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1 # Serine-ubiquitination regulates Golgi morphology and the secretory
2 # pathway upon Legionella infection

3

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1 **Abstract**

2 SidE family of *Legionella* effectors catalyze non-canonical phosphoribosyl-
3 linked ubiquitination (PR-ubiquitination) of host proteins during bacterial
4 infection. SdeA localizes predominantly to ER and partially to the Golgi
5 apparatus, and mediates serine ubiquitination of multiple ER and Golgi proteins.
6 Here we show that SdeA induces fragmentation of the Golgi stacks due to its
7 ubiquitin ligase activity. The Golgi tethering factors GRASP55 and GRASP65
8 are PR-ubiquitinated on multiple serine residues, thus preventing their ability to
9 cluster and form oligomeric structures. In addition, we found that the functional
10 consequence of Golgi fragmentation is not linked to the recruitment of Golgi
11 membranes to the growing *Legionella*-containing vacuoles. Instead, it affects
12 the secretory pathway, including cytokine release in cells. Taken together, our
13 study sheds light on the Golgi manipulation strategy by which *Legionella* hijacks
14 the secretory pathway and promotes bacterial infection.

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Introduction

Ubiquitination is a post-translational modification that is conserved from yeast to mammals. The catalysis of canonical ubiquitination is regulated via a three-enzyme cascade: firstly an ubiquitin (Ub) molecule is activated by an Ub-activating enzyme (E1) utilizing ATP; the activated Ub is linked to the catalytic cysteine of the E1 via its C-terminal Glycine and subsequently transferred to an Ub-conjugating enzyme (E2); finally, a Ub ligase (E3) links the carboxyl group of the ubiquitin's C-terminal glycine to the ε-amino group of the target lysine in the substrate protein by an isopeptide bond (Hershko et al., 2000). Protein ubiquitination virtually regulates every cellular processes, including protein quality control, protein trafficking, immunity, and DNA repair by targeting substrates to the proteasome or altering their functions (Ben-Neriah, 2002; Dikic, 2017; Donaldson et al., 2003; Rape, 2018).

Consistent with the critical roles of ubiquitination in cellular processes, emerging evidence indicates that pathogens hijack the ubiquitination machinery for efficient invasion (Bomberger et al., 2011; Hicks and Galán, 2013; Maculins et al., 2016). For example, the intracellular Gram-negative pathogen *Legionella pneumophila* secretes more than 300 effectors into host cells via its type IV secretion system (T4SS) (Hubber and Roy, 2010). Many of these toxins function as E3 ligases and are reported to manipulate host ubiquitination (Qiu and Luo, 2017). Various studies have revealed that effectors of the SidE family (SdeA, SdeB, SdeC and SidE) catalyze an NAD^+-dependent, ATP-independent

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1 type of ubiquitination without the need of E2 and E3 enzymes (Bhogaraju et al.,
2 2016; Qiu et al., 2016). Moreover, unlike the conventional ubiquitination that
3 occurs on lysine residues of substrate proteins, SidE family effectors catalyze
4 the conjugation of Ub via a phosphoribosyl moiety to serine residues of host
5 substrate proteins by a two-domain catalytic relay: a mono ADP-ribosyl
6 transferase (mART) domain that ADP-ribosylates Arg42 of Ub and a
7 phosphodiesterase (PDE) domain that cleaves the phosphodiester bond of the
8 ADP-ribosylated Ub (ADPR-Ub) and conjugates the resulting phosphoribosyl
9 ubiquitin (PR-Ub) to the serine residue of a substrate (Akturk et al., 2018; Dong
10 et al., 2018; Kalayil et al., 2018; Wang et al., 2018). PR-ubiquitination is
11 reversible, and DupA is a deubiquitinase with specific affinity for PR-
12 ubiquitinated substrates (Shin et al., 2020). SidE family effectors are crucial for
13 bacterial virulence, as a *Legionella* strain lacking SidE family members shows
14 defective growth in host cells, a phenotype that can be rescued by
15 replenishment of SdeA (Bardill et al., 2005; Qiu et al., 2016). To date, numerous
16 ER-associated proteins have been identified as PR-ubiquitination substrates of
17 SdeA, such as tubular ER protein RTN4, FAM134B, and LNP1. PR-
18 ubiquitination of these proteins is involved in regulating ER remodeling and
19 recruiting ER membranes to *Legionella* containing vacuoles (LCV) where the
20 bacteria resides and replicates (Kotewicz et al., 2017; Shin et al., 2020).
21 In our previous study, we used the catalytically dead mutant of the
22 deubiquitinase DupA as a bait to identify targets of SdeA. Besides ER-related

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1 substrates, we also identified proteins related to other cellular pathways,
2 including Golgi proteins, mitochondrial proteins and components of the
3 autophagy machinery (Shin et al., 2020). However, the biological functions of
4 PR-ubiquitination of these proteins remained unclear. In the present study, we
5 made use of biochemical and microbiological approaches to characterize the
6 PR-ubiquitination of Golgi tethering proteins GRASP55 and GRASP65 by SdeA.
7 We also provide explanations for the Golgi fragmentation induced by the PR-
8 ubiquitination of these proteins. Moreover, we demonstrate that PR-
9 ubiquitination regulates the host cellular secretory pathway during bacterial
10 infection.
11
12 **Results**
13 **SdeA is targeted to the ER and Golgi via its carboxyl terminus**
14 Previous structural and biochemical studies have revealed the structure of
15 SdeA catalytic core, and the mechanism by which SdeA ubiquitinates
16 substrates is well established (Akturk et al., 2018; Dong et al., 2018; Kalayil et
17 al., 2018; Wang et al., 2018). However, the function of the carboxyl terminal
18 (CT) region, predicted to be coiled-coil, remained unknown (Fig. 1A). Previous
19 reports suggested that coiled-coil domains are required for membrane
20 localization of many *Salmonella* type III effectors (Knodler et al., 2011). In view
21 of that SdeA co-localizes with ER protein calnexin and ubiquitinates many ER
22 proteins, such as RTN4 and FAM134B (Kotewicz et al., 2017; Qiu et al., 2017;

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1 Shin et al., 2020), we hypothesized that the CT domain of SdeA is responsible
2 for its membrane association. To analyze if the CT of SdeA contributes to its
3 membrane localization and is therefore needed for the PR-ubiquitination of
4 membrane-located substrates, we first investigated the ER localization of wild-
5 type SdeA and truncated $SdeA^{1-972}$ mutant lacking the last part of the C-terminal
6 region (Fig. 1A). COS7 cells were transfected with plasmids encoding EGFP-
7 tagged SdeA, a truncated mutant, or EGFP alone and subsequently stained for
8 the ER resident protein Calnexin. We observed that ectopically expressed SdeA
9 co-localized with ER protein Calnexin in COS7 cells, consistently with a
10 previous study (Qiu et al., 2017). In contrast and along our hypothesis that the
11 C-terminal region of SdeA is essential for its membrane localization, truncated
12 SdeA did not co-localize with Calnexin but showed a rather cytosolic distribution
13 similar to the EGFP control (Fig. 1B). In addition, we observed that part of SdeA
14 was densely localized close to the nucleus in cells (Fig. 1B). Staining with the
15 Golgi marker GM130 revealed that this part of SdeA co-localized with the Golgi
16 apparatus, while the truncated mutant $SdeA^{1-972}$ did not (Fig. 1C). We confirmed
17 that the C-terminus region of SdeA is necessary to its Golgi localization by
18 expressing the N-terminal-truncated $SdeA^{909-C}$ in cells stained with GM130.
19 $SdeA^{909