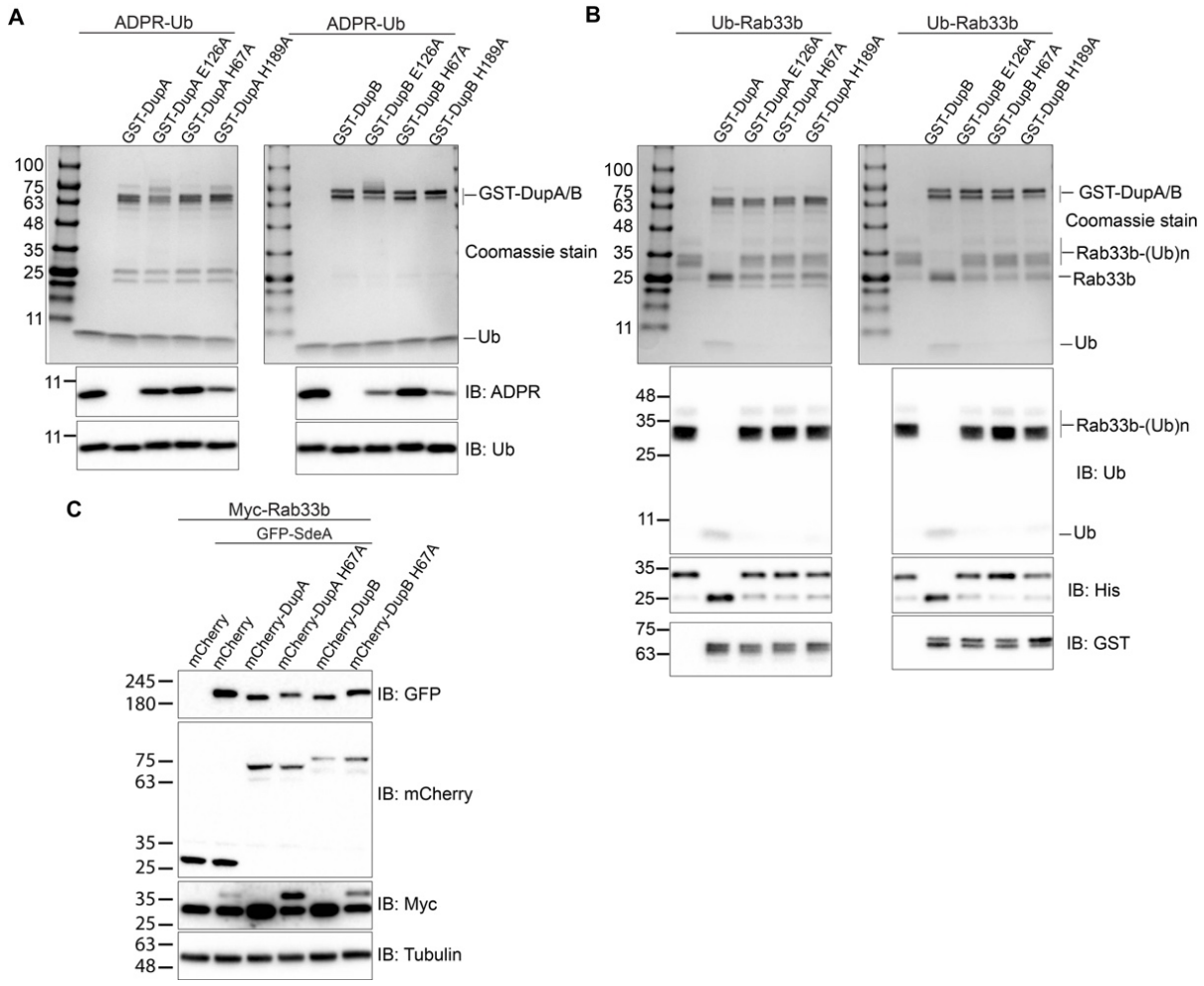


Figure 20. Conserved catalytic residues are essential for the functions of DupA and DupB .
(**A**) ADPR-Ub proteins were incubated with purified wild-type DupA/B or mutants at 37 °C for 1 hour. Reaction products were separated with SDS-PAGE followed by Coomassie blue staining or western blotting with antibodies against ubiquitin or ADP-ribose. (**B**) His_6-tagged Ub-Ra33b proteins were incubated with purified wild type DupA/B or mutants at 37 °C for 1 hour. Reaction products were separated with SDS-PAGE followed by Coomassie blue staining or western blotting with antibodies against ubiquitin or His. (**C**) Plasmids encoding Myc-Rab33b and SdeA were co-transfected with wild type DupA/B or H67A mutant plasmids. Whole cell lysates were probed with antibody against Myc tag.

7.2.3.2 DupA/B cleave the phosphodiester bond

SdeA plays its phosphodiesterase activity by cleaving the phosphodiester bond between the 2 phosphates within the ADPR-Ub, which results in the generation of PR-Ub, and linkage of this moiety via a phosphoribosylated bond to the serine residues of the substrates. We hypothesized that DupA/B work like SdeA PDE domain by cleaving the phosphodiester bond of ADPR-Ub and the same bond that links the PR-Ub and the serine of substrate. To determinate the mechanism of DupA mediated ubiquitin deconjugation of the substrates of SdeA and the ADP-

ribosylation removal of modified ubiquitin, Ub-Rab33b or ADPR-Ub were incubated with DupA/B at 37 °C. The reaction products were detected with Coomassie blue staining and the Pro-Q Diamond phosphoprotein staining. Interestingly, ADPR-Ub treated with DupA or DupB were detected by Pro-Q Diamond phosphoprotein staining, indicating that the cleavage occurs at the phosphodiester bond between 2 phosphates, which is the same as the behavior of SdeA PDE. In the case of treatment of Ub-Rab33b, similar to the PR-Ub produced from the reaction of SdeA and ubiquitin in the presence of the NAD^+, the ubiquitin products processed from Ub-Rab33b by DupA or DupB were detectable by phosphoprotein staining (Fig. 21A, B). This data suggests that DupA cleaves the phosphodiester bond that links PR-Ub to the serine of the substrates. To further confirm the cleavage site of DupA, the rest samples were separated on SDS-PAGE gel and ubiquitin species cleaved from ADPR-Ub, Ub-Rab33b were digested and prepared for mass spectrometry analysis. In agreement with the conclusion got from Pro-Q Diamond phosphoprotein staining, mass spectrometry data show that ubiquitin species cleaved from ADPR-Ub and Ub-Rab33b are PR-Ub, thus confirming that DupA/B cleave the phosphodiester bond that links PR-Ub and substrate serine (Fig. 21C, D).

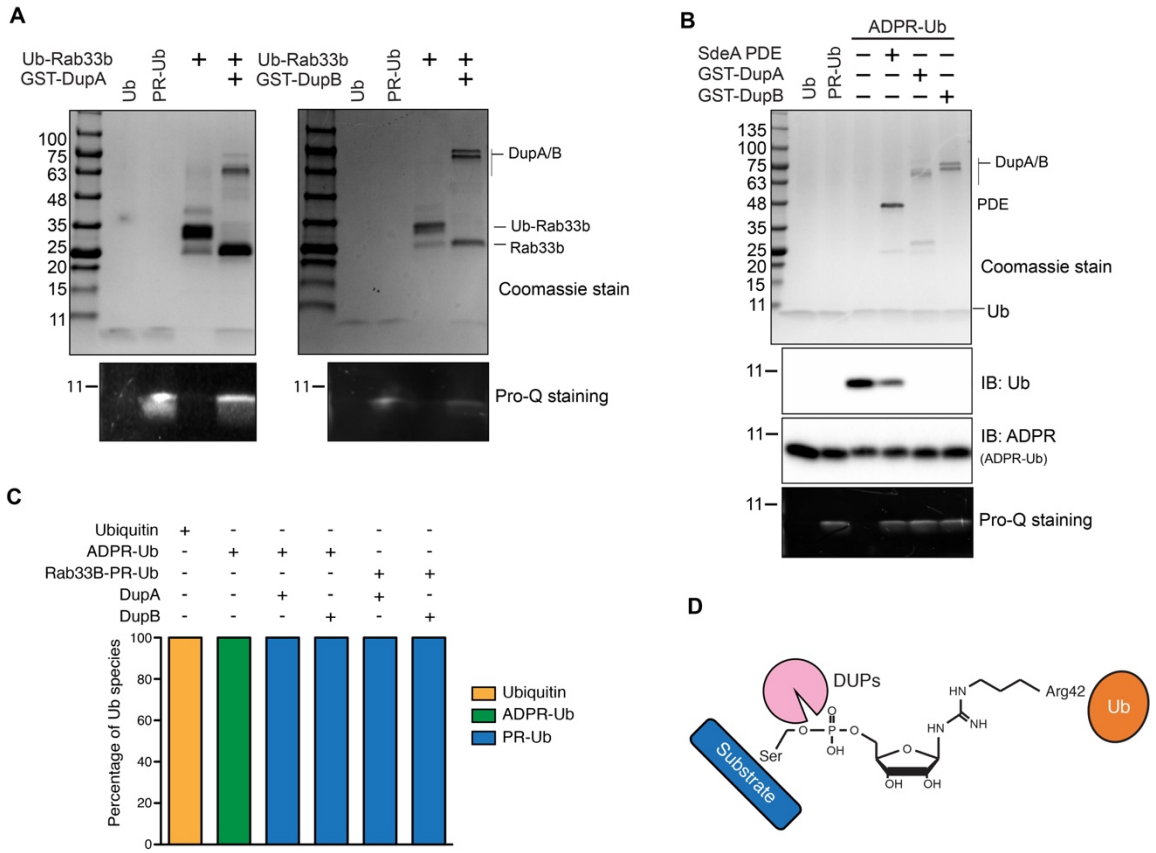


Figure 21. DupA/B cleave the phosphodiester bond between PR-Ub and the substrate serine residue.

(**A**) Ub-Rab33b was incubated with GST-DupA/B, ubiquitin cleaved from modified Rab33b was verified with Pro-Q phosphoprotein staining. Wild type ubiquitin and PR-ubiquitin produced with SdeA were used as negative and positive control respectively. (**B**) ADPR-Ub was incubated with GST-DupA/B, processed ubiquitin was probed with antibodies or verified with Pro-Q phosphoprotein staining. (**C**) Reaction products of (**A**) and (**B**) were analyzed with mass spectrometry. Ubiquitin species produced from the reactions were showed as indicated. (**D**) Schematic chemical representation of cleavage of phospho-ribose linkage between ubiquitin and substrates by DupA/B. *(MS analysis of Fig. 18C was performed by Florian Bonn).

7.2.3.3 DupA/B function differently from PDE

It is interesting that novel deubiquitinases DupA/B catalyze opposite modification with SdeA PDE in a mechanism appears to be uniform, to distinguish the differences between SdeA PDE and DupA, we compared the PDE activities of DupA with the SdeA PDE domain. Firstly, we checked the activity difference between DupA and PDE on the cleavage of the ubiquitinated substrate. We incubated Ub-Rab33b with DupA or SdeA PDE, the immunoblotting shows that only DupA removed ubiquitin from Rab33b, SdeA PDE could not reverse the ubiquitination modified by itself (Fig. 22A). Then we compared functions of DupA/B or SdeA PDE in cleavage of ADPR-Ub and transfer of PR-Ub to substrate serine. As expected, SdeA PDE cleaved the phosphodiester bond of ADPR-Ub, which is indicated by the reduced signal of ADP-ribose of ubiquitin. Interestingly, DupA showed higher activity than SdeA PDE and eliminated the ADP-ribosylation of ubiquitin completely (Fig. 22A). To make sure that the separated PDE activity is not impaired compared with the full length, we used the truncated SdeA containing both mART and PDE domain, and the full-length SdeA to verify the activities of these PDE on the cleavage of Ub-Rab33b. The longer PDE proteins broke down the ADP-ribosylation on ubiquitin, however, they did not display any activity to cleave ubiquitin from modified Rab33b (Fig. 22A, B). In the ubiquitination process mediated by SdeA, the cleavage of the phosphodiester bond is followed by the transfer of the phosphoribosylated ubiquitin to substrates. So we compared the activities of SdeA PDE and DupA on the cleavage of ADPR-Ub when the substrate Rab33b or the 11 amino acid-peptide of another substrate RTN4B that can be recognized and ubiquitinated by SdeA are present. Western blotting data showed that SdeA PDE cleaved the ADPR-Ub and transferred the PR-Ub to Rab33b

and RTN4B peptide, however, DupA did not exhibit the activity to further ubiquitinate these substrates.

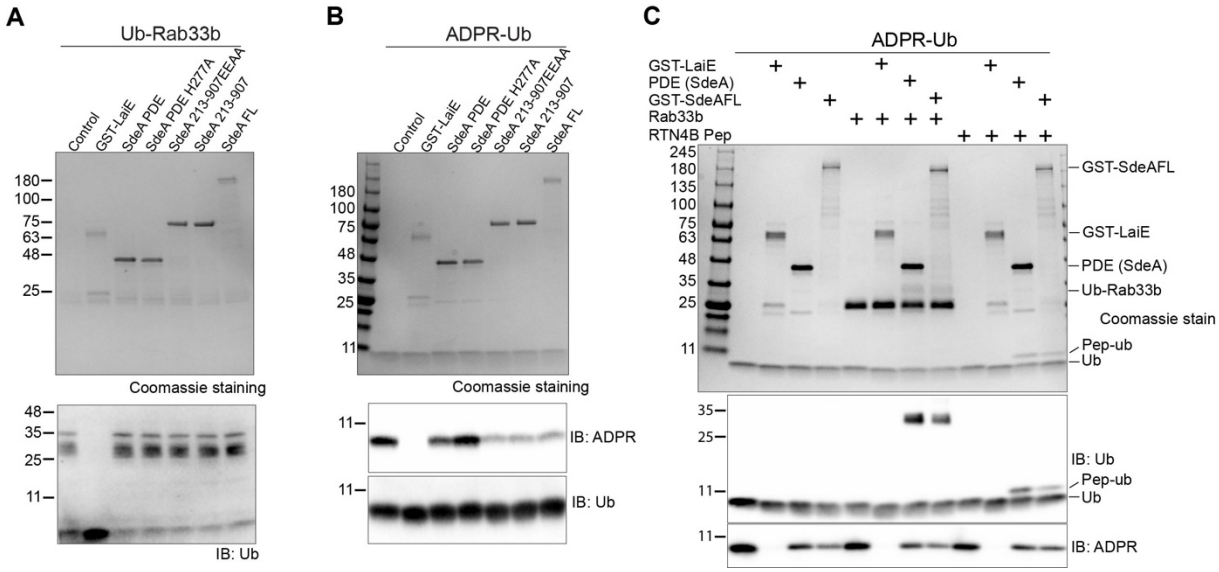


Figure 22. DupA/B function differently from SdeA PDE

(**A**) Purified Ub-Rab33b were treated with GST, GST-tagged DupA or SdeA PDE. *In vitro* reaction products were separated with SDS-PAGE and stained with Coomassie blue or blotted with antibody against ubiquitin. (**B**) Purified ADPR-Ub was treated with GST-tagged DupA or SdeA PDE. *In vitro* reaction products were separated with SDS-PAGE and stained with Coomassie blue or blotted with antibodies against ADP-ribose or ubiquitin. (**C**) Purified ADPR-Ub were treated with GST-tagged DupA or SdeA PDE in the presence of Rab33b or RTN4B peptide. *In vitro* reaction products were separated with SDS-PAGE and stained with Coomassie blue or blotted with antibodies against ADP-ribose or ubiquitin.

Unlike SdeA functioning as ubiquitin ligase that catalyzes PR-Ubiquitination of substrates, DupA/B did not show activity to transfer PR-Ub to the substrate. Our further *in vitro* reaction experiment suggests that DupA mediated ubiquitination of substrate Rab33b after a short term of incubation (5 min), this ubiquitination disappeared rapidly (Shin et al., 2020). Determination of the structure of DupA H67A and PR-Ub complex and biochemical experiments revealed that DupA has stronger binding affinity to ubiquitin, ADPR-Ub and PR-ubiquitinated substrates, because of extensive electrostatic interactions. However, SdeA PDE does not bind to PR-ubiquitinated substrates (Shin et al., 2020). These differences explain nicely why SdeA PDE does not has the deubiquitinase activity of DupA, and why DupA cleaves ADPR-Ub effectively but does not transfer PR-Ub to substrates.

7.2.4 Identification of substrates of SidE family effectors using DupA mutant

In consideration of the critical roles of SidE family effectors in bacteria infection and proliferation, it is necessary to identify the PR-ubiquitination substrates of SidE family effectors for understanding the strategy that *Legionella* adopts for efficient invasion and better understanding of biological mechanism of the regulation of cellular pathways. However, substrates of SidE effectors and subsequent functions of PR-ubiquitination were poorly understood. Given that DupA targets to PR-ubiquitinated substrates through its strong binding affinity, we hypothesized that catalytically dead mutant of DupA (H67A) that binds with modified substrates but not able to cleave down the ubiquitin from substrates could be used as a bait to isolate PR-ubiquitinated proteins from lysates of cells infected with *Legionella* for mass spectrometry analysis. To test this hypothesis and identify PR-ubiquitinated endogenous proteins, we infected HEK293T cells expressing FcγRII receptor with *Legionella* strains and incubated cell lysates with GST-tagged DupA H67A. Enriched proteins were investigated with western blotting using the antibody against ubiquitin. Interestingly, the result suggests that DupA inactive mutants H67A effectively bound and enriched many PR-ubiquitinated substrates from infected cells (Fig. 23 A).

To determinate these isolated PR-ubiquitinated proteins, we incubated the pulled down products with wild-type DupA and eluted PR-ubiquitinated proteins by cleaving the phosphodiester bond to the substrate serine. This DupA-catalyzed cleavage strategy makes it possible to elute PR-ubiquitinated proteins without contamination of unspecific interactions, such as conventionally ubiquitinated proteins. More than 1,000 proteins from cells infected with WT *Legionella* or the *ΔdupA/B* strain were identified by using the DupA H67A trapping mutant (Fig. 23A, B). Of these potential substrates, 181 proteins were reproducibly and significantly enriched from mammalian cells infected with *Legionella* strains (WT or *ΔdupA/B*) compared to control cells or cells infected with a strain lacking SidE effectors (Fig. 23C).

Interestingly, among these identified PR-ubiquitination substrate proteins, many endoplasmic reticulum (ER) resident proteins, such as FAM134C, RTN4 and TEX264, were highly enriched and scored with high ratios of dupA/B over sidEs infection (Supplementary data 1). A number of these proteins are members of reticulon-type ER membrane proteins that are involved in the modulation of ER remodeling or selective ER fragmentation and autophagy (ER-phagy) (Grumati et al., 2017). Further study

demonstrated that PR-ubiquitination catalyzed by SdeA plays a critical role in ER remodeling of host cells (Shin et al., 2020).

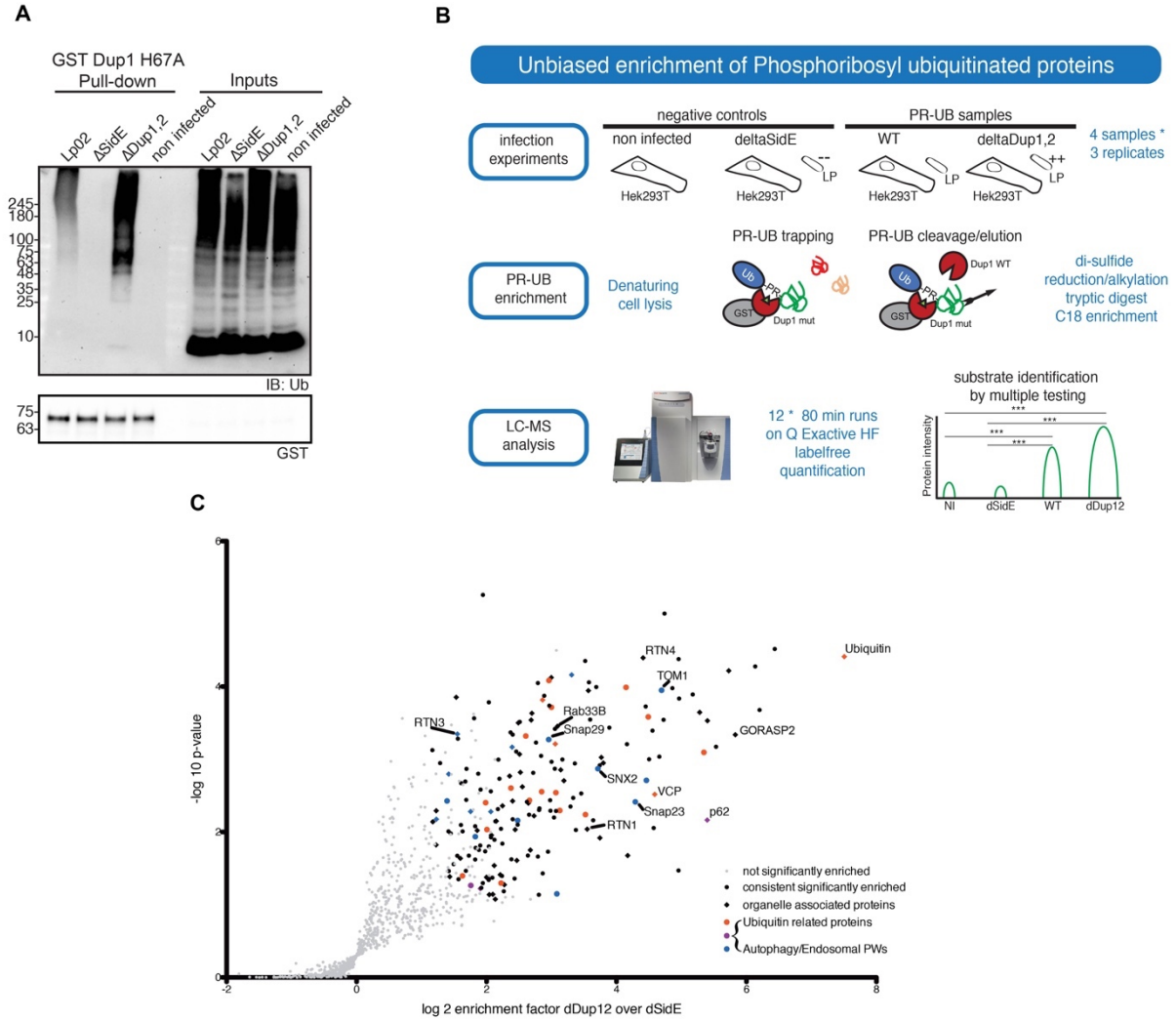


Figure 23. Identification of phosphoribosyl-linked ubiquitination substrates using DupA dead mutant.

(**A**) HEK293T cells expressing FcγRII were infected with wild type or mutant *Legionella* strains. Clarified cell lysates were used for incubation with purified GST-DupA H67A mutant protein. Isolated proteins were separated with SDS-PAGE and probed with antibodies against ubiquitin or GST. (**B**) Schematic diagram of the proteomic approach for identification of PR-ubiquitination substrates using DupA catalytically dead mutant. (**C**) Volcano plot showing identified substrates by quantitative mass spectrometry.

*(The experiments for Figure 23 were performed together with Alexis Gonzalez and Florian Bonn)

7.3 PR-ubiquitination regulates Golgi morphology and secretory pathway

Using the deubiquitinase DupA mutant that specifically binds to PR-ubiquitinated substrates as a trapping bait, we identified over 180 putative targets of SdeA.

Besides ER related proteins, Golgi and mitochondrial associated proteins were also identified (Shin et al., 2020). However the biological consequences of PR-ubiquitination of these Golgi proteins were poorly understood. To this end, we performed biochemical and microbiological experiments to validate SdeA mediated PR-ubiquitination of Golgi tethering proteins GRASP55, GRASP65 and GCP60 and explore the effects of PR-ubiquitination of these proteins on Golgi regulation.

7.3.1 SdeA localizes to ER and partially to the Golgi in cells

Biochemical studies have been previously conducted to understand the function of SdeA, which comprises of an N-terminal DUB domain spanning residues 1 to 200, PDE and mART domains that act as core catalytic region, spanning residues 213 to 907 (Bhogaraju et al., 2016; Qiu et al., 2016). Extensive researches of the structure of this catalytic core nicely explained the mechanism of how SdeA ubiquitinates substrates (Akturk et al., 2018; Dong et al., 2018; Kalayil et al., 2018; Wang et al., 2018). However the biological function of the far C-terminal coiled-coil like region of SidE family effectors remained unknown. Previous study reported that coiled-coil domains are involved in membrane localization of many *Salmonella* type III effectors (Knodler et al., 2011). Given the fact that SdeA targets and ubiquitinates numerous ER proteins, such as RTN4 and FAM134B (Kotewicz et al., 2017; Shin et al., 2020), we asked that whether C-terminus is relevant to membrane interaction of SdeA. To test this hypothesis and unveil the function of the C-terminus of SdeA in the PR-ubiquitination of substrates, we firstly checked the ER localization of wild type SdeA or truncated SdeA lacking extreme C-terminal regions (Fig. 24A). SdeA deletion constructs were designed according to the reported SdeA structure and protein sequence prediction, Cos7 cells were transfected with the plasmids encoding EGFP-tagged SdeA. 24 h after transfection, cells were fixed and stained with the antibody against ER-resident protein Calnexin. Immunostaining result suggests that exogenously expressed SdeA overlapped with ER marker Calnexin in COS7 cells, which is in agreement with a previously reported research (Qiu et al., 2017). Nevertheless SdeA deletions lacking the C-terminal region did not co-localize with Calnexin, indicating that the C-terminus region of SdeA is responsible for membrane localization (Fig. 24B).

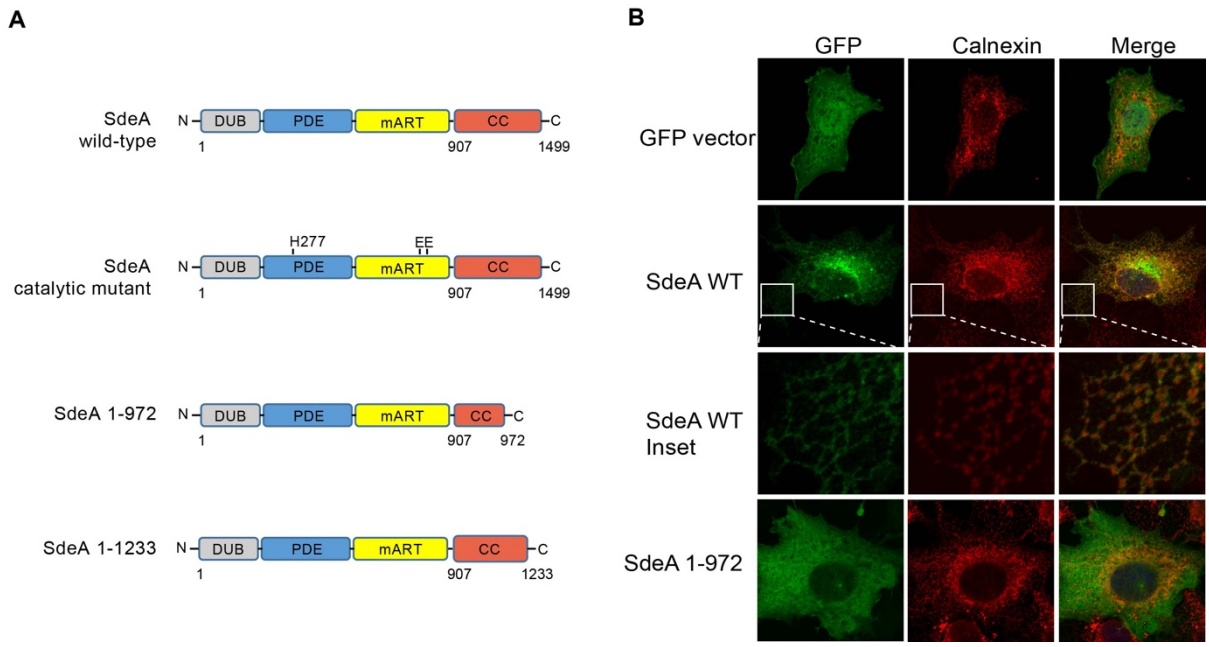


Figure 24. C-terminus region of SdeA is responsible for membrane localization.

(A) Schematic diagram of truncated SdeA constructs. (**B**) Immunostaining showing the co-localization between SdeA and ER protein Calnexin. COS7 expressing GFP-tagged wild type SdeA or truncated mutant were fixed with 4% PFA, permeabilized with 0.1% saponin, and stained with antibody against Calnexin, images were acquired with a confocal microscope.

Of note, we found that SdeA exhibited condensed signal close to the nucleus (Fig. 24B). Given that SdeA modifies Golgi proteins like Rab33b, we asked if SdeA is also localized to the Golgi, to test this hypothesis, COS7 cells were transfected with the plasmid that encodes EGFP tagged wild type SdeA, after 24 hours culture in 37 °C incubator, cells were fixed and stained with antibody against Golgi marker protein GM130. Immunofluorescence imaging suggests that SdeA partially localizes to Golgi, as overlapping was detected between SdeA and Golgi marker GM130. Whereas the truncated mutants (SdeA 1-972, SdeA 1-1233) that lack the C-terminal region of SdeA did not exhibit accumulation in the Golgi (Fig. 25A). In addition, the dispersed GM130 staining indicates that expression of wild type SdeA in cells might cause Golgi structural disruption. To further confirm if the C-terminal region of SdeA is responsible for SdeA Golgi localization, plasmid encoding SdeA C-terminus 1234-C (SdeA 1234-C) was transfected in COS7 cells, cells were cultured for 24 h and stained with GM130. Immunostaining result shows that SdeA 1234-C expressed in cells was highly co-localized with GM130, without damaging Golgi integrity (Fig. 25A). Co-localization between SdeA 1234-C and GM130 was shown by measuring intensities of GFP-tagged SdeA 1234-C and GM130 staining along the line drawn across the Golgi. Gray

values were measured with ImageJ (Fig. 25B). These data indicate that the C-terminal region of SdeA is essential for its ER and Golgi membrane localization.

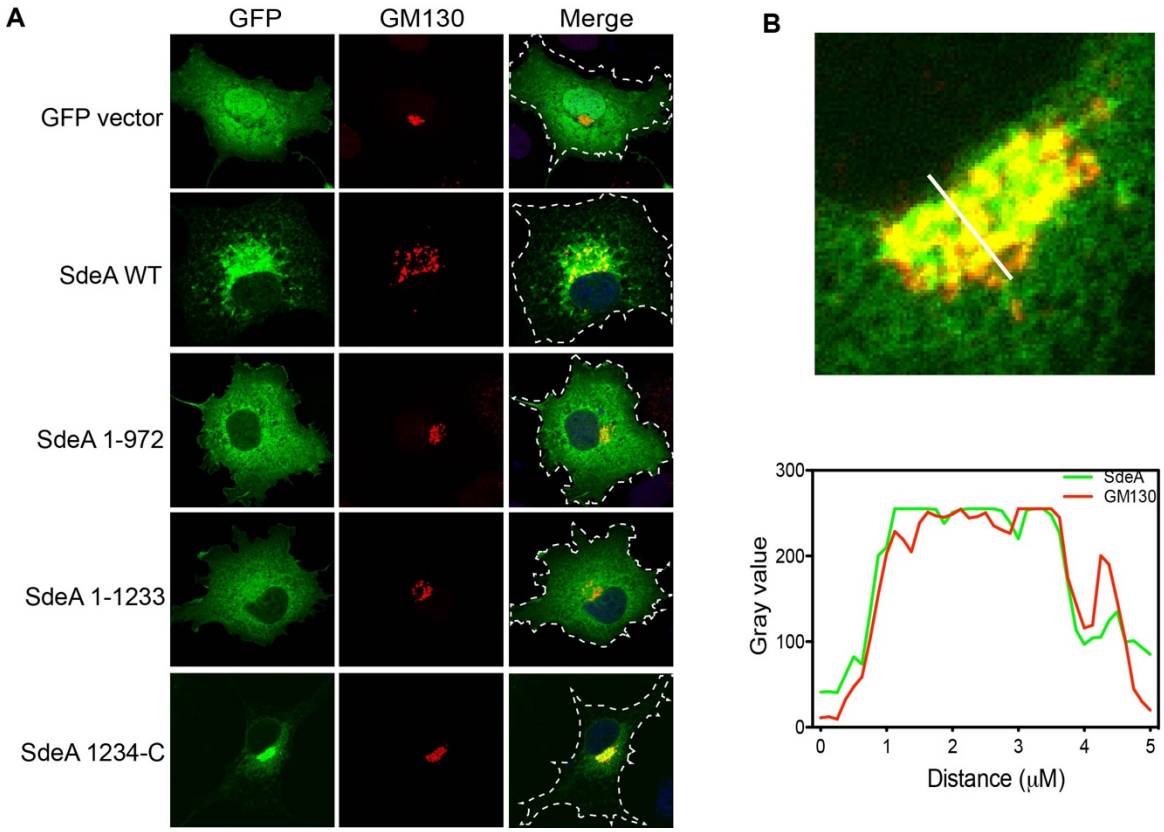


Figure 25. SdeA C terminus is targeting to the Golgi.

(**A**) Cos7 expressing GFP tagged wild type SdeA or truncated mutant were fixed with 4% PFA, permeabilized with 0.1% saponin, and stained with antibody against GM130. (**B**) image of SdeA 1234-C from (**A**) was cropped and enlarged. Co-localization between SdeA 1234-C and GM130 was shown with intensities of SdeA or GM130 along a line drawn across the Golgi. Distributions and gray values were measured with Image J.

In view of that Golgi proteins were identified as SdeA substrates, we asked if Golgi localization of SdeA is involved in its function in ubiquitinating substrates in cells. To test this hypothesis, we co-transfected cells with plasmids encoding wild type SdeA or the truncated mutants missing C-terminal region (SdeA 1-972, SdeA 1-1089, SdeA 1-1233) with Golgi associated protein Rab33b, a known substrate of SdeA, to study whether these SdeA mutants function normally to modify Golgi substrate. Western blotting result suggests that the truncated mutants missing Golgi localization could not ubiquitinate Rab33b even if they were able to modify ubiquitin (Fig. 26). These data demonstrate that C-terminus mediating localization of SdeA in the Golgi is important for its function in Golgi protein ubiquitination.

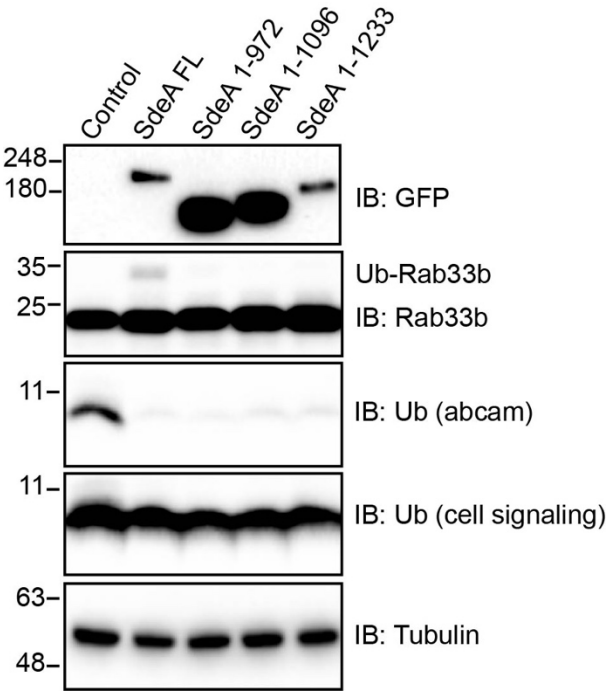


Figure 26. Ubiquitination of Golgi protein is dependent on Golgi localization of SdeA.
Effect of SdeA C-terminal region on the ubiquitination of Golgi protein substrate. HEK293T cells were transfected with plasmids encoding full length or truncated SdeA proteins lacking membrane interaction region, 24 h after transfection, cells were lysed and blotted with antibodies.

7.3.2 SdeA expression results in dispersed Golgi in cells

As mentioned above, SdeA expression in cells resulted in a scattered staining of Golgi protein GM130. To confirm whether SdeA causes Golgi morphology change in cells, we transfected cells with plasmids expressing wild type SdeA, PDE catalytically dead mutant (SdeA H277A) or mART catalytically dead mutant (SdeA EE/AA). Immunofluorescence showed that expression of wild-type SdeA highly dispersed the Golgi apparatus in COS7 cells. Of note, SdeA defective mutants that are not able to ubiquitinate substrates showed weak toxicity to Golgi integrity (Fig. 27A). Moreover, this effect of SdeA on Golgi separation was counteracted by the expression of PR-ubiquitination specific deubiquitinase DupA, but not the defective mutant DupA H67A (Fig. 27B). These data suggest that SdeA-mediated Golgi separation is dependent on its protein PR-ubiquitination activity.

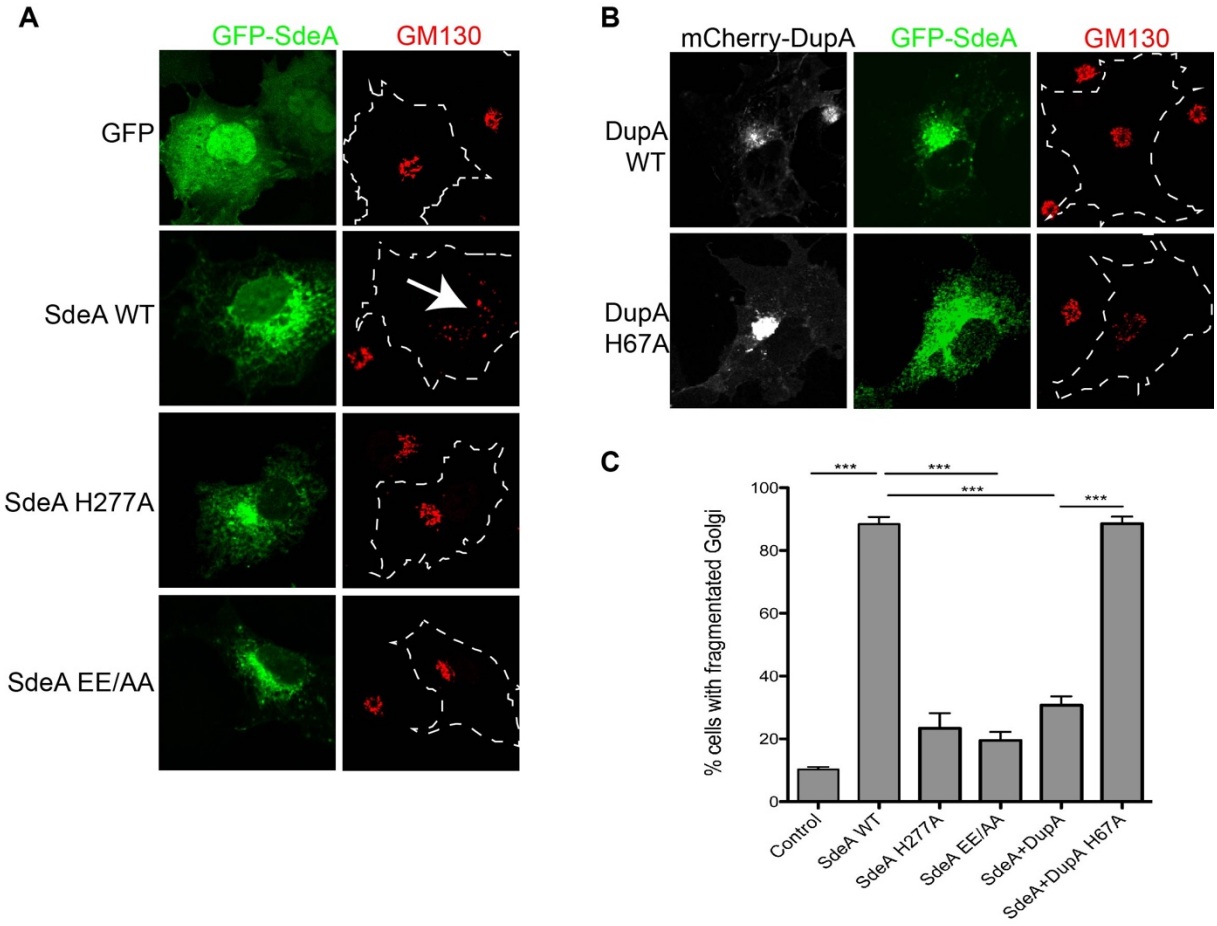


Figure 27. SdeA expression induces Golgi separation in cells

(**A**) Immunostaining images showing SdeA expression induces Golgi separation. COS7 cells were transfected with plasmids encoding GFP tagged SdeA wild type or catalytically defective mutants. After 24 h of culture at 37 °C, cells were fixed with 4% PFA and stained with antibody against Golgi marker GM130. (**B**) Immunostaining images showing SdeA expression mediated Golgi disruption is reversed by DupA. COS7 cells were co-transfected with plasmids encoding GFP-tagged wild-type SdeA and DupA. After 24 h of culture at 37 °C, cells were fixed with 4% PFA and stained with antibody against Golgi marker GM130. (**C**) Quantification of the cells with dispersed Golgi in A and B. Data are shown as means ± SEM of more than 60 cells taken from three independent experiments. ***P<0.001.

To further confirm the effect of the *Legionella* effector SdeA on Golgi integrity, we then attempted to check if SdeA effector induces Golgi disruption under physiological condition during bacteria infection. For this reason, we infected A549 cells with *Legionella* wild-type strain, a mutant strain that lacks genes encoding SidE family proteins (*ΔsidEs*) or a mutant strain that lacks genes encoding DupA and DupB (*ΔdupA/B*). 2 hours after infection, cells were washed and then stained with antibodies against *Legionella* and Golgi marker GM130. Strikingly, the immunostaining result indicates that cells infected with wild-type *Legionella* exhibited markedly Golgi integrity

impairment, compared with not infected cells or cells infected with the *Legionella ΔsidEs* strain. Moreover, *Legionella ΔDupA/B* strain without DupA/B showed stronger virulence to the Golgi, compared with the wild-type *Legionella* (Fig. 28A, B). These data indicate that *Legionella* effector SdeA causes Golgi dispersal under both exogenous protein expression in cells and physiological bacteria infection conditions via its PR-ubiquitinating activity.

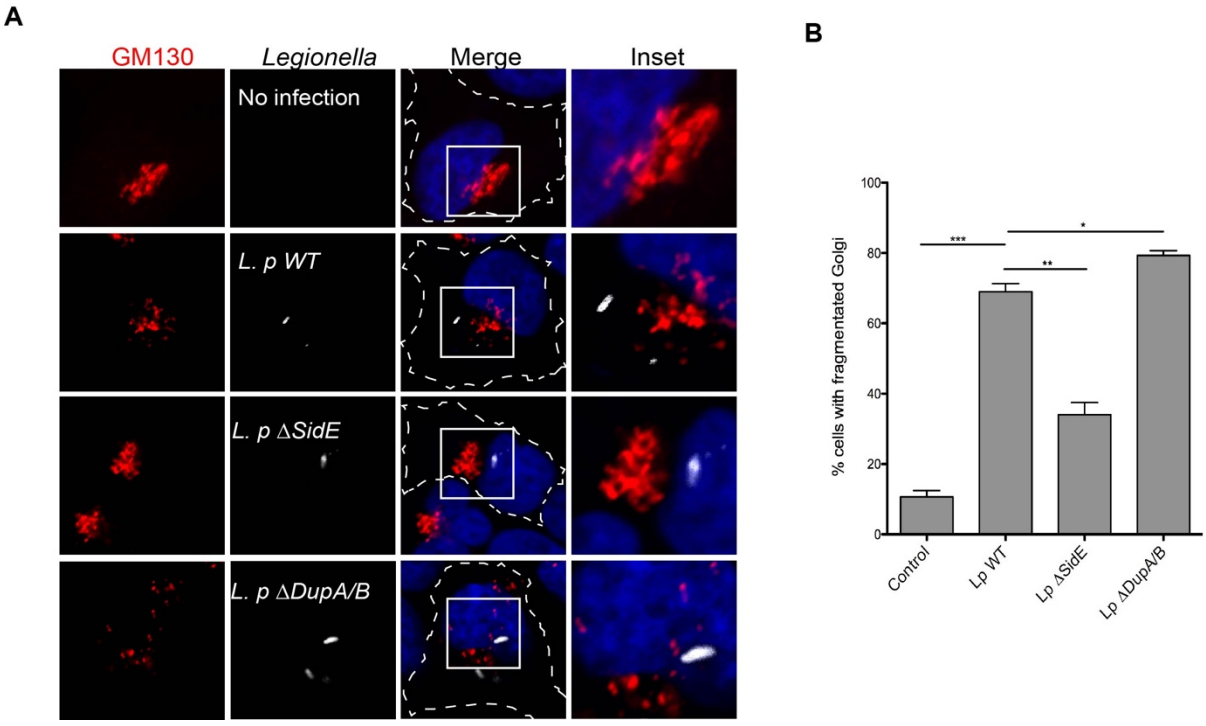


Figure 28. Legionella infection disrupts the Golgi in cells.

(**A**) Immunostaining images showing SidE effectors mediated Golgi disruption during *Legionella* infection. A549 cells were infected with a wild type *Legionella* strain, a mutant strain that lacks genes encoding SidE family proteins (*ΔsidEs*) or a mutant that lacks genes encoding DupA and DupB (*ΔdupA/B*). After 2 hours of infection, cells were washed and fixed with 4% PFA, followed by staining with antibodies against *Legionella* and GM130. (**B**) Quantification of data from A. Data were shown as means ± SEM of more than 60 cells taken from three independent experiments. ***P<0.001, *P<0.05.

7.3.3 SdeA disconnects the Golgi into mini-stacks, but does not dissociate cisternae tethering

To further confirm the effect of SdeA on Golgi morphology, we expressed SdeA in HeLa cells, then stained cells with both cis (GM130) and trans (TGN46) Golgi marker antibodies (Fig. 29). Similarly, immunostaining data showed that SdeA expression

dispersed the Golgi protein staining. Of note, cis-Golgi marker and trans-Golgi marker were colocalized, indicating that the Golgi was separated but still stacked.

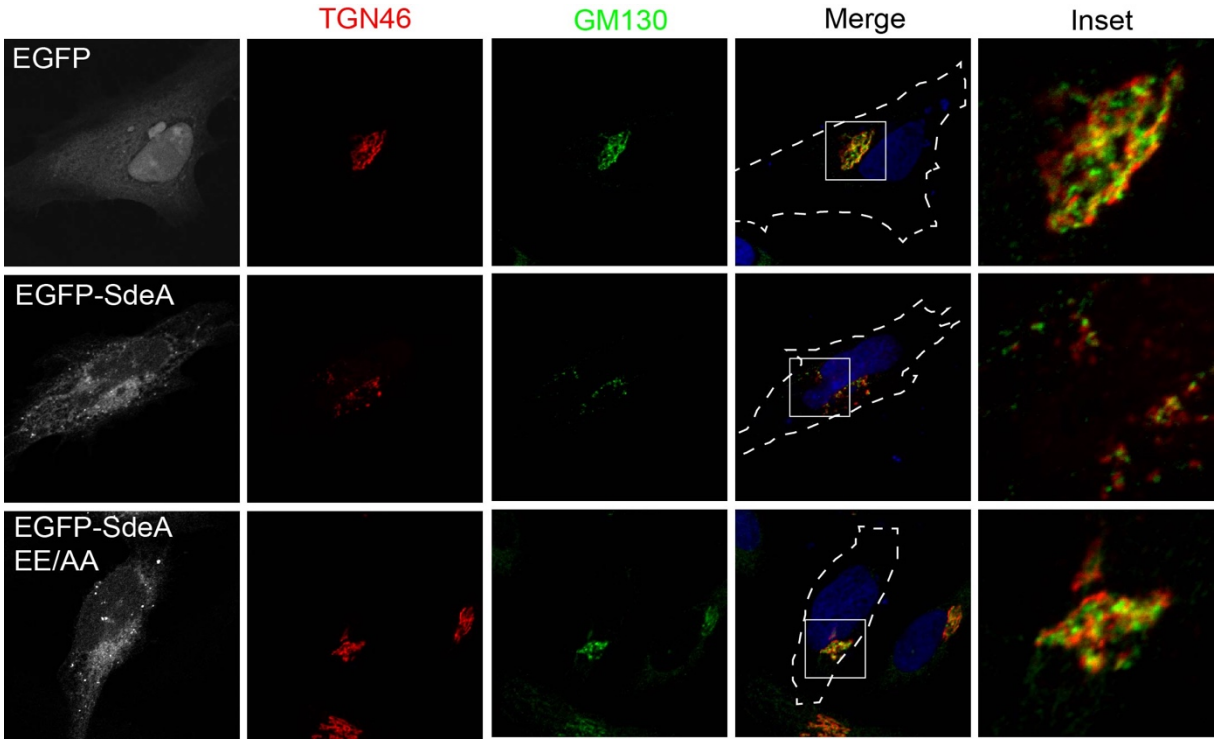


Figure 29. SdeA expression induces Golgi separation in HeLa.
Immunostaining images showing SdeA expression induces Golgi dispersal. HeLa cells were transfected with plasmids encoding GFP-tagged SdeA wild-type or catalytically defective mutants. 24 h after transfection, cells were fixed with 4% PFA and stained with antibody against the Golgi marker GM130. Images were acquired with a confocal microscopy.

Similar observation was made when cells expressing SdeA were detected with DNA-PAINT super-resolution microscopy. In control cells without SdeA transfection, cis-Golgi protein GM130 was in the vicinity of the trans-Golgi protein Golgin 97 (Fig. 30). In cells expressing SdeA, the Golgi was separated remarkably, however cis-Golgi and trans-Golgi were still connected, indicating that SdeA does not affect stacking of the Golgi. This observation is in agreement with a recent study showing that GRASP proteins are condensed at the rim of cisternae, knockout of GRASP proteins results in separated stacks, but does not lead to Golgi unstacking (Grond et al., 2020).

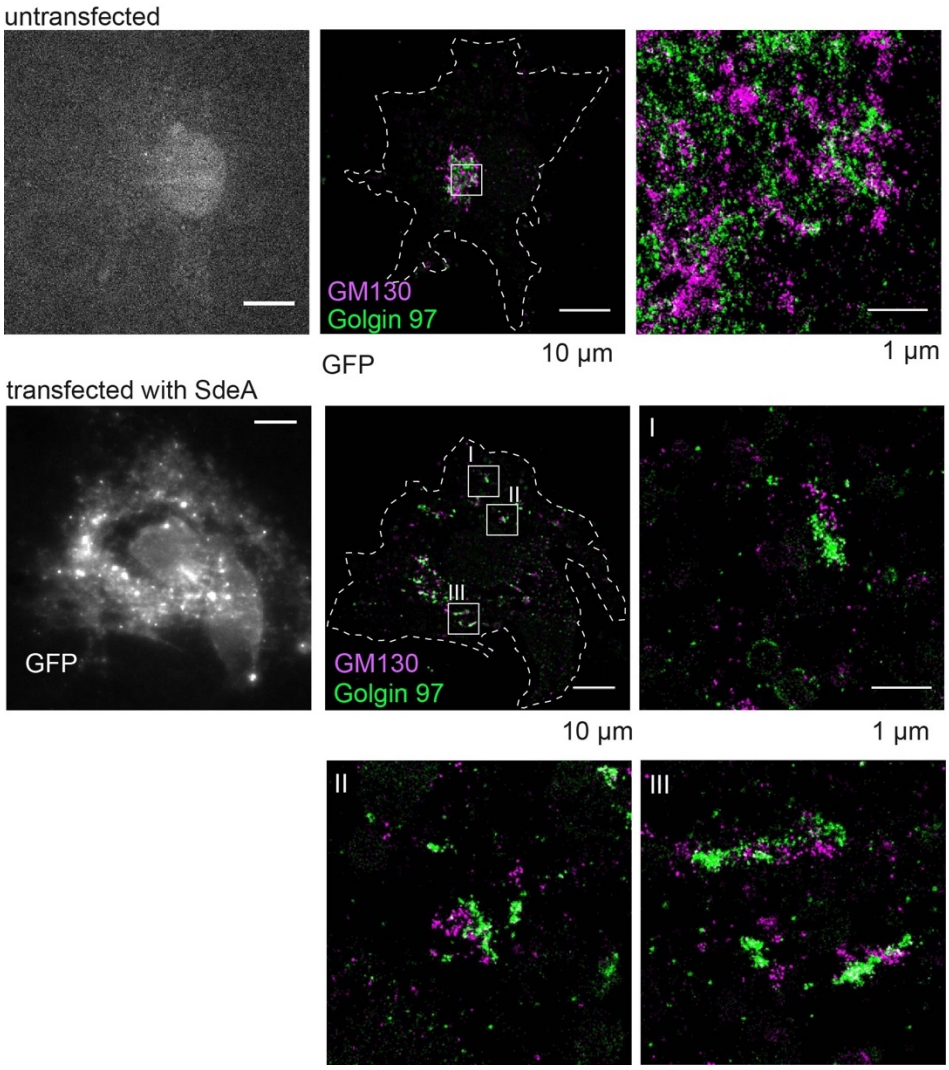


Figure 30. SdeA expression induces Golgi separation in HeLa, but does not affect Golgi stacking. Images showing SdeA expression induces Golgi dispersal but not unstacking. COS7 cells were transfected with plasmids encoding GFP-tagged SdeA. 24 h after transfection, cells were fixed with 4% PFA and stained with antibody against the cis-Golgi marker GM130 and trans-Golgi Golgin 97. Images were acquired with super-resolution microscopy.

*(DNA-PAINT super-resolution imaging was performed by Dr. Marius Glogger from Mike Heilemann Lab at Goethe University)

7.3.4 SdeA catalyzes PR-ubiquitination of Golgi proteins

Legionella deubiquitinases DupA and DupB specifically target novel protein PR-ubiquitination catalyze by SidE effectors in host cells. Given the strong binding affinity of DupA H67A dead mutant to modified substrates of SidE effectors, we used it as a trapping bait and pulled-down over 180 potential host substrate proteins of SidE effectors from cells infected with *Legionella* (Shin et al., 2020). Among these identified putative substrate proteins for PR-ubiquitination, numerous ER-resident proteins, and

Golgi associated proteins were listed with high scores, according to the intensity ratios of proteins enriched from cells infected with *Legionella ΔDupA/B* strain over proteins enriched from cells infected with *Legionella ΔSidEs* strain. This finding is consistent with the ER and Golgi localization of SdeA, which is relevant to substrate targeting. Of note, Golgi tethering factor GRASP55 was listed with the highest ratios among the putative Golgi protein substrates, another identified Golgi protein GCP60 is also involved in Golgi structure maintenance (Fig. 31A), In view of the functions of GRASP55 and GCP60 in membrane connecting of Golgi cisternae rim and interaction with Golgi resident proteins (Grond et al., 2020; Shorter et al., 1999; Sohda et al., 2001), we asked if SdeA modifies these Golgi proteins and interferes these proteins in Golgi structure maintenance, which finally results in Golgi separation. For this reason, firstly we purified recombinant GRASP55 from *E. coli* and performed *in vitro* reaction to validate that whether SdeA modifies GRASP55. We inserted cDNA of GRASP55 into E. coli expression vector to purify His_6-tagged GRASP55 His tag affinity resin. GRASP55 protein was incubated with purified SdeA at 37 °C in the presence of ubiquitin and NAD^+. *In vitro* reaction mixes were separated with SDS-PAGE followed by Coomassie blue staining or probing with antibodies against GRASP55 and ubiquitin. Western blotting result showed that SdeA ubiquitinated purified GRASP55 *in vitro* (Fig. 31B). Considering that GRASP65, the homologous protein of GRASP55, sharing high sequence similarity with GRASP55, is also involved in Golgi integrity maintenance, we asked if SdeA ubiquitinates GRASP65 as well. For this reason, we purified recombinant GRASP65 and incubated it with SdeA at 37 °C in the presence of ubiquitin and NAD^+. Similar to GRASP55, multiple shifted bands were detected from *in vitro* reaction mix on the SDS-PAGE gel, indicating that GRASP65 was ubiquitinated as well by SdeA (Fig. 31B).

A

	Protein names	dDup12/dSidE
ER proteins	STIM1	5,73
	FAM134C	4,86
	VCP	4,59
	RTN4	4,41
	SNAP23	4,29
	TEX264	3,64
	LNP	2,38
Golgi related proteins	GRASP55	5,83
	TMED8	4,72
	GCP60	2,69
	YIF1A	2,44
	RAB33B	3,09
	SNX5	2,76

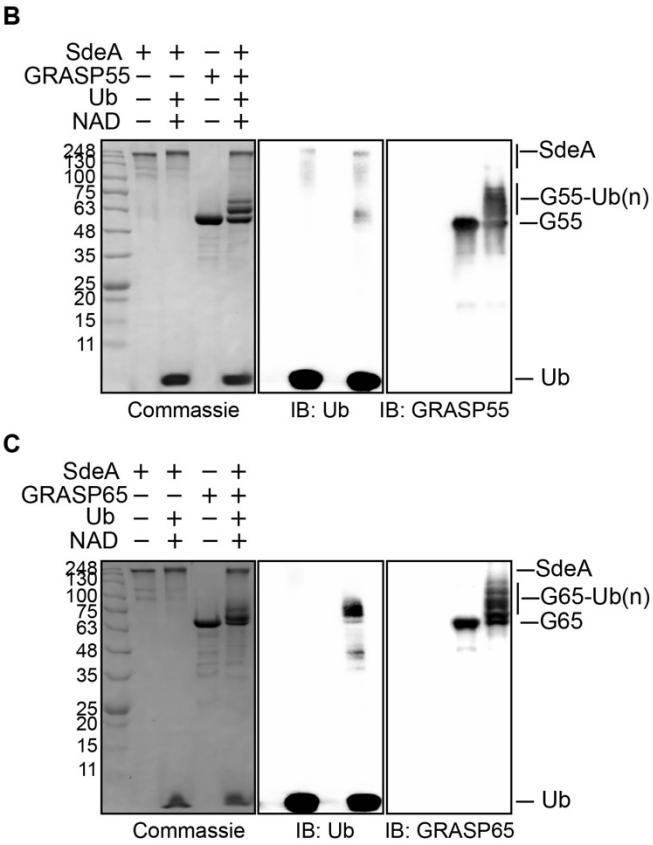


Figure 31. SdeA ubiquitinates Golgi tethering GRASP proteins.

(**A**) Putative ER and Golgi associated SdeA substrates identified by mass spectrometry analysis. Values refer to the peptide ratios between enriched samples from indicated *Legionella* infected cells. Among these candidates, Golgi tethering factor GRASP55 listed as one of the most highly ubiquitinated proteins was labeled in red. (**B**, **C**) *In vitro* assays of GRASP55 and GRASP65 ubiquitination catalyzed by SdeA. Purified recombinant GRASP55 or GRASP65 were treated with SdeA in the presence of ubiquitin and NAD^+. *In vitro* reaction mixes were separated with SDS-PAGE, followed by Coomassie blue staining or western blotting with antibodies against ubiquitin, GRASP55 or GRASP65.

To further confirm the ubiquitination of GRASP proteins catalyzed by SdeA, we next test the effect of GRASP proteins by SdeA exogenously expressed in cells. Western blot result indicates that GRASP55 was ubiquitinated in HEK293T cells transfected plasmids encoding wild-type SdeA but not in cells expressing inactive SdeA PDE mutant or mART mutant. Moreover, the shifted ubiquitination band disappeared when the PR-ubiquitination specific deubiquitinase DupA was co-expressed (Fig. 32A). Similar result was observed from samples of cells expressing GRASP65 and SdeA (Fig. 32B).

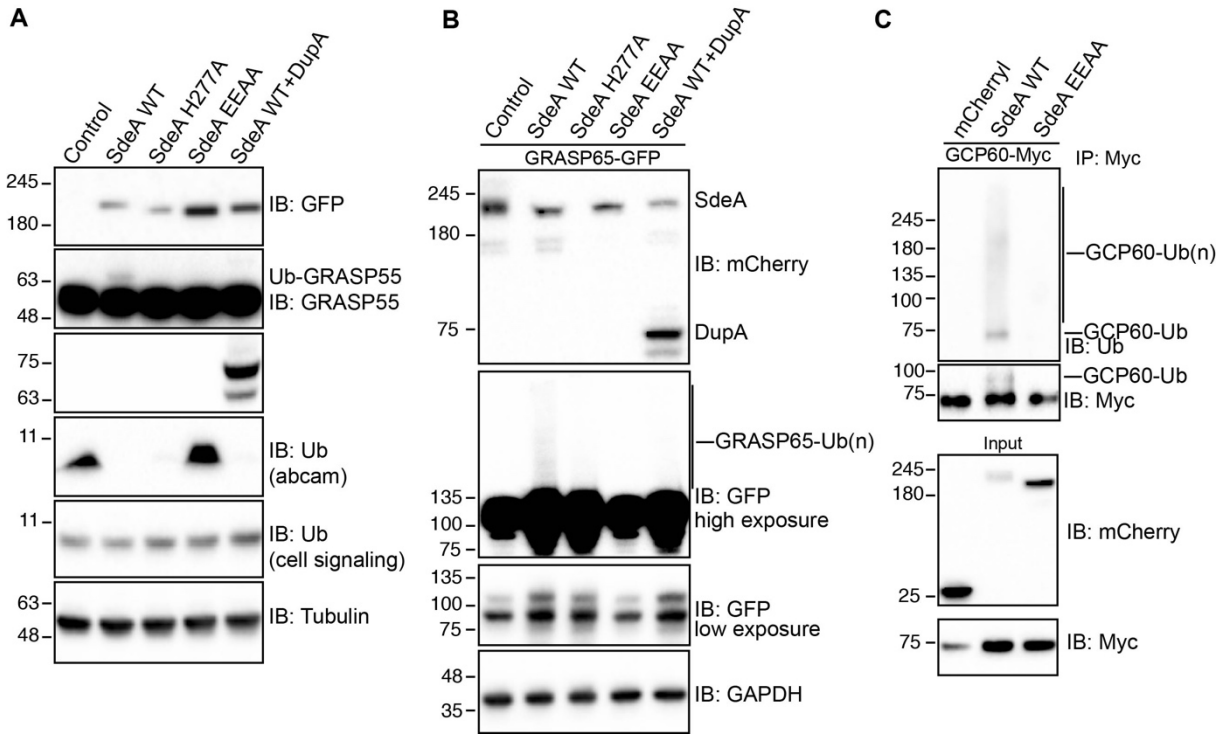


Figure 32. SdeA ubiquitinates Golgi tethering GRASP proteins in cells.

(**A**) Ubiquitination of GRASP55 by exogenously expressed SdeA in cells. HEK293T cells were transfected with plasmids encoding wild-type SdeA or indicated dead SdeA mutants. After 24 h of culture, cells were collected and lysed with lysis buffer containing 50 mM NaCl, 150 mM Tris, 0.1% SDS and protease inhibitors. Total cell lysates were separated with SDS-PAGE followed by probing with the antibody against GRASP55. (**B**) Ubiquitination of GRASP65 by exogenous SdeA in cells. HEK293T cells were transfected with plasmids encoding GFP tagged GRASP65 and wild type SdeA or indicated SdeA mutants. After 24 h of culture, cells lysed for isolation of GRASP65, which was separated with SDS-PAGE and probed with antibody. (**C**) Ubiquitination of GCP60 catalyzed by exogenous SdeA in cells. Myc-tagged GCP60 was purified from HEK293T cells transfected with plasmids encoding wild type SdeA or indicated SdeA dead mutants, followed by separation with SDS-PAGE and probing with the antibody against Myc tag.

To further validate the effect of SdeA on Golgi protein, we conducted similar experiments to test the PR-ubiquitination of GCP60 catalyzed by SdeA. Western blotting result indicates that GCP60 purified from cells expressing wild-type SdeA was ubiquitination (Fig. 32C).

To verify the SdeA localization is critical for its function, we checked the effects of SdeA truncated mutants missing Golgi localization on the PR-ubiquitination of GRASP55, western blotting result showed notably reduced activities of these mutants in modifying substrate GRASP55 (Fig. 33A), this is in agreement with the observation on PR-ubiquitination of Rab33b, further confirming that Golgi localization is essential for

substrates modification function of SdeA. Our function analysis of SdeA revealed that M408 and L411 are important residues of SdeA for binding substrates (Kalayil et al., 2018). To test if SdeA recognizes GRASP55 specifically, we performed *in vitro* reaction by incubating purified GRASP55 with wild type SdeA or SdeA ML/AA mutant. Reaction products were separated with SDS-PADE gel followed by Coomassie staining, the result shows that SdeA M/L mutant could not modify GRASP55 (Fig. 33B). Similarly, the PR-ubiquitination of GRASP55 was markedly reduced in cells expressing SdeA ML/AA mutant, compared to cells expressing wild type SdeA (Fig. 33C). We then investigated protein-protein interaction between GRASP55 and SdeA or SdeA M/L mutant. Co-IP experiment showed that the interaction with GRASP55 was much reduced for SdeA mutant compared with wild-type SdeA (Fig. 33D). These data indicate that SdeA specifically recognizes and catalyzes PR-ubiquitination of Golgi tethering protein GRASP55.

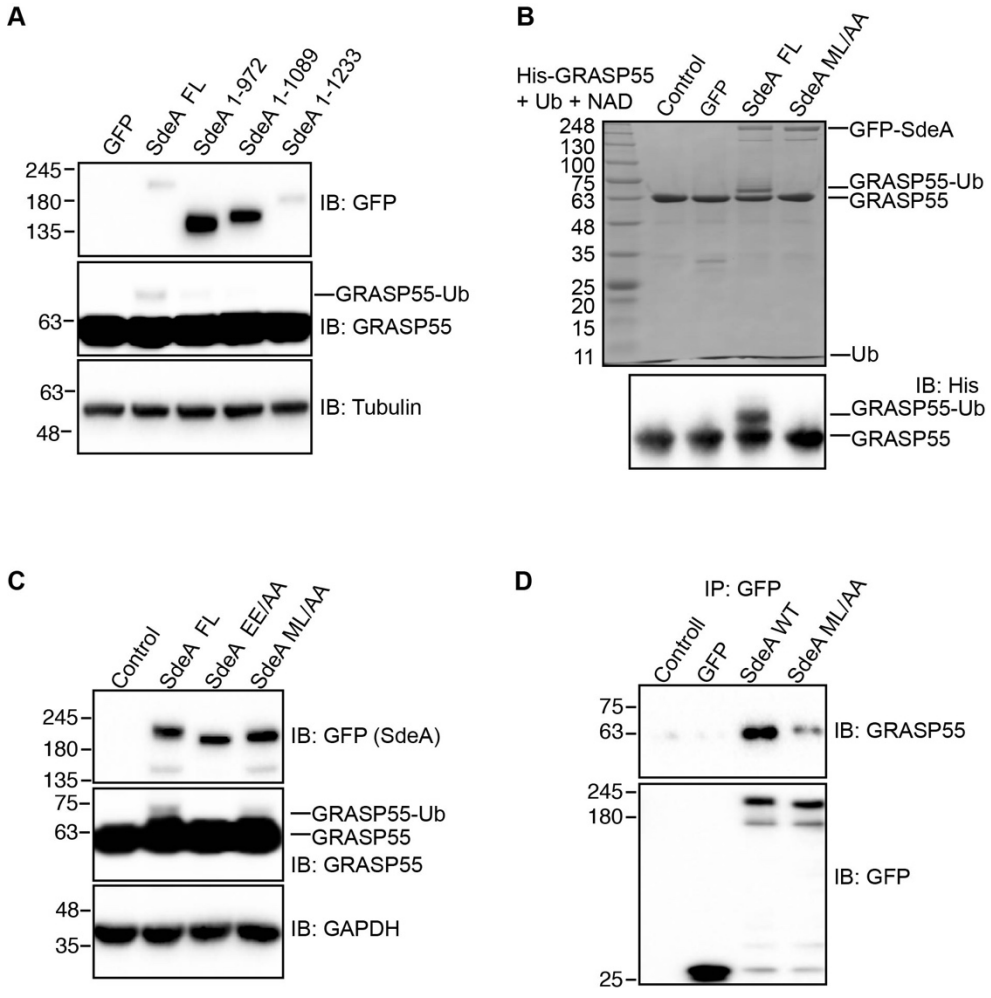


Figure 33. SdeA ubiquitinates Golgi tethering GRASP protein specifically.

(**A**) In vitro modification of GRASP55 by wild type SdeA or mutant. Purified recombinant GRASP55 was incubated with wild type SdeA or mutant ML/AA in the presence of ubiquitin and NAD^+. Reaction mixes were separated with SDS-PAGE followed by Coomassie blue staining. Shifted Ubiquitination band of GRASP55 caused by wild type SdeA was indicated. (**B**) HEK293T cells were transfected with plasmid encoding wild type SdeA or SdeA inactive mART (EE/AA), or MLAA mutant that does not bind substrate, cells were lysed after 24 h of culture. Total cell lysates were separated with SDS-PAGE and blotted with the antibody against GRASP55. (**C**) Assay of protein interaction between GRASP55 and wild type SdeA or SdeA mutant. HEK293T cells were transfected plasmids encoding GFP tagged wild type SdeA or SdeA ML/AA mutant, after 24 hours culture, cells were lysed with mild lysis buffer containing 1% NP40, SdeA proteins were isolated with GFP-trap beads. Immunoprecipitation results were separated with SDS-PAGE followed by western blotting with the antibody against GRASP55.

7.3.5 Legionella infection induces PR-ubiquitination of GRASP55 and GRASP65

To further confirm the PR-ubiquitination of GRASP proteins caused by *Legionella* SidE family effectors, we next attempted to test if these Golgi proteins are ubiquitinated during *Legionella* infection. For this reason, firstly, we transfected HEK293T cells with plasmids encoding FcγRII and GFP-tagged GRASP55 and infected cells with indicated *Legionella* strains. Infected cells were lysed with mild lysis buffer containing 1% Triton X-100, then used for purification of GRASP55 proteins with GFP-trap beads and western blotting analysis. It is suggested that GRASP55 proteins enriched from cells infected with wild-type *Legionella* strain were ubiquitinated in an infection time dependent manner. In addition, ubiquitination signal of GRASP55 protein from cells infected with the *Legionella* mutant strain *ΔdupA/B* was enhanced compared with that of GRASP55 protein from cells infected with the wild-type *Legionella* strain, indicating that more ubiquitinated GRASP55 accumulated without activities of these two deubiquitinases for PR-ubiquitination (Fig. 34A). To further confirm PR-ubiquitination of Golgi substrates catalyzed by SidE effectors during infection, similar experiment was performed to check the ubiquitination of GRASP65 from cells infected with *Legionella*. Similar to GRASP55, ubiquitination of GRASP65 was observed upon *Legionella* infection in a time-course manner, and the ubiquitination level of GRASP65 purified from cells infected with the DupA/B deleted mutant *Legionella* strain was enhanced as well compared with GRASP65 purified from cells infected with wild-type *Legionella* strain (Fig. 34B).

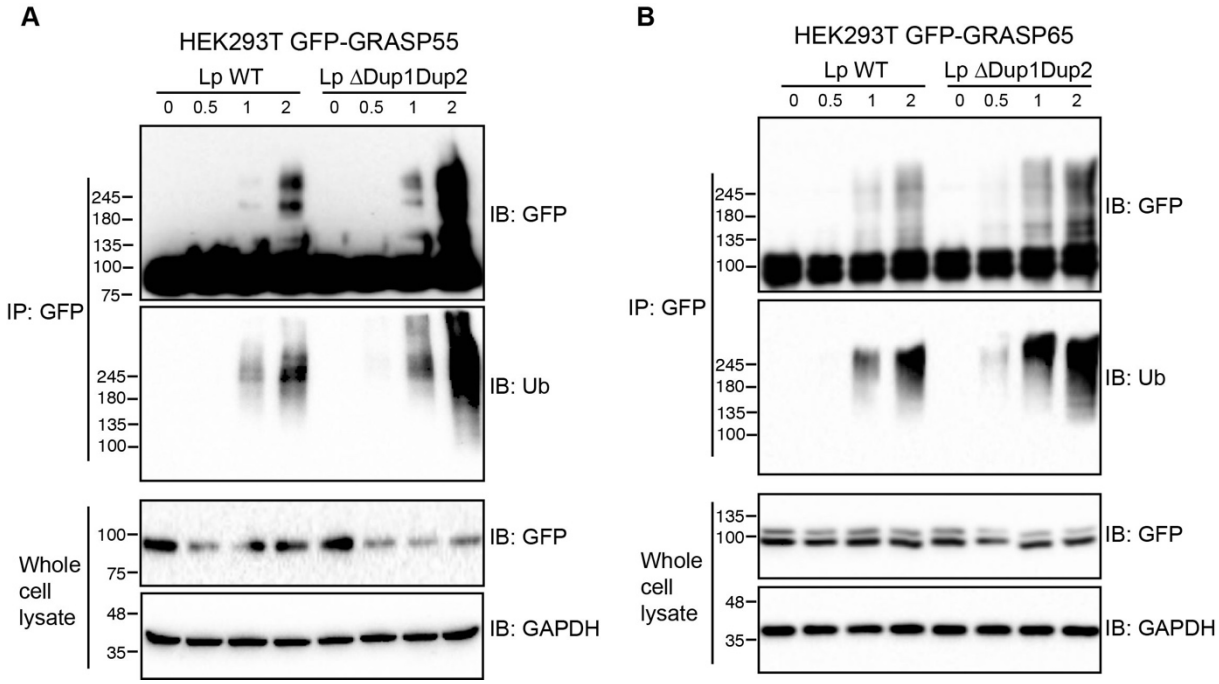


Figure 34. Legionella infection causes PR-ubiquitination of Golgi tethering GRASP proteins.
(**A**) Ubiquitination assay of GRASP55 in cells infected with *Legionella*. HEK293T cells cultured in 6-well plate were co-transfected with plasmids encoding GFP-tagged GRASP55 and FcγRII. After 24 hours, cells were infected with *Legionella* bacteria strains opsonized with the *Legionella* antibody. After the indicated times of infection, cells were lysed with mild lysis buffer containing 1% Triton X-100 for purification of GRASP55 proteins, which were then separated with SDS-PAGE followed by probing with antibodies against GFP or ubiquitin. (**B**) Ubiquitination assay of GRASP65-GFP purified from HEK293T cells infected by *Legionella* strains.

This observations were consistent with the results of infection of cells expressing GRASP55 or GRASP65 with *Legionella* lacks SidE family effectors. *Legionella* missing SidE effectors did not ubiquitinate GRASP proteins, demonstrating that ubiquitination caused by *Legionella* infection was catalyzed by SidE effectors (Fig. 35A,B). To further prove that this conclusion, we incubated isolated GRASP55 and GRASP65 with purified recombinant DupA at 37 °C. Western blotting result showed that DupA, the deubiquitinase DupA targeting PR-ubiquitination, eliminated the ubiquitination of GRASP55 and GRASP65 proteins caused by *Legionella* infection (Fig. 35C,D). These data indicate that SdeA induces PR-ubiquitination of Golgi tethering proteins GRASP55 and GRASP65 during *Legionella* infection.

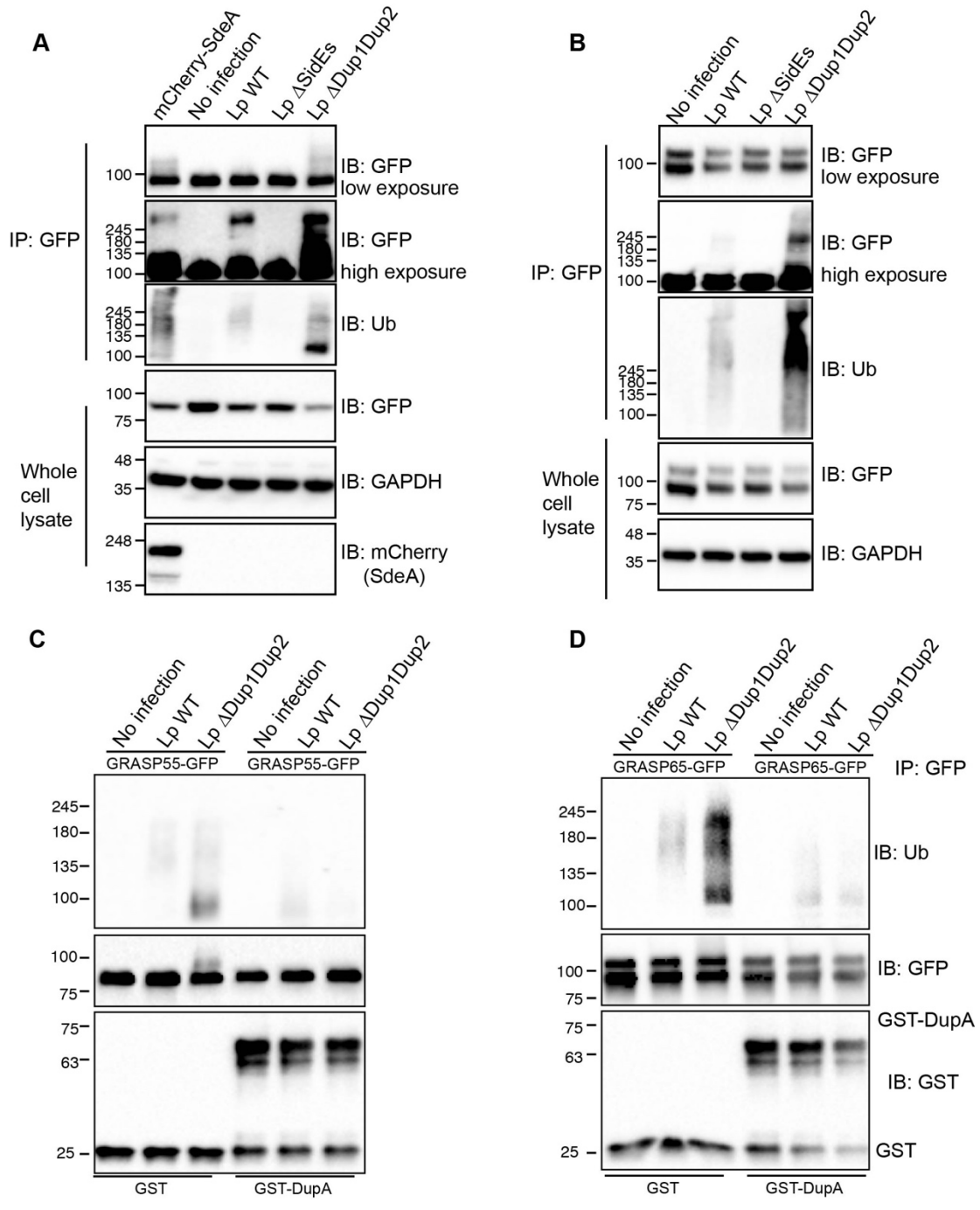


Figure 35. SidE family effectors are involved in the PR-ubiquitination of Golgi tethering GRASP proteins caused by *Legionella* infection.

(**A**) Ubiquitination assay of GRASP55 in cells infected with *Legionella*. HEK293T cells cultured in 6-well plate were co-transfected with plasmids encoding GFP tagged GRASP55 and FcγRII. After 24 hours, cells were infected with *Legionella* bacteria strains opsonized with *Legionella* antibody. After the indicated times of infection, cells were lysed with mild lysis buffer containing 1% Triton X-100 for purification of GRASP55 proteins, which were then separated with SDS-PAGE followed by probing with antibodies against GFP or ubiquitin. (**B**) Ubiquitination assay of GRASP55 in cells infected with *Legionella*. (**C**) Removal of GRASP55 and GRASP65 PR-ubiquitination caused by *Legionella* infection with purified DupA. HEK293T cells were transfected with plasmids encoding GFP-tagged GRASP55 or

GRASP65 and infected with *Legionella*. Isolated GRASP55 or GRASP65 washed with TBS and incubated with GST-tagged DupA at 37 °C.

7.3.6 Identification of GRASP55 serine targets of SdeA

Previous studies have elucidated the chemical mechanism of ubiquitination catalyzed by SdeA and revealed that SdeA ADP-ribosylates ubiquitin at Arg42 and transfers ubiquitin to serines of substrate proteins via a phosphoribosyl linker (Bhogaraju et al., 2016; Qiu et al., 2016). To gain insight into the modification of GRASP55 protein by SdeA, we ubiquitinated purified GRASP55 *in vitro* with SdeA and then prepared digested reaction products for mass spectrometry analyses to identify the ubiquitinated serines (Fig. 36A).

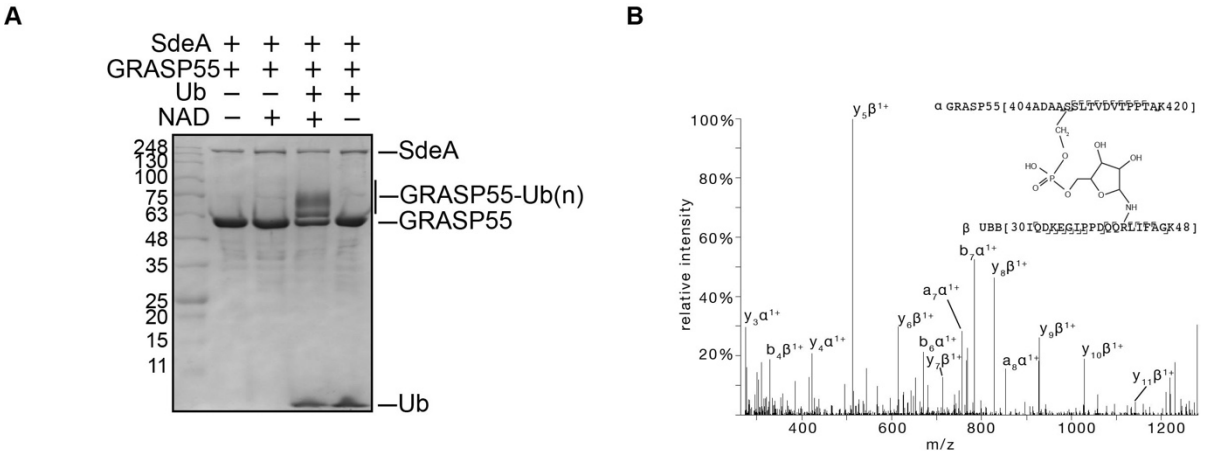


Figure 36. Identification of GRASP55 ubiquitination sites with mass spectrometry.

(**A**) Preparation of PR-ubiquitinated GRASP55 for mass spectrometry analysis. 20 µg of purified GRASP55 proteins were incubated with SdeA in the presence of ubiquitin and NAD^+. 10% of each reaction mixture was separated with SDS-PAGE followed by Coomassie blue staining or western blotting with antibodies against ubiquitin or GRASP55. The rest samples were subjected to mass spectrometry analysis. (**B**) Spectrum of GRASP-ubiquitin cross linked peptide.

*(MS analyses were performed by Florian Bonn and Thomas Colby)

Mass spectrometry analyses identified 4 serines that were modified in GRASP55 by SdeA in vitro (S3, S408, S409, S449) (Fig 36B, supplementary data2). To further confirm these serines are the main targets of SdeA, identified serines and adjacent serines S3, S4, S449, S451 were mutated to threonine to minimally effect the physio-chemical properties of these amino acids, in addition, S408, S409, S441 were mutated to alanine by site-directed mutagenesis to make GRASP55 serine mutant (GRASP55

7S*). HA-tagged wild-type GRASP55 or GRASP55 7S* were expressed with SdeA in HEK293T cells. Immunoprecipitation of GRASP55 and western blotting data showed that ubiquitination of GRASP55 mutant was hardly detected, compared with that of the the wild-type GRASP55 co-expressed with SdeA (Fig. 37A). Moreover, we asked whether replacements of serine will affect the GRASP55 ubiquitination caused by SidE effectors during *Legionella* infection. For this reason, we transfected HEK293T cells with plasmids expressing wild type or mutant GRASP55, then infected cells with wild type or *ΔsidEs* mutant *Legionella* for further analysis with western blotting. Similar to the result of the SdeA transfection experiment, GRASP55 mutant from infected cells exhibited seriously repressed ubiquitination, compared with the ubiquitination of wild-type GRASP55 (Fig. 37B).

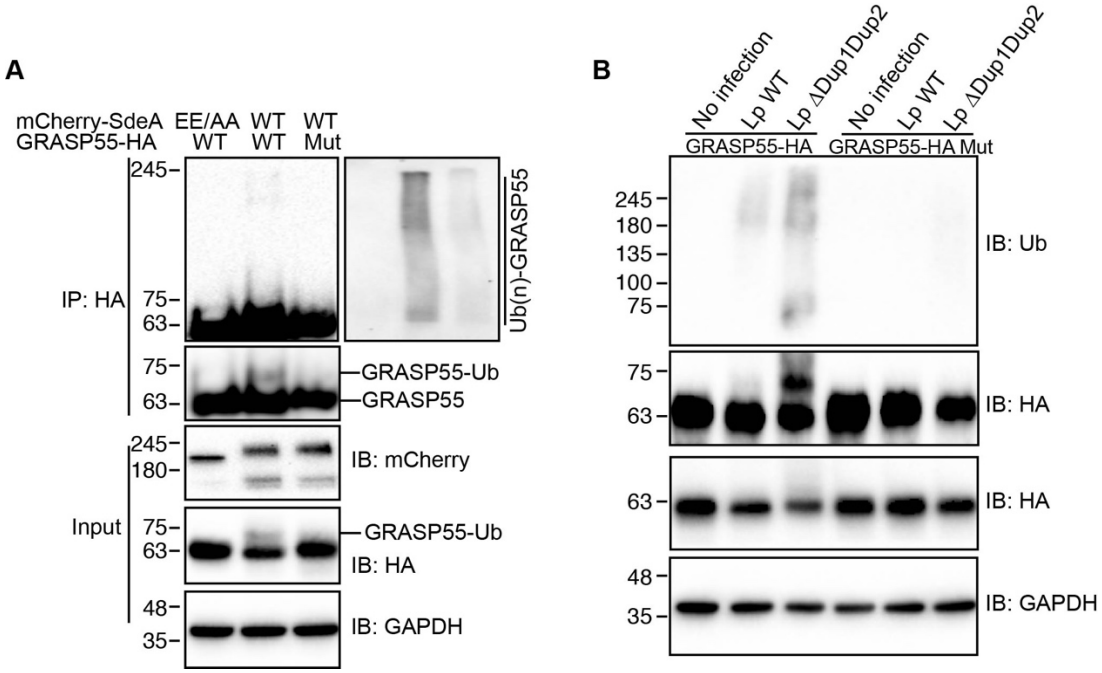


Figure 37. PR-Ubiquitination of GRASP55 Serine mutant.

(**A**) Validation of ubiquitination sites in GRASP55. Plasmids encoding HA-tagged wild type GRASP55 or GRASP55 mutant were co-transfected with SdeA in HEK293T cells. After 24 hours of transfection, cells were lysed with mild lysis buffer for HA immunoprecipitation. Isolated GRASP55-HA proteins were separated with SDS-PAGE followed by western blotting with antibodies against HA or ubiquitin. (**B**) Ubiquitination assay of wild-type GRASP55 and GRASP55 serine mutant expressed in cells infected with *Legionella*.

7.3.7 SdeA-mediated PR-ubiquitination interferes GRASP proteins interaction

Previous studies revealed that GRASP proteins play an important role in the maintenance of Golgi integrity through dimerization and binding to Golgi matrix

proteins (Jarvela and Linstedt, 2012; Rabouille and Linstedt, 2016). On the other hand, phosphorylation of serines in the C-terminal of GRASP proteins impairs dimerization of GRASP proteins and disrupts Golgi structure (Feinstein and Linstedt, 2008). We asked if SdeA mediated PR-ubiquitination of GRASP proteins affects these interactions critical for Golgi structure maintenance. For this reason, firstly, we conducted *in vitro* reaction of GRASP55-GFP and SdeA in the presence of ubiquitin and NAD^+, then incubated ubiquitinated GRASP55-GFP bound to GFP-trap beads with purified His-tagged GRASP55. Protein-protein interaction between GRASP55 proteins with different tags was analyzed with immunoprecipitation using GFP-trap beads. Western blotting result showed that GRASP55 incubated with wild-type SdeA bound to less His-GRASP55, compared with GRASP55 incubated catalytic dead SdeA H277A mutant (Fig. 38A). This indicates that SdeA mediated PR-ubiquitination of GRASP55 interferes with dimerization of GRASP55. To further confirm this, we performed co-IP to test the effect of PR-ubiquitination of GRASP55 caused by SdeA expression on protein-protein interaction between differently tagged GRASP55 in cells. Plasmids encoding HA-tagged wild-type GRASP55 or GRASP55 serine mutant were co-transfected with GFP-tagged GRASP55 serine mutant and SdeA. After 24 hours of transfection, cells were lysed and immunoprecipitation was performed to purified HA-GRASP55. Western blotting result indicates that wild-type GRASP55-HA co-expressed with SdeA showed less binding to GRASP55-GFP, compared with GRASP55-HA serine mutant resistant to SdeA. This data is in agreement with the result of *in vitro* reaction and interaction experiment (Fig. 38B).

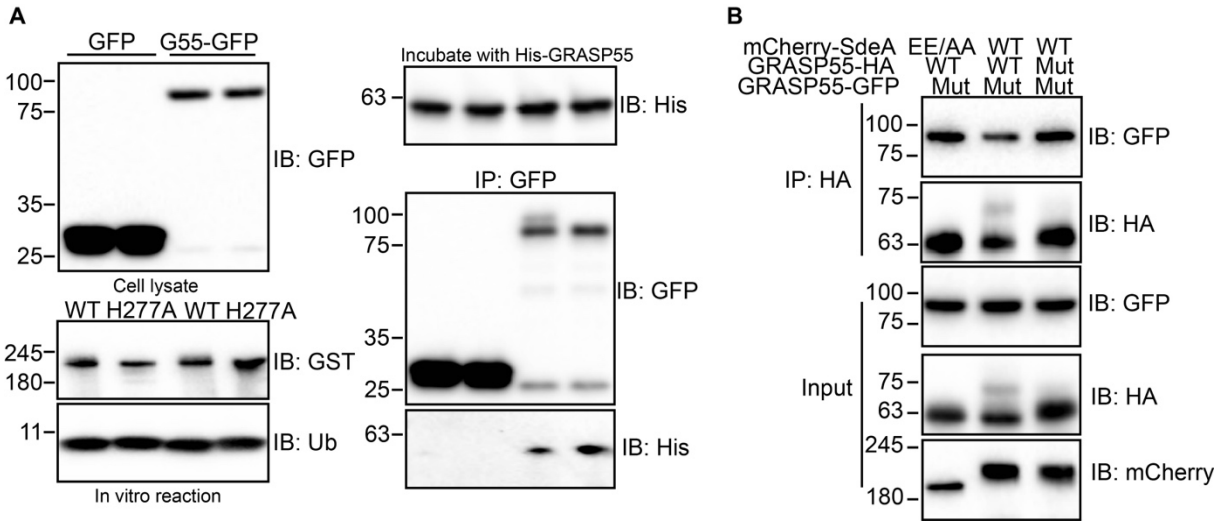


Figure 38. Serine ubiquitination impairs GRASP55 function.

(**A**) GRASP55-GFP proteins purified from HEK293T cells were modified *in vitro* by SdeA in the presence of ubiquitin and NAD^+, then GRASP55 proteins bound to GFP-trap beads were washed and incubated with His-tagged GRASP55. His-tagged GRASP55 interacting with GRASP55-GFP proteins was analyzed using western blotting. (**B**) Effect of SdeA mediated PR-ubiquitination on GRASP55 dimerization in cells. Plasmids encoding HA-tagged GRASP55 and GFP-tagged GRASP55 serine mutant were co-transfected with SdeA into HEK293T cells. Protein-protein interaction between GRASP55-HA and GRASP55 serine mutant was analyzed with co-immunoprecipitation and western blotting.

To further confirm this observation and the effect of PR-ubiquitination on GRASP55 dimerization, we reconstituted G55/G65 KO HeLa cells with wild-type GRASP55 or GRASP55 serine mutant, then checked Golgi integrity of cells expressing SdeA. Microscopy images result indicates that reconstitutions of both wild-type GRASP55 and GRASP55 serine mutant rescued Golgi from fragmentation caused by GRASP55/GRASP65 knockout (Fig. 39), in addition, the restored Golgi apparatuses were further disrupted when SdeA was expressed (Fig. 39).

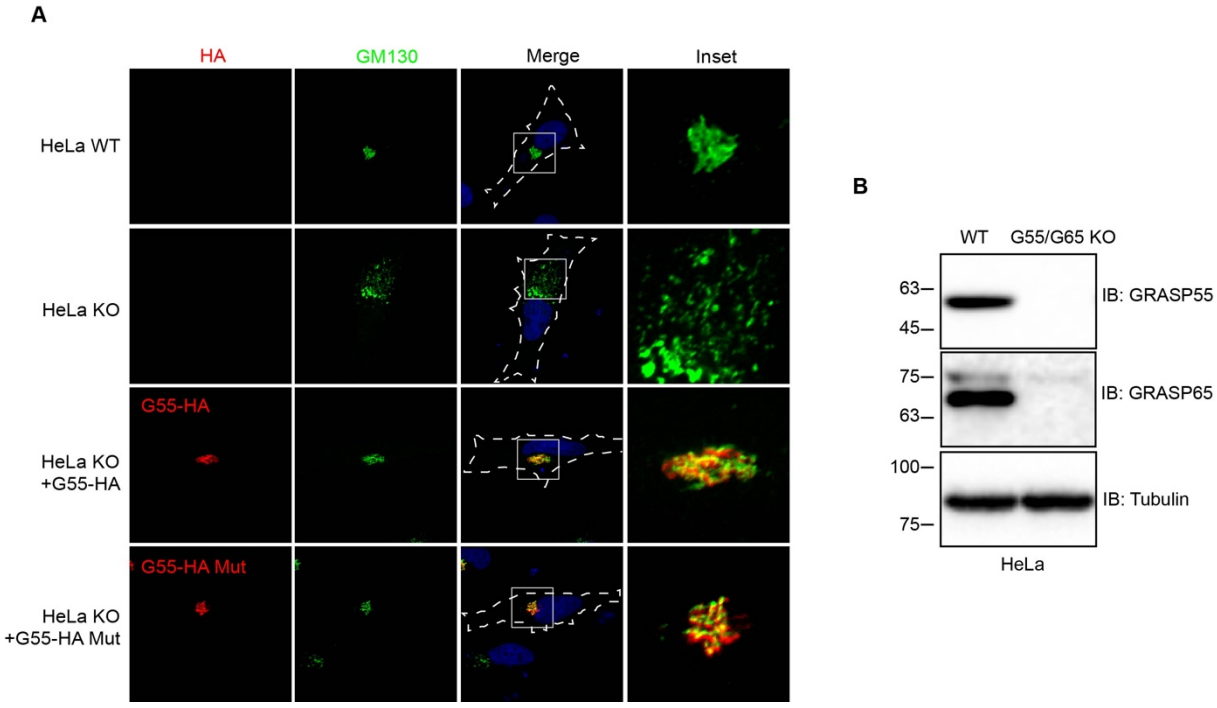


Figure 39. Replenishment of GRASP55 restores the Golgi structure.

(**A**) Immunostaining images showing exogenous wild-type GRASP55-HA or GRASP55 serine mutant rescued Golgi from damage caused by GRASP55/GRASP65 knockout. (**B**) Western blotting of samples of cell lysates from wild type HeLa cell line and G55/G65 knockout HeLa cell line. Knockout of GRASP55 and GRASP65 was validated by probing with anti-GRASP55 and anti-GRASP65 antibodies.

Interestingly, HeLa cells replenished with GRASP55 serine mutant exhibited higher tolerance to SdeA expression in Golgi structure maintenance than cells expressing wild type GRASP55 (Fig. 40A, B). These data suggest that SdeA mediated PR-ubiquitination of GRASP55 is involved in Golgi structure disruption, might via disturbing the connection between Golgi stacks.

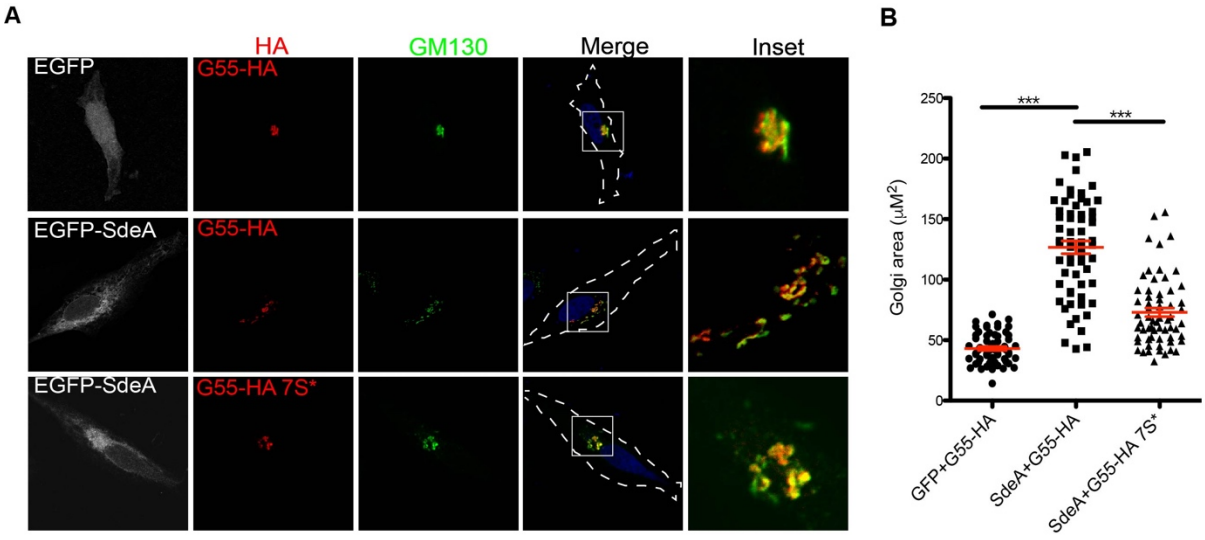


Figure 40. PR-ubiquitination of GRASP55 impairs the Golgi structure.

(**A**) Immunostaining images showing expression of GRASP55 serine mutant inhibited Golgi fragmentation caused by SdeA expression. (**B**) Quantification of areas of the Golgi apparatuses in cells expressing SdeA and wild type or serine mutant GRASP55. Data are shown as means ± SEM of more than 60 cells taken from three independent experiments. ***P<0.001, *P<0.05.

7.3.8 Legionella does not recruit Golgi vesicle to the bacterial vacuole

Extensive previous studies have revealed that numerous intracellular pathogens have marked requirements of host organelles during infection. For example, it has been reported that *Chlamydia* infection of human epithelial cells causes Golgi fragmentation and generation of Golgi ministacks, which is then recruited to bacterial inclusion and contributed to bacterial maturation (Heuer et al., 2009). As for *Legionella*, it has been reported that LCV recruits ER membranes in the presence of SidE family effectors, which results in the transformation of the *Legionella* phagosome into a specific compartment harboring features of ER membranes (Kotewicz et al., 2017; Shin et al., 2020; Xu and Luo, 2013). Moreover, it has long been known that *Legionella* containing vacuole is decorated with PI(4)P, which functions as bait to recruit bacterial effectors that possess PI(4)P binding domains during infection. One previous study reported that PI(4)P on LCV is directly recruited from the host Golgi by bacteria, but not transformed from other phospholipids (Weber et al., 2018). In addition, the

maintenance of PI(4)P on LCV requires the bacterial T4SS secretion system, but the exact effectors involved in PI(4)P retention on LCV are still unclear. We asked that if *Legionella* infection mediated Golgi disruption facilitates the fusion of Golgi vesicles with LCV, then boosts the formation of LCV and consequent intracellular replication. To this end, we infected A549 cells with *Legionella*, and stained PFA-fixed cells with antibodies that target cis-Golgi protein GM130 or trans-Golgi protein TGN46. Immunostaining images were acquired with confocal microscopy to check whether these host Golgi proteins are recruited to LCV during infection. Immunostaining result indicates that neither cis-Golgi protein nor trans-Golgi protein was recruited to LCV during *Legionella* infection (Fig. 41A, B).

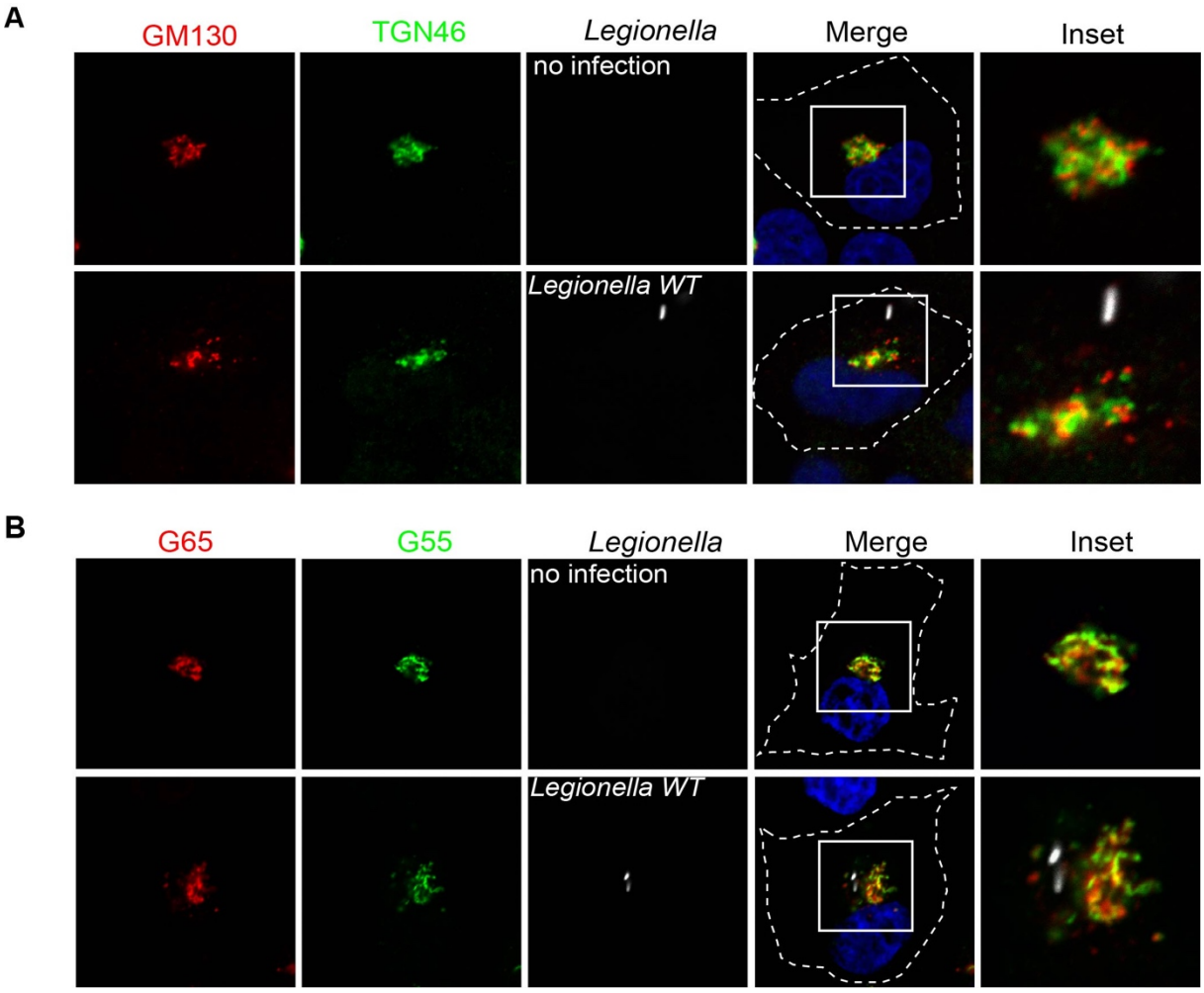


Figure 41. Legionella does not recruit Golgi proteins to LCV.

(**A**) Immunostaining images showing *Legionella* does not recruit cis-Golgi protein GM130 or trans-Golgi protein TGN46 during infection. A549 cells were infected with *Legionella* for 2 hours. Infected A549 cells were washed with PBS for 3 times to remove un-endocytosed bacteria, then fixed with 4% PFA and stained with anti-GM130 and anti-TGN46 antibodies. (**B**) Immunostaining images showing *Legionella*

does not recruit trans-Golgi protein GRASP55 or cis-Golgi protein GRASP65. A549 cells were infected with *Legionella*. Infected A549 cells were washed with PBS for 3 times to remove un-endocytosed bacteria, then fixed with 4% PFA and stained with anti-GRASP55 and anti-GRASP65 antibodies

To further confirm this observation, we infected HEK293T cells overexpressing GRASP55 and another trans-Golgi protein GalT with *Legionella* to check whether *Legionella* recruits these proteins from the Golgi. Immunostaining images indicate that both exogenous GRASP55 and GalT were recruited to LCV upon infection of wild-type *Legionella*. However, exogenously expressed GRASP55 and GalT were partially localized to ER in our study. In view of that the ER membranes can be remodeled and recruited to LCV during infection. The recruitment of GRASP55 is likely derived from the ER, instead of the dispersed Golgi (Fig. 42A, B). These data indicate that *Legionella* does not hijack Golgi derived vesicles by disrupting the Golgi.

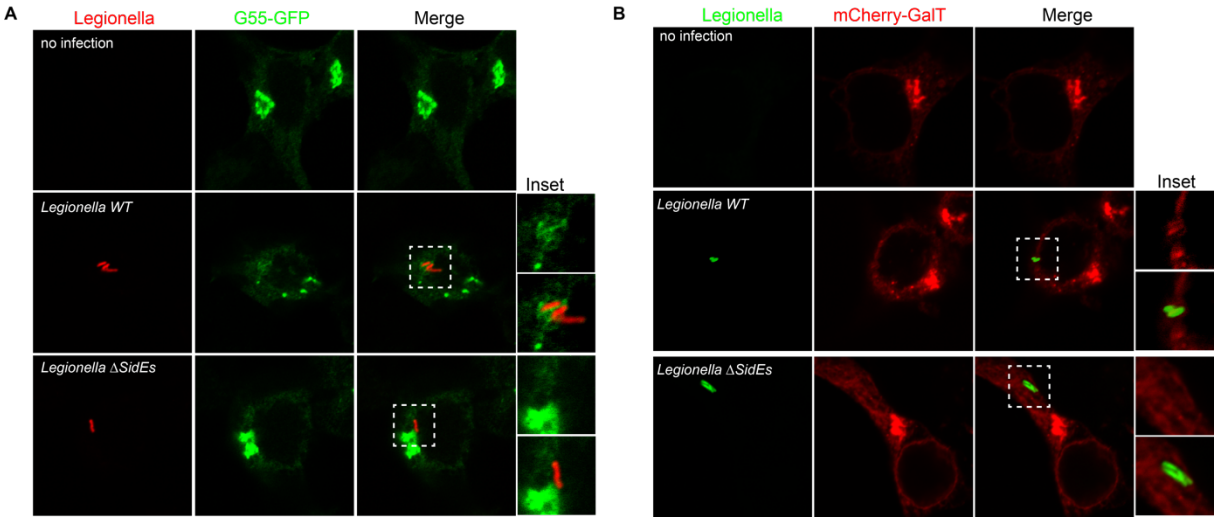


Figure 42. Legionella recruits exogenous Golgi protein GRASP55 and GalT during infection.
(**A**) HEK293T cells expressing GFP tagged GRASP55 were infected with wild type or *sidE* mutant *Legionella* for 2 hours. Infected cells were washed with PBS for 3 times to remove un-endocytosed bacteria, then fixed with 4% PFA and stained with the anti-*Legionella* antibody. (**B**) HEK293T cells expressing mCherry-tagged GalT were infected with wild type or *sidE* mutant *Legionella* for 2 hours. Infected cells were washed with PBS for 3 times to remove un-endocytosed bacteria, then fixed with 4% PFA and stained with anti-*Legionella* antibody.

7.3.9 SidE family effectors mediated PR-ubiquitination regulates secretory pathway of host cells

7.3.9.1 PR-ubiquitination regulates trafficking of VSVG

In eukaryotic cells, newly synthesized proteins are transported from the ER to the Golgi, where the proteins get modified by various protein modification enzymes, and sorted to different destinations via the trans-Golgi network. Considering the critical role of the intact Golgi in protein trafficking, we asked whether PR-ubiquitination of Golgi proteins mediated Golgi fragmentation affects the secretory pathway in cells. To this end, we attempted to monitor the trafficking pathway in cells expressing SdeA or infected with *Legionella* using the vesicular stomatitis virus glycoprotein (VSVG) tagged with GFP protein. VSVG is a transmembrane protein containing a mutation that results in its reversible misfolding and accumulation in the ER at 40 °C. When the temperature is shifted to 32°C, the protein folds correctly and is transported from the ER to the plasma membrane via the Golgi (Bergmann, 1989; Presley et al., 1997). In previous studies, VSVG protein has been used to monitor secretory protein trafficking in cells (De Jong et al., 2006; Scidmore et al., 1996). In order to investigate the effect of SdeA mediated protein PR-ubiquitination on the trafficking of VSVG, A549 cells were transfected with VSVG-GFP plasmid then cultured at 37 °C for one day to express VSVG-GFP protein before 16 h incubation at 40 °C. Cells were then infected with *Legionella* strains for 2 hours and then transferred to 32 °C, which triggers the VSVG protein release from the ER. Immunofluorescence images suggested that, in uninfected cells and cells infected with *Legionella* sidEs mutant strain, VSVG started to move to the Golgi quickly and accumulated in the Golgi, once the cells were incubated at 32 °C. In addition, almost all the VSVG proteins were transported to the Golgi after 20 min, whereas this process was slowed down in wild-type *Legionella* infected cells. Moreover, after 60 min incubation at 32 °C, more VSVG containing vesicles emerged in control cells or cells infected with *Legionella* mutant strain compared to that in the cells infected with wild-type *Legionella* (Fig. 43A, B). This result indicate that SdeA mediated PR-ubiquitination of host proteins inhibits VSVG trafficking via the Golgi.

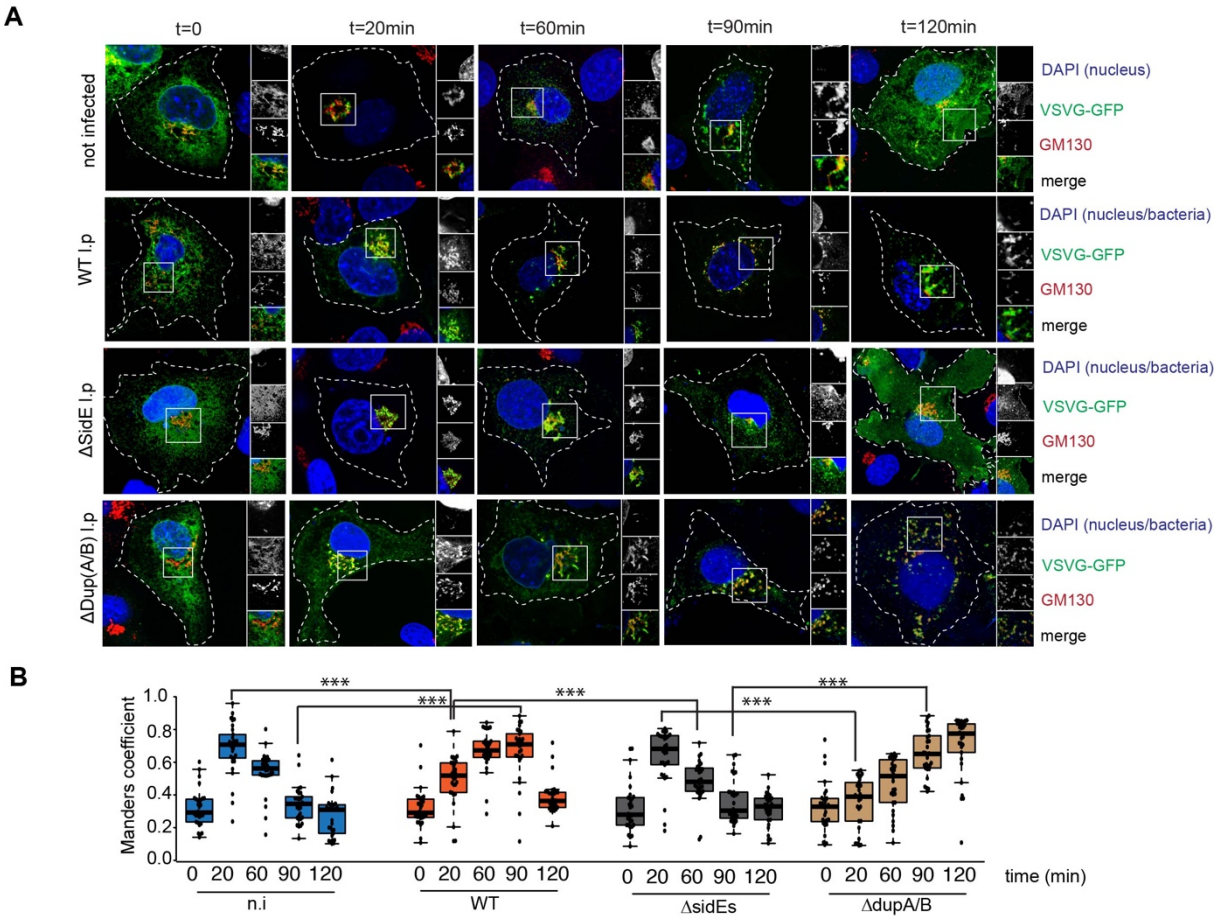


Figure 43. SidE family effectors mediated PR-ubiquitination slows down VSVG trafficking through Golgi

(**A**) Microscopy images showing the effect of SidE family effectors on VSVG trafficking during *Legionella* infection. A549 cells were transfected with VSVG-GFP plasmid and cultured at 37 degrees for 24 hours to express the protein before transferred to 40 degrees. After 16 hours of incubation at 40 degrees, cells were infected with *Legionella* for 2 hours then washed once and moved to 32 degrees for 120 min to release VSV-G from ER. Fixed cells were stained for GM130, images were acquired with immunofluorescence microscopy. (**B**) Quantitative analysis of data of Figure 33 A. Co-localization between GFP-VSVG and Golgi marker GM130 was shown as Manders coefficient. Data represents 30 cells taken from 3 independent experiments. White boxes indicate insets which are split into red, green, blue channels and displayed on the right side of the image. Center lines show the medians; box limits indicate the 25th and 75th percentiles as determined by R software; whiskers extend 1.5 times the interquartile range from the 25th and 75th percentiles, outliers are represented by dots; data points are plotted as circles.

*(Imaging of Fig. 43 was performed by Dr. Rukmini Mukherjee)

Endoglycosidase H (EndoH) is an enzyme that is capable of cleaving mannose rich (ER) but not complex (Golgi and post-Golgi) form of N-like oligosaccharides from glycoproteins. EndoH has been widely used to monitor protein trafficking by measuring

the transformation of a glycoprotein from EndoH sensitive to EndoH resistant form (Burke et al., 1984; Ernst et al., 2018). To further test the effect of PR-ubiquitination on VSVG trafficking through the Golgi, we attempted to EndoH cleavage to analyze the transformation of VSVG glycosylation. For this reason, we infected HEK293T cells expressing VSVG at 40 °C for 2 h, then the infected cells were incubated at 32 °C for indicated times. Cells were lysed with lysis buffer containing 1% SDS, resulting lysates were treated with EndoH and separated with SDS-PAGE. Western blotting results indicate that VSVG trafficking was slowed down in cells infected with wild-type *Legionella* compared to uninfected cells or cells infected with *Legionella* sidEs mutant strain. As for uninfected cells or cells infected with *Legionella* sidEs mutant strain, the EndoH resistant form of VSVG was detected in samples from cells incubated 15 min at 32 °C and increased over time. In addition, almost all VSVG were converted to EndoH resistant form after 120 min incubation at 32 °C. In cells infected with wild-type *Legionella*, the EndoH resistant form of VSVG transformed slower than that in the uninfected cells or cells infected with *Legionella* sidEs mutant strain, only around 50% of the proteins were resistant to EndoH after 120 min incubation at 32 °C (Fig. 44A, B). These data further confirm that SidE family effectors mediated PR-ubiquitination inhibits VSVG trafficking through the Golgi.

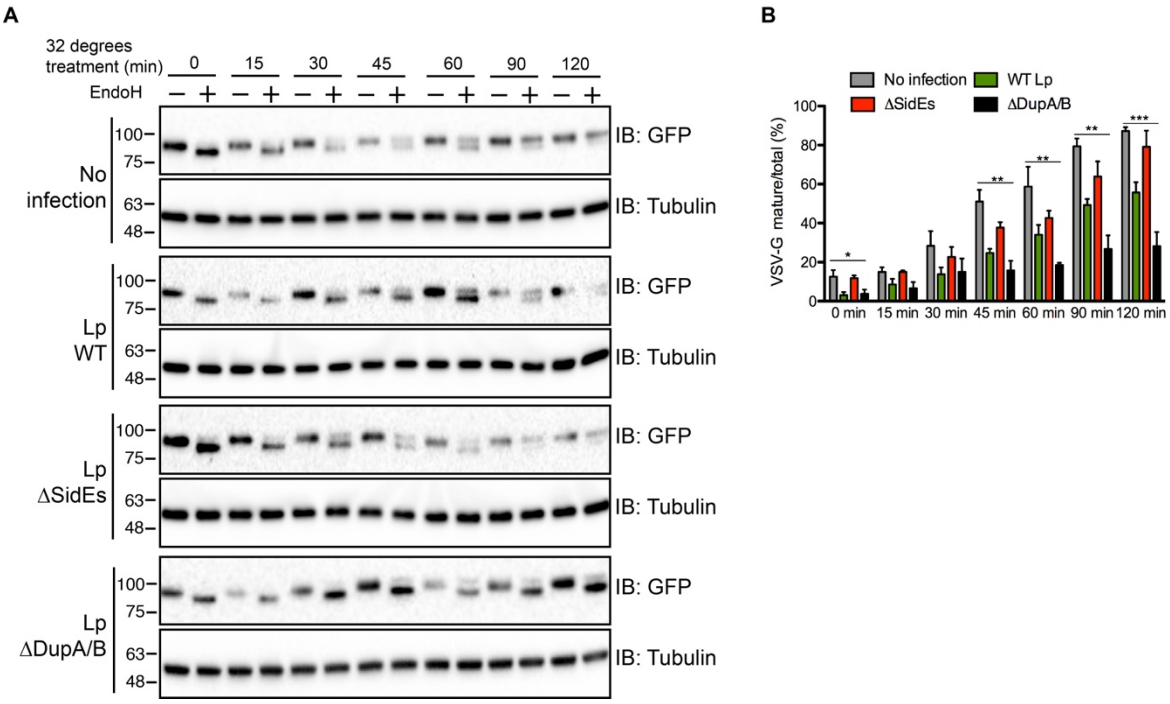


Figure 44. SidE family effectors inhibit VSVG trafficking through Golgi.

(**A**) Western blotting of VSV-G cleavage with EndoH. HEK293T cells were co-transfected with plasmids encoding VSVG-GFP and CD32 and incubated overnight at 37 °C to express exogenous proteins and

followed with 16 h of incubation at 40 °C. Cells were infected with *Legionella* for 2 hours and incubated at 32 °C for indicated times before lysed with lysis buffer containing 1% SDS. Denatured proteins were cleaved with EndoH and separated with SDS-PAGE. The upper band indicates the EndoH resistant form and the lower band indicates the EndoH sensitive form of VSVG. (**B**) Quantification of results from (**A**).

By co-expressing SdeA and VSVG in HEK293T cells, we further investigated the effect of SdeA on VSVG trafficking through the Golgi. HEK293T cells were co-transfected with VSVG-GFP and mCherry or mCherry-SdeA, 24 h at 37 °C after transfection, cells were transferred to 40 °C for 16 h incubation. Cycloheximide was added into the medium to inhibit further protein synthesis before cells were moved to 32 °C for different time points to release VSVG from ER. Microscopy result showed that GFP-tagged VSVG localized to the ER at 40 °C, 1 h after incubation at 32 °C, most of VSVG were exported to the Golgi, whereas this process was inhibited in cells expressing wild-type SdeA. Four hours after incubation at 32 °C, VSVG proteins were transported to the plasma membrane, however the VSVG in cells expressing wild-type SdeA were localized to the Golgi (Fig. 45A). This observation of the effect of SdeA on VSVG trafficking was confirmed by EndoH cleavage experiment (Fig. 45B). Cells treated similarly as the samples of Fig. 45A were processed with EndoH and then blotted with GFP antibody for monitoring the VSVG cleavage. VSVG converted into EndoH resistant form as the incubation at 32 °C extended. This process was markedly inhibited by the expression of SdeA (Fig. 45B). These data suggest that PR-ubiquitination catalyzed by SdeA decelerates protein trafficking through the Golgi.

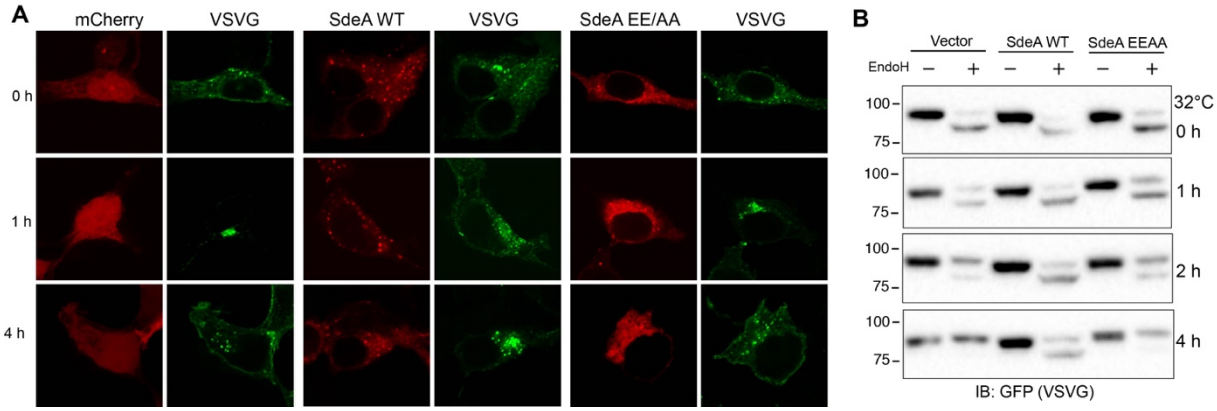


Figure 45. SdeA inhibits VSVG trafficking through Golgi.

(**A**) Microscopy result of VSVG trafficking in cells expressing SdeA. HEK293T cells expressing GFP-VSVG and mCherry-SdeA were incubated at 40 °C and transferred to 32 °C for indicated times before

being fixed with 4% PFA. (**B**) EndoH assay of samples from HEK293T cells expressing SdeA. HEK293T cells expressing GFP-VSVG and mCherry-SdeA were incubated at 40 °C and transferred to 32 °C for indicated times before being fixed.

7.3.9.2 PR-ubiquitination does not alter protein glycosylation

Then we asked whether SdeA-induced Golgi disruption affects the protein glycosylation in cells. For this reason, we transfected HEK293T cells with wild-type SdeA or SdeA EE/AA mutant. 24 h after transfection, cells were lysed and the lysates were blotted with antibody against LAMP1, which has been used as a monitor of protein glycosylation in cells (Raval et al., 2015). Western blotting data suggested that SdeA expression did not change the final glycosylation of LAMP1 in cells, as no significant band shift or mobility change on the SDS-PAGE gel was detected from LAMP1 of cells expressing SdeA (Fig. 46A). To further verify this result, we transfected HeLa cells with SdeA and stained the cells for Wheat germ agglutinin (WGA), a lectin that selectively recognizes and binds to SA and N-acetylglucosamine (GlcNAc) on the maturely glycosylated proteins. Immunostaining result suggested that the signal for WGA of cells was not affected by the expression of SdeA (Fig. 46B). These observations suggest that activity of SdeA slows down trafficking through the Golgi without completely inhibiting the function of the Golgi in protein glycosylation.

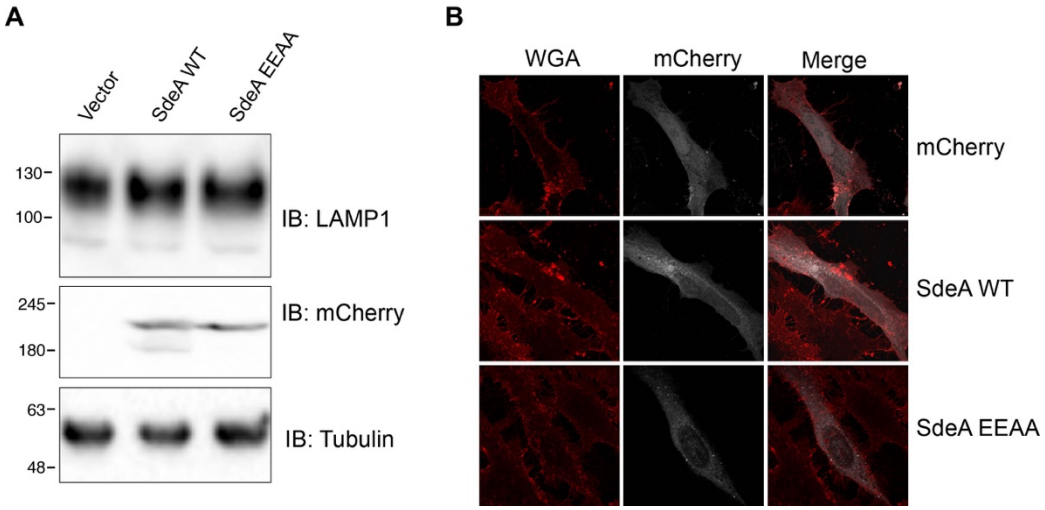


Figure 46. SdeA expression does not alter protein glycosylation.

(**A**) SdeA does not affect LAMP1 glycosylation. HEK293T cells were transfected with wild-type SdeA or SdeA EE/AA. 24 h after transfection, cells were lysed and blotted with antibody against LAMP1. (**B**) SdeA does not affect protein glycosylation in cells. HeLa cells were transfected with wild-type SdeA or SdeA EE/AA. 24 h after transfection, cells were fixed and stained for WGA.

7.3.9.3 PR-ubiquitination caused by SidE effectors inhibits cytokines secretion

As part of the immune response, macrophage cells secret cytokines upon bacterial infection. ER-to-Golgi route trafficking plays a critical role in the conventional trafficking of most of the cytokines, maintenance of Golgi integrity is important for secretion cytokines, such as TNF-α (Micaroni et al., 2013). So we asked if PR-ubiquitination caused by SidE effectors affects cytokine secretion of macrophage cells upon *Legionella* infection. In order to test this, we treated THP-1 cells with PMA to differentiate THP-1 to macrophage cells, then differentiated cells were infected with wild type or *ΔsidEs Legionella* for indicated times. Cell culture mediums were collected and filtered with a 0.22 μM filter before used for ELISA assay. ELISA data indicates that THP-1 cells infected *Legionella ΔsidEs* mutant strain secreted more TNF-α protein to medium than cells infected with wild-type *Legionella* strain or *Legionella ΔdupA/B* mutant strains (Fig. 47A). Moreover, THP-1 cells infected with *Legionella ΔdupA/B* mutant strain secreted less TNF-α protein to medium than cells infected with *Legionella ΔsidEs* mutant strains. This result indicates that SidE effectors mediated PR-ubiquitination of host proteins inhibits cytokine secretion. Unlike TNF-α, interleukin (IL)-1β secretion is independent of the conventional ER-Golgi trafficking. To verify whether the regulation of cytokine secretion by SdeA effector is specific, we measured IL-1β released with ELISA. Similar to TNF-α, secretion of IL-1β by THP-1 cells infected with the *Legionella* strain lacking SidE family effectors was enhanced, compared to cells infected with wild-type or *ΔdupA/B Legionella* strains (Fig. 47B), suggesting that both conventional and unconventional secretion processes could be affected by SidE effectors. It has been reported that GRASP55 and GRASP65 are involved in the unconventional secretion of IL-1β (Chiritoiu et al., 2019), SidE effectors may regulate IL-1β release, but possibly in a different manner than TNF-α secretion. Taken together, these data demonstrate that Golgi disruption caused by SidE effectors impairs protein secretory pathways.

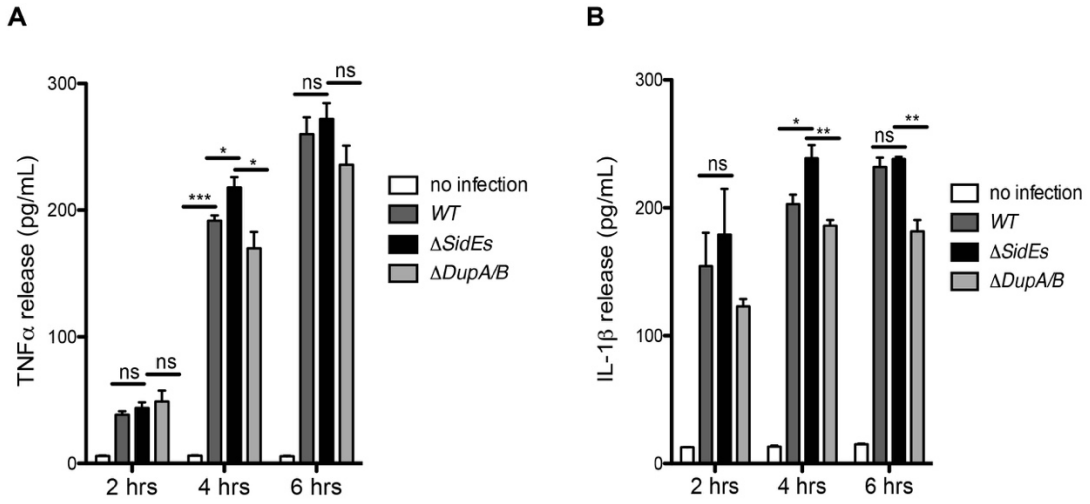


Figure 47. SdeA-catalyzed PR-ubiquitination inhibits the secretion of cytokines.

(**A**) TNFα secretion of cells infected with *Legionella*. Differentiated THP-1 cells were infected with *Legionella* strains for indicated times, the filtered medium was used for ELISA assay. (**B**) IL-1β secretion of cells infected with *Legionella*. Differentiated THP-1 cells were infected with *Legionella* strains for indicated times, the filtered medium was used for ELISA assay.

8 Discussion

To date, over 300 *Legionella* proteins have been identified as effectors that can be injected into host cells to facilitate LCV formation and bacterial replication, however, the functions of most of these effectors remain unknown. Previous studies have revealed that *Legionella* SidE family effectors including SidE, SdeA, SdeB, SdeC are required for efficient intracellular bacterial replication. These effectors share sequence homology and possibly same function as the SidE family deletion *Legionella* mutant strain (*ΔsidEs*) shows attenuated virulence against the host *Dictyostelium discoideum*, replenishment of wild-type SdeA in a *ΔsidEs* strain almost completely restored its ability to grow within the host play critical role in *Legionella* bacterial intracellular growth of during infection (Bardill et al., 2005). In addition, yeast toxicity assay shows that SdeA is extremely toxic to yeast cells as low amount expression of SdeA is able to suppress the formation and growth of the yeast colony. Even though SidE effectors are important for bacterial virulence during *Legionella* infection, the exact function of these effectors had long been unknown. Sequence BLAST analysis in a study led by Qiu identified a putative mono-ADP-ribosyltransferase (mART) domain in the central region of SdeA, which is conserved in all of SidE family effectors. This mono-ADP-ribosyltransferase domain catalyzes the ADP-ribosylation of ubiquitin on Arg42, resulting in the formation of ADP-ribose ubiquitin intermediate that is critical for the novel ubiquitination. Further studies revealed that, instead of ADP-ribolsylating other host proteins, SdeA catalyzes NAD^+ dependent ubiquitination of Rab proteins without the need of E1 and E2 (Qiu et al., 2016). Given that the conventional ubiquitination involves a three-enzyme cascade mediated formation of isopeptide bond between the ubiquitin C-terminal glycine and a lysine of substrate protein, by consuming ATP, the finding of this all-in-one ubiquitin ligase that catalyzes ADP-ribosylation of ubiquitin and consequent substrate ubiquitination expands our understanding of not only this post-translational modification and function regulation by ubiquitin, but also new cell biology about how bacteria hijacks host machinery to benefit its invasion. However, the mechanism of how ADP-ribosylated ubiquitin is transferred to substrates had been poorly understood. In addition, given the importance of SidE family effectors for *Legionella* infection, identification of more substrates of these SidE family effectors is important for answering how this novel ubiquitin ligase regulates host pathways and facilitates bacterial pathogenicity.

Our sequence analysis suggested that SdeA harbors a putative PDE domain between residues 200 and 500. *In vitro* experiment confirmed that PDE cleaves the pyrophosphate bond between two phosphates of ADP in ADP-ribosylated Ub, which leads to the generation of phosphoribosylated Ub and release of AMP. Phosphoribosylated Ub is subsequently transferred to the serine of substrate proteins, resulting in phosphoribosyl-linked serine ubiquitination. This finding answers nicely the question raised in the study of Qiu: why SdeA does not show ADP-ribosylation activity when incubated with mammalian cell lysate in the presence of radiolabeled NAD^+ (Qiu et al., 2016). Importantly, the study also improves our understanding of the mechanism of SdeA-catalyzed ubiquitination, in which Ub is first ADP-ribosylated on Arg42 by the mART domain, the ADP-ribosylated Ub is then processed to phosphoribosylated Ub and transferred to the substrates by PDE domain.

Strikingly, antibody screening assay revealed that ubiquitin treated with SdeA was not detectable by an ubiquitin antibody (Abcam), possibly due to the interference in the antibody recognition caused by SdeA-catalyzed modification on ubiquitin. Using this as a tool allows us to monitor the effect of SdeA. We found that free ubiquitin molecules of cells expressing SdeA or incubated with *Legionella* lysate were almost completely phosphoribosylated by probing with this antibody. The fact that SdeA-catalyzed phosphoribosylation of Ub makes Ub inactivated for interacting with E1 *in vitro* prompted us to investigate the effect of this SdeA-mediated modification of ubiquitin on cellular events. Firstly we addressed the question whether SdeA-mediated modification affects total ubiquitination in cells. This observation that expression of both wild-type SdeA and PDE mutant SdeA H277A suppresses the poly-ubiquitination of mammalian cells inspired us to verify the effect of SdeA on ubiquitin pool. Western blotting result showed an increase in the level of mono-Ub in cells transfected with GFP-SdeA H277A PDE mutant, but not in cells expressing WT SdeA, probably because the WT SdeA ultilizes Ub to ubiquitinate substrates. Given that ubiquitination is critical to various cellular events including protein stability, TNFα signaling, and autophagy, we checked the effect of SdeA expression on these cellular pathways in cells. In cells transfected with SdeA, protein HIF1-α that constantly undergoes ubiquitination and degradation under normal conditions was markedly accumulated. SdeA expression also suppressed nuclear translocation of p65 induced by TNFα treatment. Moreover, we found that SdeA expression inhibited mitochondria ubiquitination induced by CCCP treatment. These data reveal that SdeA-mediated

modification of ubiquitin is a robust inhibitor of host cellular Ub system and many Ub-dependent pathways. It has been characterized that ubiquitin itself can be modified and manipulated by other post-translation modifications, such as acetylation and phosphorylation (Herhaus and Dikic, 2015). The acetylation of ubiquitin has been detected on K6 and K48, two well known lysines residues that important for the formation of ubiquitin chain, K6 and K48 acetylated Ub could not be used for the synthesis of Ub chains (Ohtake et al., 2015). Selective autophagy of damaged mitochondria is induced by PINK1-activated E3 ligase Parkin. The protein kinase PINK1 has been shown to phosphorylate Ser65 of both free Ub and Ub in chains. Phosphorylation of Ub S65 results in Parkin E3 ligase activation and mitochondria translocation, but less recognition by DUB (Kane et al., 2014; Wauer et al., 2015). Moreover, besides the modifications of ubiquitin by mammalian proteins, ubiquitin is also a target of bacterial effectors. Glutamine Deamidation of ubiquitin on Gln40 by Cif, effector protein of *Enteropathogenic Escherichia coli* (EPEC), impairs ubiquitin chain synthesis (Cui et al., 2010). Taken together, our study expands the understanding of the regulation of ubiquitin molecule itself. Interestingly, even though the Ub pathway is blocked, the free Ub can be used by SdeA to ubiquitinates host proteins and manipulates related cellular events.

On the other hand, our data demonstrates that SdeA has 3 functions in host cells: one is to recycle host ubiquitin via its N-terminal DUB domain, one is to ubiquitinate substrates including Rab33b and Rab1, the third one is to modify ubiquitin and block cellular signaling. It has long been known that SdeA expression has serious toxicity in yeast in unclear mechanism, to dissect which of these two functions leads to the effects of SdeA in yeast cells, we conducted yeast toxicity assay by expressing wild-type SdeA or mART mutant EE/AA or PDE mutant H277A in yeast. As reported previously (Qiu et al., 2016), expression of wild-type SdeA completely suppressed the growth of yeast, whereas mART mutant SdeA EE/AA was not toxic to yeast. Moreover, and importantly, the PDE mutant SdeA H277A that is able to ADP-ribosylate ubiquitin but not ubiquitinate substrate proteins was still toxic to yeast. This suggests that phosphoribosylation of ubiquitin rather than ubiquitination of substrates is relevant to toxicity caused by SdeA in yeast. Virulence of *Legionella* lacking SidE family effectors *ΔsidEs* was attenuated in amoeba host *Dictyostelium discoideum*, expression of wild-type SdeA in *ΔsidEs* strain complemented such defect (Qiu et al., 2016). However, replenishment of mART mutant SdeA EE/AA or SdeA M408A/L411A that lost the ability

to bind substrates failed to restore its ability to grow in host cells (Kalayil et al., 2018). This result suggests that the PR-ubiquitintion of substrates rather than the phosphoribosylation of ubiquitin is the main function of SidE family effectors during physiological bacterial infection.

Identification of the functions of SdeA sheds light on the new strategy how bacteria benefits its invasion by hijacking ubiquitin. On the other hand, the finding of SdeA-mediated modification ubiquitin indeed expands our knowledge about post-translation modification of ubiquitin and will lead us to explore the proteins that harbor functions of mART and PDE in other species. As pathogenic bacteria can acquire the effectors via gene transfer, it is possible that proteins with similar activities exist not only in *Legionella*. It will be of great interest to identify the endogenous ADP-ribosylation or phosphoribosylation of Ub in mammalian cells in future.

As we stated in the Introduction section, during *Legionella* infection, many effectors require a healthy Ub system and host cell for the efficient intracellular replication (Xu and Luo, 2013). Given the effect of these SidE effectors on conventional ubiquitination system, it is not surprising that these SidE family effectors could be spatially and temporally regulated during infection by another effector. Continuous activation of SdeA causes growth defect in host cells, this problem is proved to be solved by inactivating Side family effectors with SidJ (Jeong et al., 2015). It has been previously reported that SidE effectors reached to highest expression level in the early stationary phase and were injected into host cells in the early stage of infection (30 min post infection), suggesting that SdiE family effectors are critical for bacterial invasion and formation of LCV (Bardill et al., 2005). SidE effectors could be also detected on the LCV at the early stage of infection and decrease as the infection proceeded. However, in cells infected with SidJ deletion strain, SidE effectors retained on LCV at late stage of infection, indicating that the functions of SidE family effectors are tightly modulated by SidJ (Jeong et al., 2015). Recently it was reported that SidJ functions as a novel ubiquitin-deconjugating enzyme that reverses SdeA-mediated PR-ubiquitination by cleaving the phosphodiester bond between serine residues of substrates and phosphoribosylated ubiquitin (Qiu et al., 2017). The ubiquitination level of Rab33b gradually decreased as the infection proceeded, in the meanwhile, the level of translocated SidJ increased, which led to the conclusion that SidJ temporally regulates SidE family via its deubiquitinase activity (Qiu et al., 2017).

By expressing SidJ in mammalian cells, we indeed proved that the presence of SidJ results in the disappearance of Rab33b ubiquitination caused by wild-type SdeA. We then incubated ubiquitinated Rab33b with lysates of wild-type *Legionella* strain or SidJ deletion strain, the data showed that *Legionella* lysates also removed ubiquitin from the modified substrate. Interestingly, this observation was also made in the absence of SidJ, indicating the existence of other *Legionella* effectors counteracting the effect of SidE effectors. To better understand how the effectors of *Legionella* control the bacterial effector mediated ubiquitination, we incubated ubiquitinated Rab33b and autoubiquitinated SdeA with purified full-length SidJ and more stable N-terminal deleted SidJ (126-C). The finding that recombinant SidJ proteins could not cleave the substrates *in vitro* suggested that reverse of PR-ubiquitination is more complicated than previously established.

Sequence alignment analysis revealed that *Legionella* has two SdeA PDE-like proteins DupA and DupB. In view of the fact that SdeA PDE cleaves the phosphodiester bond of ADP-ribosylated Ub and transfers the resulting PR-ribosylated to serine residue of substrate, we hypothesized that DupA/B can cleave the phosphodiester bond between serine residue of substrate and phosphoribosylated ubiquitin. *In vitro* reactions between ubiquitinated substrates with purified DupA or DupB demonstrated that both of these two PDE-like effectors cleave ubiquitin from modified substrates of SdeA. The identification of DupA/B led us to determine why the SidJ protein used in the previous study was active in cleavage of both canonical ubiquitination and SdeA induced serine phosphoribosl-ubiquitination, we analyzed the SidJ protein purified from *Legionella* with mass spectrometry, and the data showed that the SidJ protein was contaminated with DupA, DupB, and also SdeA, B, C proteins that contain DUB domain. SidJ protein likely displayed the DUB activities of its interactions *in vitro*. Our data suggests that SidJ does not has the activity of deubiquitinase to handle with canonical ubiquitination or SdeA catalyzed ubiquitination, SidJ probably inhibits SdeA in cells in a way without functioning as a deubiquitinase. Recently, studies of different groups reveal that SidJ inhibits SidE family by polyglutamylating them at the catalytic residue of mART domain in the presence of calmodulin (Bhogaraju et al., 2019; Black et al., 2019; Gan et al., 2019).

Unlike SdeA, DupA shows very weak activity to transfer PR-Ub to substrate *in vitro*. Determination of the structure of DupA H67A and PR-Ub complex and biochemical experiments demonstrated that DupA has stronger binding affinity to Ub, ADPR-Ub

and PR-ubiquitinated substrates, because of the extensive electrostatic interactions. However, SdeA PDE does not bind to PR-ubiquitinated substrates. These differences provide good explanation of the question why SdeA PDE does not has the deubiquitinase activity of DupA, and why DupA does not transfer PR-Ub to substrates effectively.

As shown by the yeast toxicity assay and intracellular growth determination of bacteria strains expressing wild-type SdeA or SdeA mutants, the core function of SidE family effectors to ubiquitinate proteins of host cells during infection, identification of substrates of SidE family effectors is highly necessary for better understanding of how *Legionella* manipulates cellular pathways of host cells for its benefits. To this end, a possible strategy is to express SdeA in cells or infect cells expressing tagged ubiquitin lacking C-terminal GG residues that is needed for the conventional ubiquitination but not this novel PR-ubiquitination, then isolate ubiquitinated proteins for mass spectrometry analysis. However, exogenous expression of a defective ubiquitin might be challenging for host cells, thus making the following infection occurs under abnormal condition. Given the strong binding affinity of DupA to PR-ubiquitinated substrates, using DupA H67A as a trapping bait allows us to enrich and isolate PR-ubiquitinated proteins from the lysate of cells infected with *Legionella* or expressing SdeA. The enriched proteins were further eluted by cleaving the phosphodiester bond with purified wild-type DupA, and analyzing with mass spectrometry. Over 180 proteins were significantly enriched from cells infected with *Legionella*. Among these identified proteins, a lot of protein are ER-resident proteins, such as RTN3, RTN4, FAM134C, LNP1 and TEX264, demonstrating that SdeA-mediated PR-ubiquitination might be involved in ER remodeling during bacterial infection.

In addition, proteomics analysis of enriched proteins with DupA H67A trapping mutant also identified Golgi associated proteins, including Rab33b, GCP60 and GRASP55. Interestingly, In agreement with the finding that SdeA ubiquitinates Golgi proteins, we also found that SdeA partially localizes to the Golgi in cells vis its C-terminal region possessing coiled-coil motif. Moreover, and importantly, the Golgi tethering protein GRASP55 is one of the enriched candidates scored with the highest ratios. Interestingly, extraneous expression of the full-length SdeA in cells induced structural disruption of the Golgi. This effect on Golgi structure was also observed in A549 cells infected with wild-type *Legionella* strain and was more visible in cells infected with a strain lacking DupA/B, these results suggest that SidE family effectors mediate the

regulation of Golgi structure. Of note, infection of the strain lacking SidE effectors could also induce the Golgi structural impairment, indicating the existence of other effectors involved in the modulation of Golgi integrity. It will be interesting to identify other effectors and study how these effectors synergistically regulate the morphology of the Golgi in future.

Recently extensive efforts have been made to investigate the mechanism of the novel PR-ubiquitination catalyzed by *Legionella* effector SdeA. However the functions of PR-ubiquitination of the substrates in the modulation of cellular processes are still largely unknown. This observation of Golgi dispersal caused by SdeA expression led us to ask whether SdeA catalyzes PR-ubiquitination of Golgi proteins and thus regulates the function of the Golgi.

Golgi stacking factor GRASP55 and its homolog GRASP65 have been reported to play important roles in Golgi structure maintenance (Shorter et al., 1999; Xiang and Wang, 2010). Double knockout of GRASP55 and GRASP65 *in vivo* leads to disconnection of the Golgi stacks (Grond et al., 2020). In view of the importance of GRASP proteins in preserving Golgi integrity, we asked that whether injected SidE family effectors localize to the Golgi and ubiquitinate Golgi proteins during *Legionella* infection, which affects the activities of the proteins, thus dispersing the Golgi in to mini-stacks. Both *in vitro* and *in vivo* experiments confirmed that GRASP55 and GRASP65 are ubiquitinated by SdeA. Moreover, Golgi tethering proteins GRASPs and GCP60 expressed in cells infected with wild-type *Legionella* were ubiquitinated, however infection with *Legionella ΔsidEs* mutant did not cause ubiquitination of these proteins, suggesting that this ubiquitination induced by *Legionella* infection is dependent on the ubiquitin ligase function of SidE effectors. To better understand the details of the PR-ubiquitination of GRASP55 and address the mechanism of how it affects the function of GRASP55, we applied the mass spectrometry to identify the modification sites of GRASP55 by SdeA. From mass spectrometry analyses of *in vitro* reaction sample of GRASP55 and SdeA, we detected 4 serines in GRASP55 ubiquitinated by SdeA. Interestingly, replacement of these target serines and their adjacent serines extensively abolished the ubiquitination of GRASP55 in cells expressing SdeA or infected with wild type *Legionella*. Of note, mutation of these serines did not completely suppress ubiquitination of GRASP55 in cells, suggesting that other serines in GRASP55 can be alternatively ubiquitinated by SdeA. This is in agreement with the observation made on another known SdeA substrate Rab33b, in which S154 was identified as a main target

residue of SdeA, however mutation of S154 to A did not change the PR-ubiquitination modification caused by SdeA (Bhogaraju et al., 2016).

Localization of the GRASP55 protein to the Golgi is due to its conserved N-terminal GRASP domain interaction to Golgi proteins, as well as the formation of trans-dimerization with other GRASP55 protein. It has been reported that phosphorylation of serines of the C-terminal proline rich (SPR) domain by mitotic kinases is involved in regulation of trans-dimerization of GRASP55 proteins (Feinstein and Linstedt, 2008; Wang et al., 2005). Moreover, serines in the C-terminus of GRASP55 including S408, S409, S449, which are identified as ubiquitination targets of SdeA in our study, have been reported to be modified by phosphorylation in previous studies. Phosphorylation at the C-terminal region results in function regulation of GRASP55. It has been shown that mimics of phosphorylation by changing these serines to aspartic acids disrupted GRASP55 homodimerization possibly due to protein conformational change (Kim et al., 2016; Truschel et al., 2012). The homodimerization determination indicates that interaction between GRASP55 molecules was impaired when GRASP55 was modified by SdeA, which was observed in samples from both *in vitro* reaction experiment and co-expression experiment in cells. These data provide insight into the mechanism for answering the question how PR-ubiquitination caused by bacterial effector SdeA induces Golgi fragmentation by providing an explanation that PR-ubiquitination of GRASP proteins caused by SdeA disrupts homodimerizations of GRASP proteins, thus disconnecting Golgi ribbon to stacks.

So far a number of pathogens have been described as requiring host organelles during infection for their successful invasion and intracellular bacterial proliferation. For example, it has been reported that *Chlamydia* fragments the Golgi of host cells upon infection, thus facilitating the recruitment of generated Golgi ministacks to bacterial inclusion for bacterial maturation (Heuer et al., 2009). For *Legionella*, the phagosome containing *Legionella* starts to recruit ER membranes from the early stage of infection, which facilitates the formation of an ER-like compartment where bacteria replicates (Kotewicz et al., 2017; Shin et al., 2020; Xu and Luo, 2013). In addition to the recruitment of ER membranes, it has been reported that LCV also receives PI(4)P directly from Golgi during infection of amoebae (Weber et al., 2018). From our data of the immunostaining experiment, endogenous Golgi proteins including GRASP55 or GRASP65 were not detected on the LCV or around *Legionella*, suggesting that *Legionella* does not recruit Golgi proteins. Similarly, cis Golgi marker GM130 and

trans-Golgi marker TGN46 were not detected to be recruited to LCV as well. These data indicate that *Legionella* SidE family effectors induce disconnection of the Golgi, however the dispersed Golgi is not recruited by *Legionella*. This is consistent with previous studies showing that the rare Golgi matrix proteins were identified from LCVs purified from infected host cells by proteomics approach (Schmölders et al., 2017; Urwyler et al., 2009). Recently there is another study also showing that GRASP55 is a substrate of SdeA. It is reported that PR-ubiquitinated of GRASP55 leads it to LCV during *Legionella* infection (Wan et al., 2019). Of note, in that study, the authors expressed exogenous GRASP55 in cells, overexpressed GRASP55 largely localized to the ER rather than Golgi apparatus in HEK293T cells. In view of that SidEs family effectors are involved in ER membranes recruitment to the LCV during *Legionella* infection (Shin et al., 2020), the positive detection of overexpressed GRASP55 on LCV of *Legionella* might be proteins recruited from ER. It has been shown that Golgi-derived PI(4)P-containing vesicles are related to mitochondria division (Nagashima et al., 2020). Given the fact that host mitochondrial dynamics is regulated during *Legionella* infection (Escoll et al., 2017), it is possible that *Legionella* SidE family effectors modulate mitochondria fission for efficient bacterial intracellular replication. It will be of interest to investigate the effect of PR-ubiquitination mediated Golgi disruption on mitochondria dynamics in future.

Proteins are firstly synthesized in the ER then transported to the Golgi complex for post-translational modification, and then subsequently sorted to eventual destinations via the trans-Golgi network. Given that *Legionella* needs host membrane materials for facilitating the formation of LCV, it is not surprising that the host trafficking pathway is utilized by *Legionella* during bacterial infection. Of note, multiple *Legionella* effectors have been reported to be involved in the regulation of the trafficking pathway of host cells by *Legionella* with unclear mechanism. For example, as a small GTPase of Rab family protein, Rab1 is essential for vesicle formation, tethering and membrane fusion, therefore playing critical roles in trafficking between ER and the Golgi apparatus (Stenmark, 2009). A previous study reported that host Rab1 is necessary for *Legionella* to recruit the ER-derived vesicles to the LCV during infection, inhibition of Rab1 activity results in interference in *Legionella* intracellular replication (Kagan et al., 2004). Microscopy study revealed that host Rab1 protein is detected on the LCV at the early stage of infection in a manner that is dependent on a functional Dot/Icm secretion system, this further confirms that Rab1 is needed for the formation of the LCV (Derré

and Isberg, 2004). Further studies discovered that *Legionella* effector DrrA functions as a specific GEF factor of Rab1, is involved in the recruitment of Rab1 to the LCV (Machner and Isberg, 2006). In addition to Rab1, another small GTPase Arf1 has also been identified as a target of *Legionella* effector. Arf1, a member of the ADP-ribosylation factors modulates the COPI-coated retrograde trafficking from the cis-Golgi compartment to ER (D'Souza-Schorey and Chavrier, 2006), was detected on LCV membrane, which is dependent on *Legionella* effector RalF (Nagai et al., 2002). Identification of the *Legionella* effectors that are capable of modulating the host secretory pathway will improve our understanding of both the bacterial pathogen and host cellular process. Even though extensive efforts have been made on dissecting how *Legionella* regulates host trafficking pathway, it is still needed to answer the question whether SidE family effectors mediated Golgi regulation is also involved.

Given the essential role of the Golgi apparatus in the secretory pathway, we investigated the effect of SidE family effectors mediated Golgi disruption on this network during infection. Using GFP-tagged VSVG as a tool allows us to evaluate the effect of SidE family effectors mediated PR-ubiquitination on protein trafficking in cells. The observation that *Legionella* infection decelerates VSVG trafficking through the Golgi suggests that *Legionella* SidE family effectors might regulate host secretory pathway by modulating function of the Golgi . Furthermore, this effect is enhanced in the presence of SidE family effectors. This suggests that SidE family effectors and other *Legionella* effectors contribute together to the trafficking regulation, which might be critical for *Legionella* to recruit ER membranes to form the LCV and bacterial proliferation during infection. Emerging evidence has demonstrated that GRASP55 and GRASP65 are involved in the secretion of cytokine IL-1β (Chiritoiu et al., 2019). A recent study reports that, instead of tethering the Golgi cisternae core, GRASP proteins densely localize to the rims of Golgi cisternae and function in linking of stacks. Inactivation of GRASP proteins affects the function of the lateral cisternae, which might be the source of membranes needed for unconventional secretion (Grond, R., 2020). It is possible that SdeA-catalyzed PR-ubiquitination affects GRASP protein oligomerization and thereby causes disconnection of Golgi stacks and inhibition of unconventional secretion.

Taken all together, our study reveals that modification of Ub catalyzed by SdeA shuts down the host cell Ub system, demonstrates that SdeA targets Golgi and ubiquitinates Golgi tethering proteins such as GRASP55, which results in Golgi damage and

subsequent regulation of the secretory pathway. By revealing the biological consequence of PR-ubiquitination on Golgi proteins, our study sheds light on the strategy of the Golgi regulation by which *Legionella* might utilize to benefit bacterial infection and replication in host cells.

9 References

Akturk A, Wasilko DJ, Wu X, Liu Y, Zhang Y, Qiu J, Luo ZQ, Reiter KH, Brzovic PS, Klevit RE, Mao Y. 2018. Mechanism of phosphoribosyl-ubiquitination mediated by a single legionella effector. *Nature* **557**:729-745. doi:10.1038/s41586-018-0147-6

Amerik AY, Hochstrasser M. 2004. Mechanism and function of deubiquitinating enzymes. *Biochim Biophys Acta - Mol Cell Res*. doi:10.1016/j.bbamcr.2004.10.003

Arasaki K, Mikami Y, Shames SR, Inoue H, Wakana Y, Tagaya M. 2017. Legionella effector Lpg1137 shuts down ER-mitochondria communication through cleavage of syntaxin 17. *Nat Commun*. doi:10.1038/ncomms15406

Ashida H, Kim M, Schmidt-Supprian M, Ma A, Ogawa M, Sasakawa C. 2010. A bacterial E3 ubiquitin ligase IpaH9.8 targets NEMO/IKKγ to dampen the host NF-κB-mediated inflammatory response. *Nat Cell Biol*. doi:10.1038/ncb2006

Ashida H, Nakano H, Sasakawa C. 2013. Shigella IpaH0722 E3 Ubiquitin Ligase Effector Targets TRAF2 to Inhibit PKC-NF-κB Activity in Invaded Epithelial Cells. *PLoS Pathog*. doi:10.1371/journal.ppat.1003409

Bardill JP, Miller JL, Vogel JP. 2005. IcmS-dependent translocation of SdeA into macrophages by the Legionella pneumophila type IV secretion system. *Mol Microbiol*. doi:10.1111/j.1365-2958.2005.04539.x

Bergmann JE. 1989. Using Temperature-Sensitive Mutants of VSV to Study Membrane Protein Biogenesis. *Methods Cell Biol*. doi:10.1016/S0091-679X(08)61168-1

Bernal-Bayard J, Cardenal-Muñoz E, Ramos-Morales F. 2010. The salmonella type III secretion effector, Salmonella Leucine-rich Repeat Protein (SlrP), targets the human chaperone ERdj3. *J Biol Chem*. doi:10.1074/jbc.M110.100669

Bhavsar AP, Brown NF, Stoepel J, Wiermer M, Martin DDO, Hsu KJ, Imami K, Ross CJ, Hayden MR, Foster LJ, Li X, Hieter P, Finlay BB. 2013. The Salmonella Type III Effector SspH2 Specifically Exploits the NLR Co-chaperone Activity of SGT1 to Subvert Immunity. *PLoS Pathog*. doi:10.1371/journal.ppat.1003518

Bhogaraju S, Bonn F, Mukherjee R, Adams M, Pfleiderer MM, Galej WP, Matkovic V, Lopez-Mosqueda J, Kalayil S, Shin D, Dikic I. 2019. Inhibition of bacterial ubiquitin ligases by SidJ–calmodulin catalysed glutamylation. *Nature*. doi:10.1038/s41586-019-1440-8

Bhogaraju S, Kalayil S, Liu Y, Bonn F, Colby T, Matic I, Dikic I. 2016.

Phosphoribosylation of Ubiquitin Promotes Serine Ubiquitination and Impairs Conventional Ubiquitination. *Cell* **167**:1636-1649 e13. doi:10.1016/j.cell.2016.11.019

Bhogaraju Sagar, Kalayil S, Liu Y, Bonn F, Colby T, Matic I, Dikic I. 2016a. Phosphoribosylation of Ubiquitin Promotes Serine Ubiquitination and Impairs Conventional Ubiquitination. *Cell*. doi:10.1016/j.cell.2016.11.019

Bhogaraju Sagar, Kalayil S, Liu Y, Bonn F, Colby T, Matic I, Dikic I. 2016b. Phosphoribosylation of Ubiquitin Promotes Serine Ubiquitination and Impairs Conventional Ubiquitination. *Cell* **167**:1636-1649.e13. doi:10.1016/j.cell.2016.11.019

Birmingham CL, Smith AC, Bakowski MA, Yoshimori T, Brumell JH. 2006. Autophagy controls Salmonella infection in response to damage to the Salmonella-containing vacuole. *J Biol Chem*. doi:10.1074/jbc.M509157200

Black MH, Osinski A, Gradowski M, Servage KA, Pawłowski K, Tomchick DR, Tagliabracci VS. 2019. Bacterial pseudokinase catalyzes protein polyglutamylation to inhibit the SidE-family ubiquitin ligases. *Science*. doi:10.1126/science.aaw7446

Bode AM, Dong Z. 2004. Post-translational modification of p53 in tumorigenesis. *Nat Rev Cancer*. doi:10.1038/nrc1455

Bracco E. 2018. A short evolutionary journey across the HERC Ubiquitin Ligases. *J Immunol Sci*. doi:10.29245/2578-3009/2018/1.1113

Burke B, Matlin K, Bause E, Legler G, Peyrieras N, Ploegh H. 1984. Inhibition of N-linked oligosaccharide trimming does not interfere with surface expression of certain integral membrane proteins. *EMBO J*. doi:10.1002/j.1460-2075.1984.tb01845.x

Burstein D, Zusman T, Degtyar E, Viner R, Segal G, Pupko T. 2009. Genome-scale identification of Legionella pneumophila effectors using a machine learning approach. *PLoS Pathog*. doi:10.1371/journal.ppat.1000508

Chen J, Chen ZJ. 2013. Regulation of NF-κB by ubiquitination. *Curr Opin Immunol*. doi:10.1016/j.coi.2012.12.005

Chimini G, Chavrier P. 2000. Function of Rho family proteins in actin dynamics during phagocytosis and engulfment. *Nat Cell Biol*. doi:10.1038/35036454

Chiritoiu M, Brouwers N, Turacchio G, Pirozzi M, Malhotra V. 2019. GRASP55 and UPR Control Interleukin-1β Aggregation and Secretion. *Dev Cell*.

doi:10.1016/j.devcel.2019.02.011

Choy A, Dancourt J, Mugo B, O'Connor TJ, Isberg RR, Melia TJ, Roy CR. 2012. The Legionella effector RavZ inhibits host autophagy through irreversible Atg8 deconjugation. *Science*. doi:10.1126/science.1227026

Creasey EA, Isberg RR. 2012. The protein SdhA maintains the integrity of the Legionella-containing vacuole. *Proc Natl Acad Sci U S A* **109**:3481–3486. doi:10.1073/pnas.1121286109

Cui J, Yao Q, Li S, Ding X, Lu Q, Mao H, Liu L, Zheng N, Chen S, Shao F. 2010. Glutamine deamidation and dysfunction of ubiquitin/NEDD8 induced by a bacterial effector family. *Science*. doi:10.1126/science.1193844

D'Souza-Schorey C, Chavrier P. 2006. ARF proteins: Roles in membrane traffic and beyond. *Nat Rev Mol Cell Biol*. doi:10.1038/nrm1910

Damgaard RB, Walker JA, Marco-Casanova P, Morgan N V., Titheradge HL, Elliott PR, McHale D, Maher ER, McKenzie ANJ, Komander D. 2016. The Deubiquitinase OTULIN Is an Essential Negative Regulator of Inflammation and Autoimmunity. *Cell*. doi:10.1016/j.cell.2016.07.019

Daniels CM, Ong SE, Leung AKL. 2014. Phosphoproteomic approach to characterize protein mono and polyADP-ribosylation sites from cells. *J Proteome Res*. doi:10.1021/pr401032q

Dantuma NP, Groothuis TAM, Salomons FA, Neefjes J. 2006. A dynamic ubiquitin equilibrium couples proteasomal activity to chromatin remodeling. *J Cell Biol*. doi:10.1083/jcb.200510071

De Jong AS, Visch HJ, De Mattia F, Van Dommelen MM, Swarts HG, Luyten T, Callewaert G, Melchers WJ, Willems PH, Van Kuppeveld FJ. 2006. The coxsackievirus 2B protein increases efflux of ions from the endoplasmic reticulum and Golgi, thereby inhibiting protein trafficking through the Golgi. *J Biol Chem*. doi:10.1074/jbc.M511766200

De Jong MF, Liu Z, Chen D, Alto NM. 2016. Shigella flexneri suppresses NF-κB activation by inhibiting linear ubiquitin chain ligation. *Nat Microbiol*. doi:10.1038/nmicrobiol.2016.84

Derré I, Isberg RR. 2004. Legionella pneumophila Replication Vacuole Formation Involves Rapid Recruitment of Proteins of the Early Secretory System. *Infect Immun*. doi:10.1128/IAI.72.5.3048-3053.2004

Diao J, Zhang Y, Huibregtse JM, Zhou D, Chen J. 2008. Crystal structure of SopA, a

Salmonella effector protein mimicking a eukaryotic ubiquitin ligase. *Nat Struct Mol Biol*. doi:10.1038/nsmb1346

Diederen BMW. 2008. Legionella spp. and Legionnaires' disease. *J Infect*. doi:10.1016/j.jinf.2007.09.010

Dong Y, Mu Y, Xie Y, Zhang Y, Han Y, Zhou Y, Wang W, Liu Z, Wu M, Wang H, Pan M, Xu N, Xu CQ, Yang M, Fan S, Deng H, Tan T, Liu X, Liu L, Li J, Wang J, Fang X, Feng Y. 2018. Structural basis of ubiquitin modification by the Legionella effector SdeA /631/80/458/582 /631/326/41/2536 /631/535 /631/45 /82/80 /82/83 /82/29 /82/16 /101/58 article. *Nature* **557**:674–678. doi:10.1038/s41586-018-0146-7

Doyle HA, Mamula MJ. 2001. Post-translational protein modifications in antigen recognition and autoimmunity. *Trends Immunol*. doi:10.1016/S1471-4906(01)01976-7

Eisenreich W, Heuner K. 2016. The life stage-specific pathometabolism of Legionella pneumophila. *FEBS Lett*. doi:10.1002/1873-3468.12326

Elliott PR, Komander D. 2016. Regulation of Met1-linked polyubiquitin signalling by the deubiquitinase OTULIN. *FEBS J*. doi:10.1111/febs.13547

Ensminger A.W., Isberg R.R. 3905. E3 ubiquitin ligase activity and targeting of BAT3 by multiple Legionella pneumophila translocated substrates. *Infect Immun*.

Ensminger AW. 2016. Legionella pneumophila, armed to the hilt: Justifying the largest arsenal of effectors in the bacterial world. *Curr Opin Microbiol*. doi:10.1016/j.mib.2015.11.002

Ensminger AW, Isberg RR. 2010. E3 ubiquitin ligase activity and targeting of BAT3 by multiple Legionella pneumophila translocated substrates. *Infect Immun*. doi:10.1128/IAI.00344-10

Ernst AM, Syed SA, Zaki O, Bottanelli F, Zheng H, Hacke M, Xi Z, Rivera-Molina F, Graham M, Rebane AA, Björkholm P, Baddeley D, Toomre D, Pincet F, Rothman JE. 2018. S-Palmitoylation Sorts Membrane Cargo for Anterograde Transport in the Golgi. *Dev Cell*. doi:10.1016/j.devcel.2018.10.024

Escoll P, Song OR, Viana F, Steiner B, Lagache T, Olivo-Marin JC, Impens F, Brodin P, Hilbi H, Buchrieser C. 2017. Legionella pneumophila Modulates Mitochondrial Dynamics to Trigger Metabolic Repurposing of Infected Macrophages. *Cell Host Microbe*. doi:10.1016/j.chom.2017.07.020

Feinstein TN, Linstedt AD. 2008. GRASP55 regulates Golgi ribbon formation. *Mol Biol*

Cell. doi:10.1091/mbc.E07-11-1200

Fiil BK, Damgaard RB, Wagner SA, Keusekotten K, Fritsch M, Bekker-Jensen S, Mailand N, Choudhary C, Komander D, Gyrd-Hansen M. 2013. OTULIN Restricts Met1-Linked Ubiquitination to Control Innate Immune Signaling. *Mol Cell*. doi:10.1016/j.molcel.2013.06.004

Fiskin E, Bhogaraju S, Herhaus L, Kalayil S, Hahn M, Dikic I. 2017. Structural basis for the recognition and degradation of host TRIM proteins by Salmonella effector SopA. *Nat Commun*. doi:10.1038/ncomms14004

Fu Y, Galán JE. 1999. A Salmonella protein antagonizes Rac-1 and Cdc42 to mediate host-cell recovery after bacterial invasion. *Nature*. doi:10.1038/45829

Furtado AR, Essid M, Perrinet S, Balañá ME, Yoder N, Dehoux P, Subtil A. 2013. The chlamydial OTU domain-containing protein ChlaOTU is an early type III secretion effector targeting ubiquitin and NDP52. *Cell Microbiol*. doi:10.1111/cmi.12171

Galán JE. 2009. Common Themes in the Design and Function of Bacterial Effectors. *Cell Host Microbe*. doi:10.1016/j.chom.2009.04.008

Gan N, Guan H, Huang Y, Yu T, Fu J, Nakayasu ES, Puvar K, Das C, Wang D, Ouyang S, Luo Z. 2020. Legionella pneumophila regulates the activity of UBE 2N by deamidase-mediated deubiquitination . *EMBO J*. doi:10.15252/embj.2019102806

Gan N, Nakayasu ES, Hollenbeck PJ, Luo ZQ. 2019. Legionella pneumophila inhibits immune signalling via MavC-mediated transglutaminase-induced ubiquitination of UBE2N. *Nat Microbiol*. doi:10.1038/s41564-018-0282-8

Gan N, Zhen X, Liu Yao, Xu X, He C, Qiu J, Liu Yancheng, Fujimoto GM, Nakayasu ES, Zhou B, Zhao L, Puvar K, Das C, Ouyang S, Luo ZQ. 2019. Regulation of phosphoribosyl ubiquitination by a calmodulin-dependent glutamylase. *Nature*. doi:10.1038/s41586-019-1439-1

Gaspar AH, Machner MP. 2014. VipD is a Rab5-activated phospholipase a1 that protects Legionella pneumophila from endosomal fusion. *Proc Natl Acad Sci U S A* **111**:4560–4565. doi:10.1073/pnas.1316376111

Ge J, Xu H, Li T, Zhou Y, Zhang Z, Li S, Liu L, Shao F. 2009. A Legionella type IV effector activates the NF-κB pathway by phosphorylating the IκB family of inhibitors. *Proc Natl Acad Sci U S A*. doi:10.1073/pnas.0907200106

Gerlach B, Cordier SM, Schmukle AC, Emmerich CH, Rieser E, Haas TL, Webb AI, Rickard JA, Anderton H, Wong WWL, Nachbur U, Gangoda L, Warnken U, Purcell AW, Silke J, Walczak H. 2011. Linear ubiquitination prevents inflammation and

regulates immune signalling. *Nature*. doi:10.1038/nature09816

Gomez-Valero L, Rusniok C, Carson D, Mondino S, Pérez-Cobas AE, Rolando M, Pasricha S, Reuter S, Demirtas J, Crumbach J, Descorps-Declere S, Hartland EL, Jarraud S, Dougan G, Schroeder GN, Frankel G, Buchrieser C. 2019. More than 18,000 effectors in the Legionella genus genome provide multiple, independent combinations for replication in human cells . *Proc Natl Acad Sci*. doi:10.1073/pnas.1808016116

Grond, R., Veenendaal, T., Duran, J. M., Raote, I., van Es, J. H., Corstjens, S., Delfgou, L., El Haddouti, B., Malhotra, V., & Rabouille C. 2020. The function of GORASPs in Golgi apparatus organization in vivo. *J Cell Biol*. doi:10.1083/jcb.202004191

Gruenberg J, Van Der Goot FG. 2006. Mechanisms of pathogen entry through the endosomal compartments. *Nat Rev Mol Cell Biol*. doi:10.1038/nrm1959

Grumati P, Dikic I. 2018. Ubiquitin signaling and autophagy. *J Biol Chem*. doi:10.1074/jbc.TM117.000117

Grumati P, Morozzi G, Hölper S, Mari M, Harwardt MLIE, Yan R, Müller S, Reggiori F, Heilemann M, Dikic I. 2017. Full length RTN3 regulates turnover of tubular endoplasmic reticulum via selective autophagy. *Elife*. doi:10.7554/eLife.25555

Haraga A, Miller SI. 2006. A Salmonella type III secretion effector interacts with the mammalian serine/threonine protein kinase PKN1. *Cell Microbiol*. doi:10.1111/j.1462-5822.2005.00670.x

Hardt WD, Chen LM, Schuebel KE, Bustelo XR, Galán JE. 1998. S. typhimurium Encodes an activator of Rho GTPases that induces membrane ruffling and nuclear responses in host cells. *Cell*. doi:10.1016/S0092-8674(00)81442-7

Hatakeyama S, Yada M, Matsumoto M, Ishida N, Nakayama KI. 2001. U Box Proteins as a New Family of Ubiquitin-Protein Ligases. *J Biol Chem*. doi:10.1074/jbc.M102755200

Herhaus L, Dikic I. 2015. Expanding the ubiquitin code through post-translational modification. *EMBO Rep*. doi:10.15252/embr.201540891

Hershko A, Ciechanover A, Varshavsky A. 2000. The ubiquitin system. *Nat Med*. doi:10.1038/80384

Heuer D, Lipinski AR, Machuy N, Karlas A, Wehrens A, Siedler F, Brinkmann V, Meyer TF. 2009. Chlamydia causes fragmentation of the Golgi compartment to ensure reproduction. *Nature* **457**:731-735. doi:10.1038/nature07578

Hicks SW, Galán JE. 2013. Exploitation of eukaryotic subcellular targeting

mechanisms by bacterial effectors. *Nat Rev Microbiol*. doi:10.1038/nrmicro3009

Higgins JJ, Pucilowska J, Lombardi RQ, Rooney JP. 2004. A mutation in a novel ATP-dependent Lon protease gene in a kindred with mild mental retardation. *Neurology*. doi:10.1212/01.WNL.0000146196.01316.A2

Hock AK, Vousden KH. 2014. The role of ubiquitin modification in the regulation of p53. *Biochim Biophys Acta - Mol Cell Res*. doi:10.1016/j.bbamcr.2013.05.022

Hodge CD, Spyracopoulos L, Mark Glover JN. 2016. Ubc13: The Lys63 ubiquitin chain building machine. *Oncotarget*. doi:10.18632/oncotarget.10948

Horwitz MA, Silverstein SC. 1980. Legionnaires' disease bacterium (Legionella pneumophila) multiples intracellularly in human monocytes. *J Clin Invest*. doi:10.1172/JCI109874

Hsu F, Zhu W, Brennan L, Tao L, Luo ZQ, Mao Y. 2012. Structural basis for substrate recognition by a unique Legionella phosphoinositide phosphatase. *Proc Natl Acad Sci U S A* **109**:13567–13572. doi:10.1073/pnas.1207903109

Hsu FS, Luo X, Qiu J, Teng Y Bin, Jin J, Smolka MB, Luo ZQ, Mao Y. 2014. The Legionella effector SidC defines a unique family of ubiquitin ligases important for bacterial phagosomal remodeling. *Proc Natl Acad Sci U S A*. doi:10.1073/pnas.1402605111

Huang TT, D'Andrea AD. 2006. Regulation of DNA repair by ubiquitylation. *Nat Rev Mol Cell Biol*. doi:10.1038/nrm1908

Hubber A, Kubori T, Nagai H. 2013. Modulation of the ubiquitination machinery by Legionella. *Curr Top Microbiol Immunol*. doi:10.1007/82-2013-343

Hubber A, Roy CR. 2010. Modulation of Host Cell Function by Legionella pneumophila Type IV Effectors . *Annu Rev Cell Dev Biol*. doi:10.1146/annurev-cellbio-100109-104034

Ikeda F, Dikic I. 2008. Atypical ubiquitin chains: New molecular signals. "Protein Modifications: Beyond the Usual Suspects" Review Series. *EMBO Rep*. doi:10.1038/embor.2008.93

Isberg RR, O'Connor TJ, Heidtman M. 2009. The Legionella pneumophila replication vacuole: Making a cosy niche inside host cells. *Nat Rev Microbiol*. doi:10.1038/nrmicro1967

Ito T, Ando H, Suzuki T, Ogura T, Hotta K, Imamura Y, Yamaguchi Y, Handa H. 2010. Identification of a primary target of thalidomide teratogenicity. *Science (80-)*. doi:10.1126/science.1177319

Jarvela T, Linstedt AD. 2012. Golgi GRASPs: Moonlighting membrane tethers. *Cell Health Cytoskelet*. doi:10.2147/CHC.S21849

Jeng EE, Bhadkamkar V, Ibe NU, Gause H, Jiang L, Chan J, Jian R, Jimenez-Morales D, Stevenson E, Krogan NJ, Swaney DL, Snyder MP, Mukherjee S, Bassik MC. 2019. Systematic Identification of Host Cell Regulators of Legionella pneumophila Pathogenesis Using a Genome-wide CRISPR Screen. *Cell Host Microbe*. doi:10.1016/j.chom.2019.08.017

Jeong KC, Sexton JA, Vogel JP. 2015. Spatiotemporal Regulation of a Legionella pneumophila T4SS Substrate by the Metaeffector SidJ. *PLoS Pathog*. doi:10.1371/journal.ppat.1004695

Kagan JC, Stein MP, Pypaert M, Roy CR. 2004. Legionella Subvert the Functions of Rab1 and Sec22b to Create a Replicative Organelle. *J Exp Med*. doi:10.1084/jem.20031706

Kalayil S, Bhogaraju S, Bonn F, Shin D, Liu Y, Gan N, Basquin J, Grumati P, Luo ZQ, Dikic I. 2018. Insights into catalysis and function of phosphoribosyl-linked serine ubiquitination. *Nature* **557**:734–738. doi:10.1038/s41586-018-0145-8

Kane LA, Lazarou M, Fogel AI, Li Y, Yamano K, Sarraf SA, Banerjee S, Youle RJ. 2014. PINK1 phosphorylates ubiquitin to activate parkin E3 ubiquitin ligase activity. *J Cell Biol*. doi:10.1083/jcb.201402104

Khaminets A, Behl C, Dikic I. 2016. Ubiquitin-Dependent And Independent Signals In Selective Autophagy. *Trends Cell Biol*. doi:10.1016/j.tcb.2015.08.010

Khweek AA, Caution K, Akhter A, Abdulrahman BA, Tazi M, Hassan H, Majumdar N, Doran A, Guirado E, Schlesinger LS, Shuman H, Amer AO. 2013. A bacterial protein promotes the recognition of the Legionella pneumophila vacuole by autophagy. *Eur J Immunol*. doi:10.1002/eji.201242835

Kim DW, Lenzen G, Page AL, Legrain P, Sansonetti PJ, Parsot C. 2005. The Shigella flexneri effector OspG interferes with innate immune responses by targeting ubiquitin-conjugating enzymes. *Proc Natl Acad Sci U S A*. doi:10.1073/pnas.0504466102

Kim J, Noh SH, Piao H, Kim DH, Kim K, Cha JS, Chung WY, Cho HS, Kim JY, Lee MG. 2016. Monomerization and ER Relocalization of GRASP Is a Requisite for Unconventional Secretion of CFTR. *Traffic*. doi:10.1111/tra.12403

Kim W, Kaelin WG. 2003. The von Hippel-Lindau tumor suppressor protein: New insights into oxygen sensing and cancer. *Curr Opin Genet Dev*.

doi:10.1016/S0959-437X(02)00010-2

Kimura Y, Tanaka K. 2010. Regulatory mechanisms involved in the control of ubiquitin homeostasis. *J Biochem*. doi:10.1093/jb/mvq044

Knodler LA, Ibarra JA, Pérez-Rueda E, Yip CK, Steele-Mortimer O. 2011. Coiled-coil domains enhance the membrane association of Salmonella type III effectors. *Cell Microbiol*. doi:10.1111/j.1462-5822.2011.01635.x

Komander D, Clague MJ, Urbé S. 2009. Breaking the chains: Structure and function of the deubiquitinases. *Nat Rev Mol Cell Biol*. doi:10.1038/nrm2731

Kotewicz KM, Ramabhadran V, Sjoblom N, Vogel JP, Haenssler E, Zhang M, Behringer J, Scheck RA, Isberg RR. 2017a. A Single Legionella Effector Catalyzes a Multistep Ubiquitination Pathway to Rearrange Tubular Endoplasmic Reticulum for Replication. *Cell Host Microbe* **21**:169–181. doi:10.1016/j.chom.2016.12.007

Kotewicz KM, Ramabhadran V, Sjoblom N, Vogel JP, Haenssler E, Zhang M, Behringer J, Scheck RA, Isberg RR. 2017b. A Single Legionella Effector Catalyzes a Multistep Ubiquitination Pathway to Rearrange Tubular Endoplasmic Reticulum for Replication. *Cell Host Microbe* **21**:169–181. doi:10.1016/j.chom.2016.12.007

Krönke J, Udeshi ND, Narla A, Grauman P, Hurst SN, McConkey M, Svinkina T, Heckl D, Comer E, Li X, Ciarlo C, Hartman E, Munshi N, Schenone M, Schreiber SL, Carr SA, Ebert BL. 2014. Lenalidomide causes selective degradation of IKZF1 and IKZF3 in multiple myeloma cells. *Science*. doi:10.1126/science.1244851

Kubbutat MHG, Jones SN, Vousden KH. 1997. Regulation of p53 stability by Mdm2. *Nature*. doi:10.1038/387299a0

Kubori T, Galán JE. 2003. Temporal regulation of Salmonella virulence effector function by proteasome-dependent protein degradation. *Cell*. doi:10.1016/S0092-8674(03)00849-3

Kubori T, Hyakutake A, Nagai H. 2008. Legionella translocates an E3 ubiquitin ligase that has multiple U-boxes with distinct functions. *Mol Microbiol*. doi:10.1111/j.1365-2958.2008.06124.x

Kubori T, Kitao T, Ando H, Nagai H. 2018. LotA, a Legionella deubiquitinase, has dual catalytic activity and contributes to intracellular growth. *Cell Microbiol*. doi:10.1111/cmi.12840

Kubori T, Shinzawa N, Kanuka H, Nagai H. 2010. Legionella metaeffector exploits host proteasome to temporally regulate cognate effector. *PLoS Pathog*. doi:10.1371/journal.ppat.1001216

Kumar S, Barouch-Bentov R, Xiao F, Schor S, Pu S, Biquand E, Lu A, Lindenbach BD, Jacob Y, Demeret C, Einav S. 2019. MARCH8 Ubiquitinates the Hepatitis C Virus Nonstructural 2 Protein and Mediates Viral Envelopment. *Cell Rep*. doi:10.1016/j.celrep.2019.01.075

Lad SP, Li J, Da Silva Correia J, Pan Q, Gadwal S, Ulevitch RJ, Li E. 2007. Cleavage of p65/RelA of the NF-κB pathway by Chlamydia. *Proc Natl Acad Sci U S A*. doi:10.1073/pnas.0608393104

Laguna RK, Creasey EA, Li Z, Valtz N, Isberg RR. 2006. A Legionella pneumophila-translocated substrate that is required for growth within macrophages and protection from host cell death. *Proc Natl Acad Sci U S A*. doi:10.1073/pnas.0609012103

Le Negrate G, Krieg A, Faustin B, Loeffler M, Godzik A, Krajewski S, Reed JC. 2008. Chla Dub1 of Chlamydia trachomatis suppresses NF-κB activation and inhibits IκBα ubiquitination and degradation. *Cell Microbiol*. doi:10.1111/j.1462-5822.2008.01178.x

Le NG, Krieg A, Faustin B, Loeffler M, Godzik A, Krajewski S, Reed JC. 2008. ChlaDub1 of Chlamydia trachomatis suppresses NF-kappaB activation and inhibits IkappaBalpha ubiquitination and degradation. *Cell Microbiol*.

Lenschow DJ, Lai C, Frias-Staheli N, Giannakopoulos N V., Lutz A, Wolff T, Osiak A, Levine B, Schmidt RE, García-Sastre A, Leib DA, Pekosz A, Knobeloch KP, Horak I, Virgin IV HW. 2007. IFN-stimulated gene 15 functions as a critical antiviral molecule against influenza, herpes, and Sindbis viruses. *Proc Natl Acad Sci U S A*. doi:10.1073/pnas.0607038104

Li M, Chen D, Shiloh A, Luo J, Nikolaev AY, Qin J, Gu W. 2002. Deubiquitination of p53 by HAUSP is an important pathway for p53 stabilization. *Nature*. doi:10.1038/nature737

Lin YH, Doms AG, Cheng E, Kim B, Evans TR, Machner MP. 2015. Host cell-catalyzed S-palmitoylation mediates Golgi targeting of the legionella ubiquitin ligase GobX. *J Biol Chem*. doi:10.1074/jbc.M115.637397

Lin YH, Lucas M, Evans TR, Abascal-Palacios G, Doms AG, Beauchene NA, Rojas AL, Hierro A, Machner MP. 2018. RavN is a member of a previously unrecognized group of Legionella pneumophila E3 ubiquitin ligases. *PLoS Pathog*. doi:10.1371/journal.ppat.1006897

Lu H, Clarke M. 2005. Dynamic properties of Legionella-containing phagosomes in

Dictyostelium amoebae. *Cell Microbiol* **7**:995–1007. doi:10.1111/j.1462-5822.2005.00528.x

Luo ZQ, Isberg RR. 2004. Multiple substrates of the Legionella pneumophila Dot/Icm system identified by interbacterial protein transfer. *Proc Natl Acad Sci U S A*. doi:10.1073/pnas.0304916101

Luzio JP, Pryor PR, Bright NA. 2007. Lysosomes: Fusion and function. *Nat Rev Mol Cell Biol*. doi:10.1038/nrm2217

Ma K, Zhen X, Zhou B, Gan N, Cao Y, Fan C, Ouyang S, Luo ZQ, Qiu J. 2020. The bacterial deubiquitinase Ceg23 regulates the association of Lys-63-linked polyubiquitin molecules on the Legionella phagosome. *J Biol Chem*. doi:10.1074/jbc.RA119.011758

Machner MP, Isberg RR. 2006. Targeting of Host Rab GTPase Function by the Intravacuolar Pathogen Legionella pneumophila. *Dev Cell*. doi:10.1016/j.devcel.2006.05.013

Maculins T, Fiskin E, Bhogaraju S, Dikic I. 2016. Bacteria-host relationship: Ubiquitin ligases as weapons of invasion. *Cell Res*. doi:10.1038/cr.2016.30

Marchès O, Ledger TN, Boury M, Ohara M, Tu X, Goffaux F, Mainil J, Rosenshine I, Sugai M, De Rycke J, Oswald E. 2003. Enteropathogenic and enterohaemorrhagic Escherichia coli deliver a novel effector called Cif, which blocks cell cycle G2/M transition. *Mol Microbiol*. doi:10.1046/j.1365-2958.2003.03821.x

Mattoo S, Lee YM, Dixon JE. 2007. Interactions of bacterial effector proteins with host proteins. *Curr Opin Immunol*. doi:10.1016/j.coi.2007.06.005

Medvar B, Raghuram V, Pisitkun T, Sarkar A, Knepper MA. 2016. Comprehensive database of human E3 ubiquitin ligases: Application to aquaporin-2 regulation. *Physiol Genomics*. doi:10.1152/physiolgenomics.00031.2016

Meierhofer D, Wang X, Huang L, Kaiser P. 2008. Quantitative analysis of global ubiquitination in HeLa cells by mass spectrometry. *J Proteome Res*. doi:10.1021/pr800468j

Mesquita FS, Thomas M, Sachse M, Santos AJM, Figueira R, Holden DW. 2012. The Salmonella deubiquitinase SseI inhibits selective autophagy of cytosolic aggregates. *PLoS Pathog*. doi:10.1371/journal.ppat.1002743

Micaroni M, Stanley AC, Khromykh T, Venturato J, Wong CXF, Lim JP, Marsh BJ, Storrie B, Gleeson PA, Stow JL. 2013. Rab6a/a' Are Important Golgi Regulators

of Pro-Inflammatory TNF Secretion in Macrophages. *PLoS One* **8**. doi:10.1371/journal.pone.0057034

Mills IG, Urbé S, Clague MJ. 2001. Relationships between EEA1 binding partners and their role in endosome fusion. *J Cell Sci*.

Misaghi S, Balsara ZR, Catic A, Spooner E, Ploegh HL, Starnbach MN. 2006. Chlamydia trachomatis-derived deubiquitinating enzymes in mammalian cells during infection. *Mol Microbiol*. doi:10.1111/j.1365-2958.2006.05199.x

Morales DJ, Lenschow DJ. 2013. The antiviral activities of ISG15. *J Mol Biol*. doi:10.1016/j.jmb.2013.09.041

Moreau P, Masszi T, Grzasko N, Bahlis NJ, Hansson M, Pour L, Sandhu I, Ganly P, Baker BW, Jackson SR, Stoppa AM, Simpson DR, Gimsing P, Palumbo A, Garderet L, Cavo M, Kumar S, Touzeau C, Buadi FK, Laubach JP, Berg DT, Lin J, Di Bacco A, Hui AM, Van De Velde H, Richardson PG, Kupperman E, Berger A, Dick L, Williamson M, Chauhan D, Anderson K, Esseltine DL, Niculescu L, Taraskiewicz S, Stecklair J, Lonial S, Rajkumar V, Miquel JS, Einsele H, Orlowski R. 2016. Oral Ixazomib, lenalidomide, and dexamethasone for multiple myeloma. *N Engl J Med*. doi:10.1056/NEJMoa1516282

Mu Y, Wang Y, Huang Y, Li D, Han Y, Chang M, Fu J, Xie Y, Ren J, Wang H, Zhang Y, Luo ZQ, Feng Y. 2020. Structural insights into the mechanism and inhibition of transglutaminase-induced ubiquitination by the Legionella effector MavC. *Nat Commun*. doi:10.1038/s41467-020-15645-7

Mukherjee S, Keitany G, Li Y, Wang Y, Ball HL, Goldsmith EJ, Orth K. 2006. Yersinia YopJ acetylates and inhibits kinase activation by blocking phosphorylation. *Science*. doi:10.1126/science.1126867

Nagai H, Kagan JC, Zhu X, Kahn RA, Roy CR. 2002. A bacterial guanine nucleotide exchange factor activates ARF on Legionella phagosomes. *Science*. doi:10.1126/science.1067025

Nagashima S, Tábara LC, Tilokani L, Paupe V, Anand H, Pogson JH, Zunino R, McBride HM, Prudent J. 2020. Golgi-derived PI(4)P-containing vesicles drive late steps of mitochondrial division. *Science*. doi:10.1126/science.aax6089

Nakatogawa H, Ichimura Y, Ohsumi Y. 2007. Atg8, a Ubiquitin-like Protein Required for Autophagosome Formation, Mediates Membrane Tethering and Hemifusion. *Cell*. doi:10.1016/j.cell.2007.05.021

O'Brien KM, Lindsay EL, Starai VJ. 2015. The Legionella pneumophila effector protein,

LegC7, alters yeast endosomal trafficking. *PLoS One*. doi:10.1371/journal.pone.0116824

O'Connor TJ, Adepoju Y, Boyd D, Isberg RR. 2011. Minimization of the Legionella pneumophila genome reveals chromosomal regions involved in host range expansion. *Proc Natl Acad Sci U S A*. doi:10.1073/pnas.1111678108

Ogawa M, Yoshimori T, Suzuki T, Sagara H, Mizushima N, Sasakawa C. 2005. Escape of intracellular Shigella from autophagy. *Science*. doi:10.1126/science.1106036

Ohtake F, Saeki Y, Sakamoto K, Ohtake K, Nishikawa H, Tsuchiya H, Ohta T, Tanaka K, Kanno J. 2015. Ubiquitin acetylation inhibits polyubiquitin chain elongation. *EMBO Rep*. doi:10.15252/embr.201439152

Okuda J, Toyotome T, Kataoka N, Ohno M, Abe H, Shimura Y, Seyedarabi A, Pickersgill R, Sasakawa C. 2005. Shigella effector IpaH9.8 binds to a splicing factor U2AF35 to modulate host immune responses. *Biochem Biophys Res Commun*. doi:10.1016/j.bbrc.2005.05.145

Orth K, Xu Z, Mudgett MB, Bao ZQ, Palmer LE, Bliska JB, Mangel WF, Staskawicz B, Dixon JE. 2000. Disruption of signaling by yersinia effector YopJ, a ubiquitin-like protein protease. *Science*. doi:10.1126/science.290.5496.1594

Paiva SL, Crews CM. 2019. Targeted protein degradation: elements of PROTAC design. *Curr Opin Chem Biol*. doi:10.1016/j.cbpa.2019.02.022

Pan ZQ, Kentsis A, Dias DC, Yamoah K, Wu K. 2004. Nedd8 on cullin: Building an expressway to protein destruction. *Oncogene*. doi:10.1038/sj.onc.1207414

Peng J, Schwartz D, Elias JE, Thoreen CC, Cheng D, Marsischky G, Roelofs J, Finley D, Gygi SP. 2003. A proteomics approach to understanding protein ubiquitination. *Nat Biotechnol*. doi:10.1038/nbt849

Petzold G, Fischer ES, Thomä NH. 2016. Structural basis of lenalidomide-induced CK1α degradation by the CRL4 CRBN ubiquitin ligase. *Nature*. doi:10.1038/nature16979

Pickart CM. 2001. Mechanisms Underlying Ubiquitination. *Annu Rev Biochem*. doi:10.1146/annurev.biochem.70.1.503

Piscatelli H, Kotkar SA, McBee ME, Muthupalani S, Schauer DB, Mandrell RE, Leong JM, Zhou D. 2011. The ehec type iii effector nlel is an e3 ubiquitin ligase that modulates pedestal formation. *PLoS One*. doi:10.1371/journal.pone.0019331

Presley JF, Cole NB, Schroer TA, Hirschberg K, Zaal KJM, Lippincott-Schwartz J. 1997. ER-to-Golgi transport visualized in living cells. *Nature*. doi:10.1038/38001

Price CT, Al-Khodor S, Al-Quadan T, Santic M, Habyarimana F, Kalia A, Kwaik YA. 2009. Molecular mimicry by an F-box effector of Legionella pneumophila hijacks a conserved polyubiquitination machinery within macrophages and protozoa. *PLoS Pathog*. doi:10.1371/journal.ppat.1000704

Price CTD, Al-Quadan T, Santic M, Rosenshine I, Abu Kwaik Y. 2011. Host proteasomal degradation generates amino acids essential for intracellular bacterial growth. *Science*. doi:10.1126/science.1212868

Qiu J, Luo ZQ. 2017a. Legionella and Coxiella effectors: Strength in diversity and activity. *Nat Rev Microbiol*. doi:10.1038/nrmicro.2017.67

Qiu J, Luo ZQ. 2017b. Hijacking of the host ubiquitin network by Legionella pneumophila. *Front Cell Infect Microbiol*. doi:10.3389/fcimb.2017.00487

Qiu J, Sheedlo MJ, Yu K, Tan Y, Nakayasu ES, Das C, Liu X, Luo ZQ. 2016. Ubiquitination independent of E1 and E2 enzymes by bacterial effectors. *Nature* **533**:120–124. doi:10.1038/nature17657

Qiu J, Yu K, Fei X, Liu Y, Nakayasu ES, Piehowski PD, Shaw JB, Puvar K, Das C, Liu X, Luo ZQ. 2017. A unique deubiquitinase that deconjugates phosphoribosyl-linked protein ubiquitination. *Cell Res*. doi:10.1038/cr.2017.66

Rabouille C, Linstedt AD. 2016. GRASP: A multitasking tether. *Front Cell Dev Biol* **4**:1–8. doi:10.3389/fcell.2016.00001

Ragaz C, Pietsch H, Urwyler S, Tiaden A, Weber SS, Hilbi H. 2008. The Legionella pneumophila phosphatidylinositol-4 phosphate-binding type IV substrate SidC recruits endoplasmic reticulum vesicles to a replication-permissive vacuole. *Cell Microbiol*. doi:10.1111/j.1462-5822.2008.01219.x

Raval KK, Tao R, White BE, De Lange WJ, Koonce CH, Yu J, Kishnani PS, Thomson JA, Mosher DF, Ralphe JC, Kamp TJ. 2015. Pompe disease results in a Golgi-based glycosylation deficit in human induced pluripotent stem cell-derived cardiomyocytes. *J Biol Chem*. doi:10.1074/jbc.M114.628628

Redman KL, Rechsteiner M. 1989. Identification of the long ubiquitin extension as ribosomal protein S27a. *Nature*. doi:10.1038/338438a0

Rinaldo S, Paiardini A, Stelitano V, Brunotti P, Cervoni L, Fernicola S, Protano C, Vitali M, Cutruzzolà F, Giardina G. 2015. Structural basis of functional diversification of the HD-GYP domain revealed by the Pseudomonas aeruginosa PA4781 protein, which displays an unselective bimetallic binding site. *J Bacteriol*. doi:10.1128/JB.02606-14

Rohde JR, Breitkreutz A, Chenal A, Sansonetti PJ, Parsot C. 2007. Type III Secretion Effectors of the IpaH Family Are E3 Ubiquitin Ligases. *Cell Host Microbe*. doi:10.1016/j.chom.2007.02.002

Rolando M, Sanulli S, Rusniok C, Gomez-Valero L, Bertholet C, Sahr T, Margueron R, Buchrieser C. 2013. Legionella pneumophila effector RomA uniquely modifies host chromatin to repress gene expression and promote intracellular bacterial replication. *Cell Host Microbe*. doi:10.1016/j.chom.2013.03.004

Rothmeier E, Pfaffinger G, Hoffmann C, Harrison CF, Grabmayr H, Repnik U, Hannemann M, Wölke S, Bausch A, Griffiths G, Müller-Taubenberger A, Itzen A, Hilbi H. 2013. Activation of Ran GTPase by a Legionella Effector Promotes Microtubule Polymerization, Pathogen Vacuole Motility and Infection. *PLoS Pathog*. doi:10.1371/journal.ppat.1003598

Rowbotham TJ. 1980. Preliminary report on the pathogenicity of Legionella pneumophila for freshwater and soil amoebae. *J Clin Pathol*. doi:10.1136/jcp.33.12.1179

Rubinsztein DC. 2006. The roles of intracellular protein-degradation pathways in neurodegeneration. *Nature*. doi:10.1038/nature05291

Rytkönen A, Poh J, Garmendia J, Boyle C, Thompson A, Liu M, Freemont P, Hinton JCD, Holden DW. 2007. SseL, a Salmonella deubiquitinase required for macrophage killing and virulence. *Proc Natl Acad Sci U S A*. doi:10.1073/pnas.0610095104

Sanada T, Kim M, Mimuro H, Suzuki M, Ogawa M, Oyama A, Ashida H, Kobayashi T, Koyama T, Nagai S, Shibata Y, Gohda J, Inoue JI, Mizushima T, Sasakawa C. 2012. The Shigella flexneri effector OspI deamidates UBC13 to dampen the inflammatory response. *Nature*. doi:10.1038/nature10894

Schmölders J, Manske C, Otto A, Hoffmann C, Steiner B, Welin A, Becher D, Hilbi H. 2017. Comparative proteomics of purified pathogen vacuoles correlates intracellular replication of Legionella pneumophila with the small GTPase ras-related protein 1 (Rap1). *Mol Cell Proteomics*. doi:10.1074/mcp.M116.063453

Schulman BA, Wade Harper J. 2009. Ubiquitin-like protein activation by E1 enzymes: The apex for downstream signalling pathways. *Nat Rev Mol Cell Biol*. doi:10.1038/nrm2673

Scidmore MA, Fischer ER, Hackstadt T. 1996. Sphingolipids and glycoproteins are differentially trafficked to the Chlamydia trachomatis inclusion. *J Cell Biol*.

doi:10.1083/jcb.134.2.363

Sheedlo MJ, Qiu J, Tan Y, Paul LN, Luo ZQ, Das C. 2015. Structural basis of substrate recognition by a bacterial deubiquitinase important for dynamics of phagosome ubiquitination. *Proc Natl Acad Sci U S A*. doi:10.1073/pnas.1514568112

Sheng X, You Q, Zhu H, Chang Z, Li Q, Wang Haifeng, Wang C, Wang Hongyan, Hui L, Du C, Xie X, Zeng R, Lin A, Shi D, Ruan K, Yan J, Gao GF, Shao F, Hu R. 2017. Bacterial effector NleL promotes enterohemorrhagic E. coli-induced attaching and effacing lesions by ubiquitylating and inactivating JNK. *PLoS Pathog*. doi:10.1371/journal.ppat.1006534

Shin D, Mukherjee R, Liu Yaobin, Gonzalez A, Bonn F, Liu Yan, Rogov V V., Heinz M, Stolz A, Hummer G, Dötsch V, Luo ZQ, Bhogaraju S, Dikic I. 2020. Regulation of Phosphoribosyl-Linked Serine Ubiquitination by Deubiquitinases DupA and DupB. *Mol Cell*. doi:10.1016/j.molcel.2019.10.019

Shorter J, Watson R, Giannakou ME, Clarke M, Warren G, Barr FA. 1999. GRASP55, a second mammalian GRASP protein involved in the stacking of Golgi cisternae in a cell-free system. *EMBO J*. doi:10.1093/emboj/18.18.4949

Shpilka T, Weidberg H, Pietrokovski S, Elazar Z. 2011. Atg8: An autophagy-related ubiquitin-like protein family. *Genome Biol*. doi:10.1186/gb-2011-12-7-226

Sohda M, Misumi Y, Yamamoto A, Yano A, Nakamura N, Ikehara Y. 2001. Identification and Characterization of a Novel Golgi Protein, GCP60, that Interacts with the Integral Membrane Protein Giantin. *J Biol Chem*. doi:10.1074/jbc.M108961200

Stebbins CE, Galán JE. 2000. Modulation of host signaling by a bacterial mimic: Structure of the Salmonella effector SptP bound to Rac1. *Mol Cell*. doi:10.1016/S1097-2765(00)00141-6

Steiner B, Swart AL, Welin A, Weber S, Personnic N, Kaech A, Freyre C, Ziegler U, Klemm RW, Hilbi H. 2017. ER remodeling by the large GTPase atlastin promotes vacuolar growth of Legionella pneumophila. *EMBO Rep* **18**:1817–1836. doi:10.15252/embr.201743903

Stenmark H. 2009. Rab GTPases as coordinators of vesicle traffic. *Nat Rev Mol Cell Biol*. doi:10.1038/nrm2728

Stewart MD, Ritterhoff T, Klevit RE, Brzovic PS. 2016. E2 enzymes: More than just middle men. *Cell Res*. doi:10.1038/cr.2016.35

Stolz A, Ernst A, Dikic I. 2014. Cargo recognition and trafficking in selective autophagy.

Nat Cell Biol. doi:10.1038/ncb2979

Suzuki R, Moriishi K, Fukuda K, Shirakura M, Ishii K, Shoji I, Wakita T, Miyamura T, Matsuura Y, Suzuki T. 2009. Proteasomal Turnover of Hepatitis C Virus Core Protein Is Regulated by Two Distinct Mechanisms: a Ubiquitin-Dependent Mechanism and a Ubiquitin-Independent but PA28γ-Dependent Mechanism. *J Virol*. doi:10.1128/jvi.01690-08

Suzuki S, Suzuki T, Mimuro H, Mizushima T, Sasakawa C. 2018. Shigella hijacks the glomulin– cIAP s–inflammasome axis to promote inflammation . *EMBO Rep*. doi:10.15252/embr.201643841

Thibaudeau TA, Smith DM. 2019. A practical review of proteasome pharmacology. *Pharmacol Rev*. doi:10.1124/pr.117.015370

Tobe T, Beatson SA, Taniguchi H, Abe H, Bailey CM, Fivian A, Younis R, Matthews S, Marches O, Frankel G, Hayashi T, Pallen MJ. 2006. An extensive repetoire of type III secretion effectors in Escherichia coli O157 and the role of lambdoid phages in their dissemination. *Proc Natl Acad Sci U S A*. doi:10.1073/pnas.0604891103

Tokunaga F, Sakata SI, Saeki Y, Satomi Y, Kirisako T, Kamei K, Nakagawa T, Kato M, Murata S, Yamaoka S, Yamamoto M, Akira S, Takao T, Tanaka K, Iwai K. 2009. Involvement of linear polyubiquitylation of NEMO in NF-κB activation. *Nat Cell Biol*. doi:10.1038/ncb1821

Toulabi L, Wu X, Cheng Y, Mao Y. 2013. Identification and structural characterization of a Legionella phosphoinositide phosphatase. *J Biol Chem*. doi:10.1074/jbc.M113.474239

Truschel ST, Zhang M, Bachert C, Macbeth MR, Linstedt AD. 2012. Allosteric regulation of GRASP protein-dependent golgi membrane tethering by mitotic phosphorylation. *J Biol Chem*. doi:10.1074/jbc.M111.326256

Tsolis RM, Townsend SM, Miao EA, Miller SI, Ficht TA, Adams LG, Bäumler AJ. 1999. Identification of a putative Salmonella enterica serotype typhimurium host range factor with homology to IpaH and YopM by signature-tagged mutagenesis. *Infect Immun*. doi:10.1128/iai.67.12.6385-6393.1999

Urbanus ML, Quaile AT, Stogios PJ, Morar M, Rao C, Di Leo R, Evdokimova E, Lam M, Oatway C, Cuff ME, Osipiuk J, Michalska K, Nocek BP, Taipale M, Savchenko A, Ensminger AW. 2016. Diverse mechanisms of metaeffector activity in an intracellular bacterial pathogen, Legionella pneumophila . *Mol Syst Biol*. doi:10.15252/msb.20167381

Urwyler S, Nyfeler Y, Ragaz C, Lee H, Mueller LN, Aebersold R, Hilbi H. 2009. Proteome analysis of Legionella vacuoles purified by magnetic immunoseparation reveals secretory and endosomal GTPases. *Traffic*. doi:10.1111/j.1600-0854.2008.00851.x

Valleau D, Quaile AT, Cui H, Xu X, Evdokimova E, Chang C, Cuff ME, Urbanus ML, Houliston S, Arrowsmith CH, Ensminger AW, Savchenko A. 2018. Discovery of Ubiquitin Deamidases in the Pathogenic Arsenal of Legionella pneumophila. *Cell Rep*. doi:10.1016/j.celrep.2018.03.060

Verlhac P, Viret C, Faure M. 2015. Dual function of CALCOCO2/NDP52 during xenophagy. *Autophagy*. doi:10.1080/15548627.2015.1046672

Vittal V, Stewart MD, Brzovic PS, Klevit RE. 2015. Regulating the regulators: Recent revelations in the control of E3 ubiquitin ligases. *J Biol Chem*. doi:10.1074/jbc.R115.675165

Von Muhlinen N, Thurston T, Ryzhakov G, Bloor S, Randow F. 2010. NDP52, a novel autophagy receptor for ubiquitin-decorated cytosolic bacteria. *Autophagy*. doi:10.4161/auto.6.2.11118

Wan M, Sulpizio AG, Akturk A, Beck WHJ, Lanz M, Faça VM, Smolka MB, Vogel JP, Mao Y. 2019. Deubiquitination of phosphoribosyl-ubiquitin conjugates by phosphodiesterase-domain–containing Legionella effectors. *Proc Natl Acad Sci U S A*. doi:10.1073/pnas.1916287116

Wang F, Jiang Z, Li Y, He X, Zhao J, Yang X, Zhu L, Yin Z, Li X, Wang X, Liu W, Shang W, Yang Z, Wang S, Zhen Q, Zhang Z, Yu Y, Zhong H, Ye Q, Huang L, Yuan J. 2013. Shigella flexneri T3SS effector IpaH4.5 modulates the host inflammatory response via interaction with NF-κB p65 protein. *Cell Microbiol*. doi:10.1111/cmi.12052

Wang Y, Luo Z, Pan Y, Wang W, Zhou X, Jeong LS, Chu Y, Liu J, Jia L. 2015. Targeting protein neddylation with an NEDD8-activating enzyme inhibitor MLN4924 induced apoptosis or senescence in human lymphoma cells. *Cancer Biol Ther*. doi:10.1080/15384047.2014.1003003

Wang Y, Satoh A, Warren G. 2005. Mapping the functional domains of the Golgi stacking factor GRASP65. *J Biol Chem*. doi:10.1074/jbc.M412407200

Wang Y, Shi M, Feng H, Zhu Y, Liu S, Gao A, Gao P. 2018. Structural Insights into Non-canonical Ubiquitination Catalyzed by SidE. *Cell* **173**:1231-1243.e16. doi:10.1016/j.cell.2018.04.023

Wauer T, Swatek KN, Wagstaff JL, Gladkova C, Pruneda JN, Michel MA, Gersch M, Johnson CM, Freund SM, Komander D. 2015. Ubiquitin Ser65 phosphorylation affects ubiquitin structure, chain assembly and hydrolysis. *EMBO J*. doi:10.15252/embj.201489847

Weber S, Hilbi H. 2014. Live cell imaging of phosphoinositide dynamics during Legionella infection. *Methods Mol Biol*. doi:10.1007/978-1-4939-1261-2_9

Weber S, Steiner B, Welin A, Hilbi H. 2018. Legionella-Containing Vacuoles Capture PtdIns(4)P-Rich Vesicles Derived from the Golgi Apparatus. *MBio*. doi:10.1128/mBio.02420-18

Wilkinson KD. 2000. Ubiquitination and deubiquitination: Targeting of proteins for degradation by the proteasome. *Semin Cell Dev Biol*. doi:10.1006/scdb.2000.0164

Wong JJY, Pung YF, Sze NSK, Chin KC. 2006. HERC5 is an IFN-induced HECT-type E3 protein ligase that mediates type I IFN-induced ISGylation of protein targets. *Proc Natl Acad Sci U S A*. doi:10.1073/pnas.0600397103

Wong K, Kozlov G, Zhang Y, Gehring K. 2015. Structure of the Legionella Effector, lpg1496, Suggests a Role in Nucleotide Metabolism. *J Biol Chem* **290**:24727–24737. doi:10.1074/jbc.M115.671263

Wood MW, Jones MA, Watson PR, Siber AM, McCormick BA, Hedges S, Rosqvist R, Wallis TS, Galyov EE. 2000. The secreted effector protein of Salmonella dublin, SopA, is translocated into eukaryotic cells and influences the induction of enteritis. *Cell Microbiol*. doi:10.1046/j.1462-5822.2000.00054.x

Woods MW, Tong JG, Tom SK, Szabo PA, Cavanagh PC, Dikeakos JD, Haeryfar SMM, Barr SD. 2014. Interferon-induced HERC5 is evolving under positive selection and inhibits HIV-1 particle production by a novel mechanism targeting Rev/RRE-dependent RNA nuclear export. *Retrovirology*. doi:10.1186/1742-4690-11-27

Wu B, Skarina T, Yee A, Jobin MC, Dileo R, Semesi A, Fares C, Lemak A, Coombes BK, Arrowsmith CH, Singer AU, Savchenko A. 2010. NleG type 3 effectors from enterohaemorrhagic Escherichia coli are U-Box E3 ubiquitin ligases. *PLoS Pathog*. doi:10.1371/journal.ppat.1000960

Wu M, El Qaidi S, Hardwidge PR. 2018. SseL deubiquitinates RPS3 to inhibit its nuclear translocation. *Pathogens*. doi:10.3390/pathogens7040086

Xiang Y, Wang Y. 2010. GRASP55 and GRASP65 play complementary and essential

roles in Golgi cisternal stacking. *J Cell Biol* **188**:237–251. doi:10.1083/jcb.200907132

Xu G, Jiang X, Jaffrey SR. 2013. A mental retardation-linked nonsense mutation in cereblon is rescued by proteasome inhibition. *J Biol Chem*. doi:10.1074/jbc.M113.472092

Xu L, Luo ZQ. 2013. Cell biology of infection by Legionella pneumophila. *Microbes Infect*. doi:10.1016/j.micinf.2012.11.001

Xueyang Jia, Hongyu Ren, Xudong Nie, Yinan Li, Jianguo Li TQ. 2019. Antibiotic Resistance and Azithromycin Resistance Mechanism of Legionella pneumophila Serogroup 1 in China. *Antimicrob Agents Chemother* **63**:1–11.

Yan F, Huang C, Wang X, Tan J, Cheng S, Wan M, Wang Z, Wang S, Luo S, Li A, Guo X, Feng M, Liu X, Zhu Y, Zhou Y. 2020. Threonine ADP-Ribosylation of Ubiquitin by a Bacterial Effector Family Blocks Host Ubiquitination. *Mol Cell*. doi:10.1016/j.molcel.2020.03.016

Yau R, Rape M. 2016. The increasing complexity of the ubiquitin code. *Nat Cell Biol*. doi:10.1038/ncb3358

Youle RJ, Narendra DP. 2011. Mechanisms of mitophagy. *Nat Rev Mol Cell Biol*. doi:10.1038/nrm3028

Yuan J, Luo K, Zhang L, Cheville JC, Lou Z. 2010. USP10 Regulates p53 Localization and Stability by Deubiquitinating p53. *Cell*. doi:10.1016/j.cell.2009.12.032

Zhang Y, Higashide WM, McCormick BA, Chen J, Zhou D. 2006. The inflammation-associated Salmonella SopA is a HECT-like E3 ubiquitin ligase. *Mol Microbiol*. doi:10.1111/j.1365-2958.2006.05407.x

Zheng YT, Shahnazari S, Brech A, Lamark T, Johansen T, Brumell JH. 2009. The Adaptor Protein p62/SQSTM1 Targets Invading Bacteria to the Autophagy Pathway. *J Immunol*. doi:10.4049/jimmunol.0900441

Zheng Z, Wei C, Guan K, Yuan Y, Zhang Y, Ma S, Cao Y, Wang F, Zhong H, He X. 2016. Bacterial E3 Ubiquitin Ligase IpaH4.5 of Shigella flexneri Targets TBK1 To Dampen the Host Antibacterial Response . *J Immunol* **196**:1199–1208. doi:10.4049/jimmunol.1501045

Zhou Y, Zhu Y. 2015. Diversity of bacterial manipulation of the host ubiquitin pathways. *Cell Microbiol* **17**:26–34. doi:10.1111/cmi.12384

Zhu W, Banga S, Tan Y, Zheng C, Stephenson R, Gately J, Luo ZQ. 2011. Comprehensive identification of protein substrates of the Dot/Icm type IV

transporter of Legionella pneumophila. *PLoS One*. doi:10.1371/journal.pone.0017638

Zhu Y, Li H, Hu L, Wang J, Zhou Y, Pang Z, Liu L, Shao F. 2008. Structure of a Shigella effector reveals a new class of ubiquitin ligases. *Nat Struct Mol Biol*. doi:10.1038/nsmb.1517

10 Supplementary data

1. Identified PR-ubiquitination substrates using DupA trapping mutant

Majority protein ID	Gene names	log2 enrichment factor dDupAB over dSidE	-Log10 p-value dDupAB over dSidE	log2 enrichment factor dDupAB over NI	-Log10 p-value dDupAB over NI	log2 enrichment factor WT over NI	-Log10 p-value WT over NI	log2 enrichment factor WT over dSidE	-Log10 p-value WT over dSidE
P0CG47	UBB	7,51	4,41	7,29	4,84	5,97	4,51	6,19	4,09
Q9Y2J2	EPB41L3	6,44	4,52	6,76	4,11	6,17	4,14	5,85	4,70
O43491	EPB41L2	6,20	3,68	4,73	1,69	5,00	1,79	6,47	4,09
Q02952	AKAP12	6,13	4,28	7,19	4,57	7,41	4,85	6,36	4,56
Q9H8Y8	GORASP2	5,83	3,34	5,59	4,17	4,65	4,65	4,90	3,24
Q13586	STIM1	5,73	4,22	5,72	4,41	5,69	6,01	5,69	5,16
P11171	EPB41	5,40	3,53	5,81	4,65	5,55	5,71	5,15	3,67
Q13501	SQSTM1	5,40	2,16	5,47	2,11	4,87	2,27	4,80	2,36
Q99618	CDCA3	5,35	3,10	4,43	3,28	2,35	2,02	3,27	2,16
P50402	EMD	5,28	3,65	5,92	4,02	4,65	5,32	4,01	4,21
Q13443	ADAM9	5,17	3,89	5,08	5,12	3,16	4,41	3,26	3,14
Q99755	PIP5K1A	4,96	3,84	4,79	4,46	5,29	4,28	5,47	3,83
Q14160	SCRIB	4,96	4,38	4,16	3,46	2,70	3,10	3,50	4,87
Q9H2G2	SLK	4,96	1,47	5,05	2,36	5,02	2,52	4,93	1,50
Q86VR2	FAM134C	4,86	3,98	4,15	3,11	3,00	2,64	3,71	3,66
Q8NEN9	PDZD8	4,74	5,00	4,29	4,16	3,35	3,35	3,81	3,91
Q6PL24	TMED8	4,72	3,54	4,84	3,42	4,89	3,58	4,78	3,74
O60784	TOM1	4,69	3,95	4,77	2,96	4,83	3,20	4,75	5,35
O60716	CTNND1	4,66	3,04	4,07	2,63	4,04	2,97	4,63	3,53
P55072	VCP	4,59	2,52	3,74	2,59	4,73	2,76	5,59	2,70
Q9UK76	HN1	4,57	2,06	4,47	3,23	3,94	3,45	4,04	1,95
Q9UKG1	APPL1	4,55	3,39	4,24	3,58	4,25	4,73	4,57	4,00
Q9UBC2	EPS15L1	4,50	3,00	4,72	4,07	4,91	4,61	4,69	3,18
P42566	EPS15	4,49	3,58	4,50	3,67	4,46	3,93	4,45	3,81
P61586	RHOA	4,46	2,71	5,41	3,88	6,51	3,85	5,55	2,93
P56937	HSD17B7	4,45	3,73	4,83	4,28	3,23	3,77	2,86	3,09
Q9NQC3	RTN4	4,41	4,40	3,77	4,06	3,30	4,75	3,94	5,26
O00161	SNAP23	4,29	2,41	4,86	3,04	4,51	2,79	3,94	2,21
O00767	SCD	4,17	1,68	1,61	4,34	1,72	3,59	4,28	1,70
Q5JTV8	TOR1AIP1	4,16	3,21	4,34	2,80	3,22	2,35	3,05	2,73
Q15018	FAM175B	4,15	3,99	4,20	3,13	4,20	2,80	4,14	3,25
Q96KG9	SCYL1	3,80	2,95	3,91	2,87	3,11	3,16	3,00	3,44
O75323	GBAS	3,77	3,03	3,98	3,01	3,46	3,55	3,25	3,74

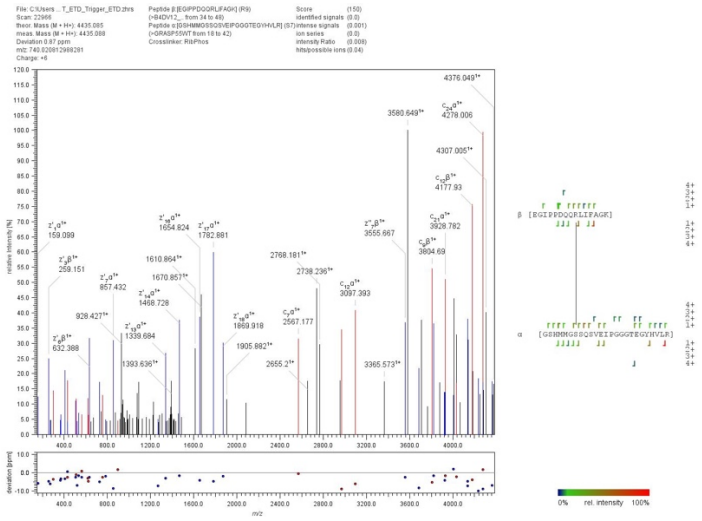
Q7Z434	MAVS	3,75	1,92	3,18	2,06	2,44	1,68	3,00	1,62
O60749	SNX2	3,72	2,87	4,34	3,11	4,59	3,40	3,97	3,17
P78536	ADAM17	3,69	3,99	3,39	2,92	3,27	2,96	3,56	4,27
Q9Y6I9	TEX264	3,64	2,16	2,34	4,60	2,23	5,52	3,52	2,12
Q9H0E2	TOLLIP	3,60	3,55	3,53	2,69	4,02	2,99	4,09	4,06
Q9UH99	SUN2	3,58	4,06	4,62	4,73	4,04	4,82	3,00	3,98
P15260	IFNGR1	3,57	3,94	3,18	2,63	2,82	2,50	3,21	4,06
Q16799	RTN1	3,55	2,04	3,53	3,92	3,17	3,85	3,19	1,90
P46736	BRCC3	3,52	2,24	3,42	3,25	3,99	3,02	4,09	2,32
Q04917	YWHAH	3,52	4,35	2,93	3,72	2,01	3,13	2,60	3,88
Q86XL3	ANKLE2	3,38	2,03	2,93	4,12	2,31	3,20	2,75	1,72
Q9Y6N7	ROBO1	3,36	2,94	3,59	3,37	2,54	2,93	2,31	2,43
O00151	PDLIM1	3,34	2,47	3,71	3,90	3,07	3,85	2,70	2,19
Q9NX40	OCIAD1	3,31	4,16	3,26	3,66	2,00	2,58	2,05	2,90
O75496	GMNN	3,29	2,35	3,06	1,80	1,85	1,48	2,08	2,63
O95168	NDUFB4	3,19	2,71	2,81	3,29	2,63	3,65	3,02	2,76
Q92597	NDRG1	3,17	3,00	3,40	2,96	3,26	2,67	3,02	2,66
Q14139	UBE4A	3,13	2,30	2,91	2,60	2,45	2,33	2,67	2,06
Q9H082	RAB33B	3,09	3,45	3,12	3,81	2,45	3,04	2,42	2,79
Q7Z3T8	ZFYVE16	3,08	1,15	5,33	3,81	5,11	4,00	2,86	1,09
Q59FP8	NEO1	3,07	2,29	2,48	2,24	1,44	1,49	2,04	1,69
Q6NXT6	TAPT1	3,05	3,42	2,77	3,92	2,02	4,03	2,30	3,18
Q96RT1	ERBB2IP	3,05	2,87	3,74	3,27	2,88	3,49	2,20	2,90
Q8N0X7	SPG20	3,00	3,71	2,61	2,88	2,63	2,87	3,02	3,69
O60271	SPAG9	2,98	2,29	2,12	2,09	2,51	2,20	3,37	2,38
Q9C0C2	TNKS1BP1	2,98	2,44	4,36	2,16	4,34	2,24	2,96	2,72
Q8WVP7	LMBR1	2,97	3,74	2,70	1,77	2,08	1,44	2,35	3,23
Q9UGV2	NDRG3	2,95	1,73	3,65	2,44	3,32	2,07	2,61	1,47
Q9BSJ8	ESYT1	2,89	3,87	3,59	2,14	3,67	2,14	2,97	3,61
Q8TEY7	USP33	2,86	3,82	2,40	3,12	1,35	2,36	1,82	3,33
Q6FIF0	ZFAND6	2,85	2,55	3,36	2,23	2,90	2,21	2,39	2,83
Q16513	PKN2	2,84	3,05	2,84	3,04	3,37	3,65	3,37	3,66
P14923	JUP	2,81	2,30	2,06	1,45	1,76	1,23	2,51	2,00
O43169	CYB5B	2,81	1,14	2,87	1,26	3,09	1,36	3,03	1,23
P23634	ATP2B4	2,79	4,05	2,65	2,67	3,08	2,90	3,22	4,27
Q13740	ALCAM	2,79	1,66	2,74	1,59	2,89	1,55	2,93	1,61
Q96S55	WRNIP1	2,72	2,81	2,59	2,89	4,24	3,61	4,37	3,52
Q9P246	STIM2	2,70	3,63	2,85	3,32	2,48	3,38	2,33	3,91
Q9H3P7	ACBD3	2,69	3,54	2,36	2,58	2,08	2,33	2,40	3,22

P43307	SSR1	2,68	1,75	2,20	2,00	2,43	2,02	2,90	1,81
O75438	NDUFB1	2,65	2,24	2,23	1,65	2,25	1,73	2,67	2,39
Q15043	SLC39A14	2,64	2,40	2,43	1,95	3,19	2,94	3,40	4,24
P53992	SEC24C	2,64	1,68	2,93	2,82	2,37	2,64	2,08	1,42
P15311	EZR	2,62	2,42	3,29	2,45	4,69	2,82	4,03	2,82
Q9BXB4	OSBPL11	2,55	2,62	2,87	2,49	2,20	2,18	1,89	2,30
Q99442	SEC62	2,52	2,82	2,97	3,45	1,99	3,09	1,54	2,23
Q86UE4	MTDH	2,51	3,07	2,36	1,70	1,77	1,46	1,92	4,60
Q9Y512	SAMM50	2,49	3,50	2,35	2,34	2,59	2,36	2,73	3,23
O75844	ZMPSTE24	2,48	2,10	2,21	1,91	2,34	2,02	2,60	2,20
Q14C86	GAPVD1	2,48	2,16	1,83	2,34	1,77	2,66	2,42	2,25
Q14697	GANAB	2,47	1,61	1,97	1,66	1,82	1,39	2,31	1,42
Q14126	DSG2	2,46	3,11	2,47	3,29	2,58	3,01	2,56	2,88
O95070	YIF1A	2,44	2,03	1,77	2,28	1,96	2,49	2,64	2,16
P55011	SLC12A2	2,43	1,36	1,85	2,52	2,17	3,02	2,76	1,52
Q96T51	RUFY1	2,40	2,18	2,60	1,62	1,70	1,19	1,51	1,66
P56962	STX17	2,39	3,17	2,69	2,83	2,76	2,99	2,47	3,45
Q9C0E8	LNP	2,38	1,63	2,20	2,52	2,32	2,60	2,50	1,69
Q9UKV5	AMFR	2,38	2,60	3,05	3,39	3,75	3,78	3,09	3,05
P42167	TMPO	2,37	3,37	2,66	3,31	1,88	3,11	1,58	3,27
P07919	UQCRH	2,27	1,28	1,45	1,37	1,35	1,27	2,17	1,22
O43676	NDUFB3	2,27	1,19	1,75	1,14	1,83	1,21	2,35	1,24
Q15436	SEC23A	2,25	2,36	2,67	1,62	2,04	1,29	1,61	1,81
P60953	CDC42	2,24	1,84	2,70	2,40	2,70	2,56	2,24	1,93
P54727	RAD23B	2,22	1,30	1,74	1,15	2,52	1,51	3,00	1,62
Q9Y6M9	NDUFB9	2,21	1,41	2,09	1,77	2,53	2,25	2,65	1,72
Q16625	OCLN	2,20	2,73	1,91	1,78	2,65	2,31	2,94	3,41
Q9BXK5	BCL2L13	2,15	2,15	2,42	1,81	2,17	1,62	1,89	1,89
P61158	ACTR3	2,14	2,15	2,66	2,65	2,76	2,23	2,24	1,84
Q96G23	CERS2	2,14	1,08	2,99	3,79	3,11	3,61	2,26	1,12
Q96HY6	DDRGK1	2,10	1,14	1,83	1,23	1,74	1,16	2,01	1,09
Q9UNK0	STX8	2,07	2,28	1,62	2,16	1,73	2,16	2,18	2,28
Q8NFQ8	TOR1AIP2	2,04	1,14	1,70	1,26	1,96	1,38	2,30	1,24
Q9BVC6	TMEM109	2,04	3,78	1,60	3,76	1,52	3,70	1,95	3,73
Q9NVH1	DNAJC11	2,03	1,34	2,19	2,52	2,30	2,66	2,14	1,41
P34932	HSPA4	2,03	1,36	1,88	1,29	1,78	1,21	1,92	1,29
Q9UNL2	SSR3	3,07	4,50	0,72	1,22	1,41	1,91	3,76	4,33
Q9BVK6	TMED9	2,24	1,03	1,03	1,98	1,45	2,37	2,66	1,18
H7BXI1	ESYT2	2,06	2,00	1,16	1,42	1,82	1,62	2,73	2,05

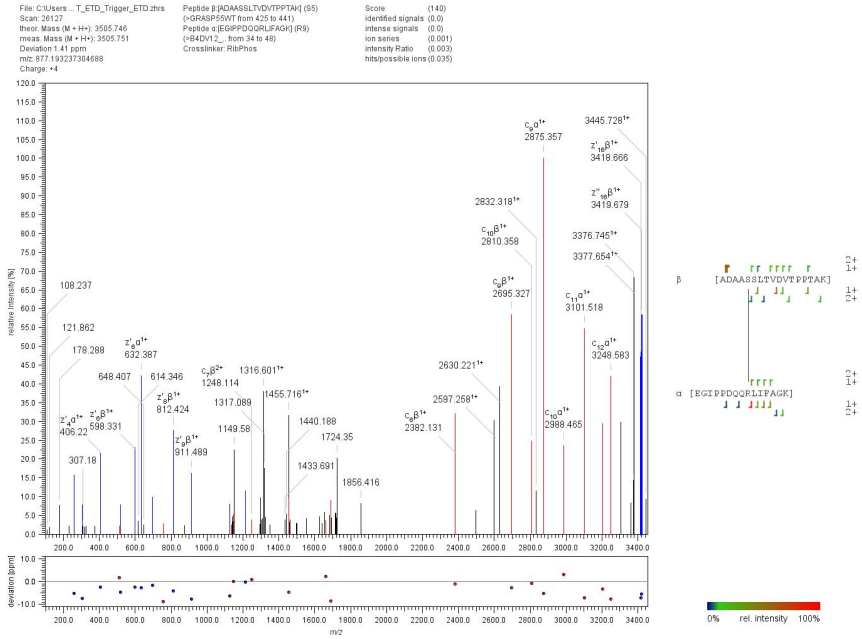
Q9Y2U8	LEMD3	3,89	3,44	3,24	1,70	1,65	1,01	2,30	2,80
Q9UHD9	UBQLN2	3,74	2,92	1,71	1,66	1,06	1,48	3,09	3,28
Q6NXS1	PPP1R2;PPP1R2P3	3,46	2,53	3,03	1,95	1,33	1,04	1,76	1,62
Q86X29	LSR	3,04	2,20	2,34	1,56	1,32	0,97	2,02	1,59
O75976	CPD	3,00	2,96	2,56	2,96	1,13	1,60	1,58	1,86
O95721	SNAP29	2,96	3,27	3,43	3,87	1,42	3,14	0,94	1,89
P18850	ATF6	2,73	2,22	1,86	1,70	0,86	1,48	1,73	2,41
Q96SU4	OSBPL9	2,36	2,77	1,62	1,73	1,02	1,37	1,76	2,92
Q8N766	EMC1	2,24	1,40	1,57	1,25	1,34	1,10	2,00	1,29
Q5HYI8	RABL3	2,18	2,31	3,45	2,90	2,03	2,55	0,75	1,43
A4D1S0	KLRG2	2,12	1,89	3,71	3,50	2,46	2,55	0,88	0,90
Q9BPU6	DPYSL5	2,07	2,56	3,20	2,20	1,61	1,31	0,47	0,86
Q96J02	ITCH	1,99	2,40	2,30	4,17	3,97	3,74	3,65	2,99
P54920	NAPA	1,98	1,73	1,79	2,63	2,14	2,37	2,32	1,81
P30519	HMOX2	1,94	5,26	1,86	2,65	2,28	2,87	2,37	4,43
Q5QNY5	PEX19	1,94	1,30	2,40	1,29	2,05	1,07	1,60	1,02
Q13308	PTK7	1,92	1,23	1,85	1,31	1,91	1,31	1,98	1,24
Q8NHP6	MOSPD2	1,91	2,58	2,05	3,29	2,90	3,42	2,76	2,94
Q9UMX0	UBQLN1	1,91	1,22	2,28	1,62	2,31	2,06	1,94	1,45
O43491	EPB41L2	1,90	1,82	2,30	1,83	2,16	2,41	1,76	3,25
O14745	SLC9A3R1	1,88	1,75	1,87	1,60	2,51	2,33	2,53	2,64
P84077	ARF1;ARF3	1,87	2,80	1,42	2,04	1,15	1,78	1,60	2,61
O96008	TOMM40	1,86	2,02	2,39	1,75	2,54	1,85	2,01	2,17
Q6UW68	TMEM205	1,83	1,78	1,90	1,44	1,78	1,28	1,71	1,53
Q9H444	CHMP4B	1,83	1,94	2,65	2,11	2,32	1,95	1,50	1,72
Q9UNH7	SNX6	1,83	2,08	1,42	2,20	1,77	2,09	2,17	2,08
Q4KMP7	TBC1D10B	1,83	1,62	2,21	1,62	2,87	1,97	2,49	2,05
Q96ER3	SAAL1	1,81	3,56	1,62	4,18	1,05	3,36	1,24	2,90
P45974	USP5	1,76	1,27	1,87	2,09	1,68	1,90	1,57	1,15
Q8NB49	ATP11C	1,75	2,28	1,73	1,55	1,94	1,68	1,97	2,41
Q6P1M0	SLC27A4	1,73	1,68	1,55	1,48	1,34	1,30	1,51	1,50
P08195	SLC3A2	1,69	2,87	1,67	3,30	2,35	3,30	2,38	3,05
P26038	MSN	1,69	2,44	1,98	2,78	3,11	2,40	2,82	2,23
P67812	SEC11A	1,68	1,47	1,76	1,14	1,69	1,10	1,61	1,40
P35241	RDX	1,67	2,69	1,94	2,37	2,43	2,06	2,17	2,07
P49006	MARCKSL1	1,63	2,01	1,84	1,80	1,54	1,82	1,34	2,30
P20618	PSMB1	1,63	1,40	2,12	1,30	2,42	1,54	1,92	1,82
P09543	CNP	1,61	2,67	1,18	2,49	1,42	3,11	1,85	3,10
O94804	STK10	1,61	2,10	2,04	1,81	1,34	1,58	0,91	3,14

Q13190	STX5	1,60	1,64	1,51	2,82	1,63	4,22	1,72	1,83
Q15042	RAB3GAP1	1,57	1,35	1,90	3,40	2,01	3,10	1,67	1,39
Q14739	LBR	1,56	3,28	1,41	2,65	1,27	2,12	1,42	2,51
O95197	RTN3	1,55	3,35	1,61	3,16	1,56	2,77	1,50	2,86
Q9H0U3	MAGT1	1,55	1,67	1,51	1,39	1,70	1,58	1,74	1,93
Q5VV42	CDKAL1	1,53	3,85	2,51	3,05	2,45	2,94	1,47	3,36
Q9Y679	AUP1	1,49	1,56	0,99	3,48	1,11	3,02	1,62	1,63
P49257	LMAN1	1,45	1,98	1,43	2,40	2,07	2,28	2,09	2,08
Q15006	EMC2	1,43	1,39	1,98	1,70	1,85	1,60	1,31	1,27
P35222	CTNNB1	1,42	1,46	1,73	2,33	1,86	2,31	1,56	1,53
Q12974	PTP4A1;PTP4A2	1,42	2,80	1,35	2,57	1,87	3,15	1,94	3,38
P35221	CTNNA1	1,42	2,01	1,32	1,66	1,33	1,69	1,43	2,06
P05556	ITGB1	1,39	2,43	1,14	3,15	1,50	3,97	1,75	2,87
Q01650	SLC7A5	1,29	2,95	1,26	2,80	1,28	3,11	1,30	3,33
Q9UEU0	VTI1B	1,23	2,18	2,02	1,98	1,99	1,84	1,20	1,76
Q9P035	HACD3	1,23	2,29	1,34	2,12	1,87	1,92	1,76	1,92
P21796	VDAC1	1,22	1,82	1,56	2,49	1,88	3,18	1,55	2,36
Q9BTV4	TMEM43	1,17	2,14	1,26	2,22	1,78	2,34	1,69	2,28
P20020	ATP2B1	1,17	3,13	1,15	2,70	1,22	2,79	1,23	3,23
Q5JSH3	WDR44	1,83	3,26	1,16	1,41	1,66	1,83	2,33	3,54
P25786	PSMA1	1,71	1,12	1,15	1,18	1,26	1,37	1,82	1,22
P11233	RALA	1,46	2,13	1,03	1,51	1,53	2,09	1,96	2,67
Q8TF71	SLC16A10	1,39	1,46	0,90	1,07	1,30	1,43	1,80	1,77
P41440	SLC19A1	1,38	1,04	1,84	1,43	2,17	1,64	1,71	1,24
Q9NX14	NDUFB11	1,37	1,38	2,47	1,44	2,70	1,52	1,60	1,50
Q14247	CTTN	1,30	1,39	1,44	1,69	1,48	1,94	1,35	1,56
Q8NE01	CNNM3	1,22	2,80	0,68	1,68	1,24	2,33	1,78	3,12
Q9Y277	VDAC3	1,14	1,27	1,47	2,22	1,76	2,60	1,43	1,54
Q8IZ07	ANKRD13A	1,13	1,41	1,47	2,46	2,89	2,45	2,55	1,98
Q8TC07	TBC1D15	1,13	1,99	1,18	1,91	1,53	2,03	1,48	2,08
Q8NBJ4	GOLM1	1,10	1,23	1,65	2,59	2,01	2,89	1,46	1,54
P45880	VDAC2	1,08	1,66	1,50	2,43	1,88	2,70	1,46	2,02
P49755	TMED10	1,08	2,71	0,90	2,09	1,11	2,88	1,28	4,06
Q5QPK2	DPM1	0,93	1,39	1,08	2,65	1,55	2,28	1,40	1,64

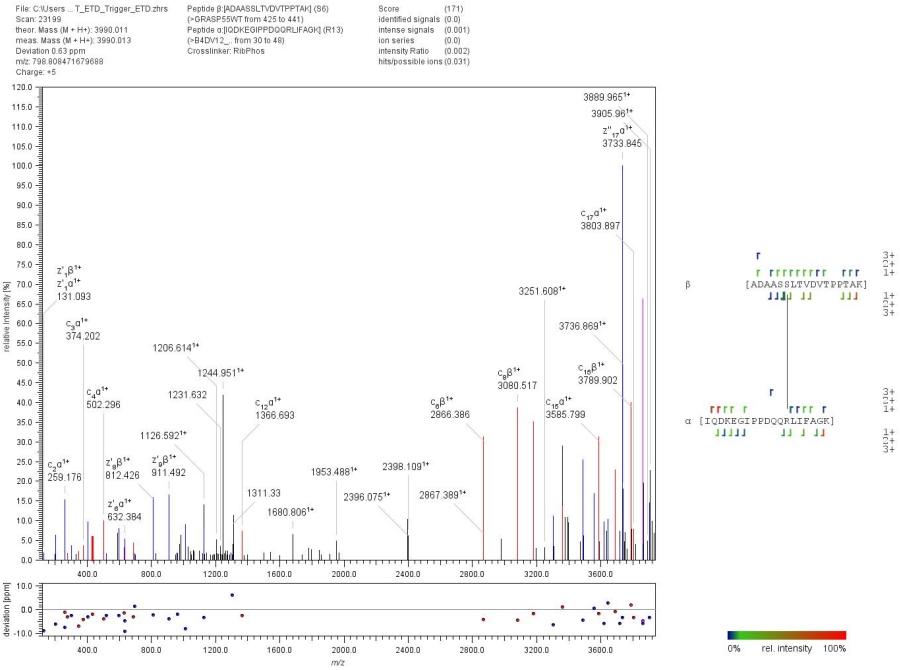
2. High resolution ETD spectrum of ubiquitin cross linked with serines of GRASP55



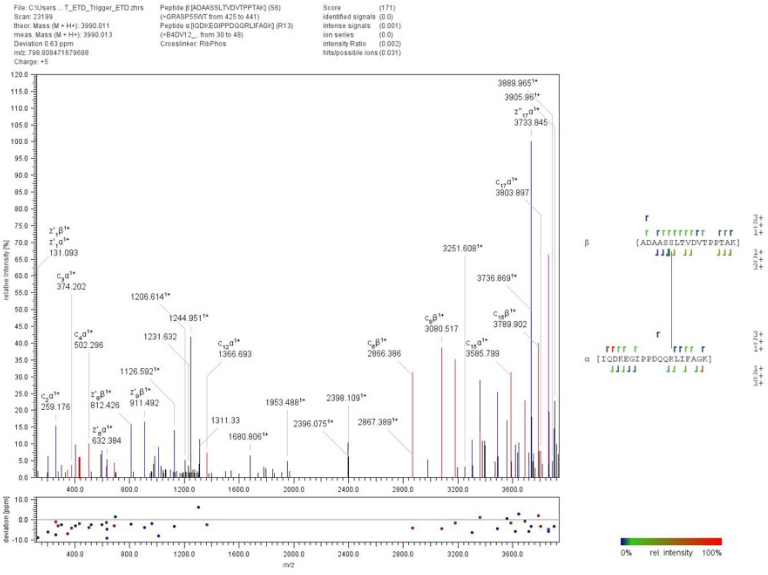
High resolution ETD spectrum of ubiquitin cross linked Serine 3 of GRASP55.



High resolution ETD spectrum of ubiquitin cross linked Serine 408 of GRASP55.



High resolution ETD spectrum of ubiquitin cross linked Serine 409 of GRASP55.



High resolution ETD spectrum of ubiquitin cross linked Serine 449 of GRASP55.

11 Publications

1. Bhogaraju S, Kalayil S, **Liu Y**, et al. Phosphoribosylation of ubiquitin promotes serine ubiquitination and impairs conventional ubiquitination. *Cell*, 2016
2. Kalayil S, Bhogaraju S, Bonn F, Shin D, **Liu Y**, Gan N, Basquin J, Grumati P, Luo Z and Dikic I. Insights into catalysis and function of phosphoribosyl-linked serine ubiquitination. *Nature, 2018*
3. Shin D[#], Mukherjee R[#], **Liu Y**[#], Gonzalez A, Bonn F, Liu Y, ... Dötsch V & Dikic I. (2019). Regulation of Phosphoribosyl-Linked Serine Ubiquitination by Deubiquitinases DupA and DupB. *Molecular cell*. (#: co-first author)
4. **Liu Y**, Mukherjee R, Bonn F, Colby T, Matic I, & Dikic I. Serine-ubiquitination regulates Golgi morphology and the secretory pathway upon Legionella infection. *bioRxiv*, 2020.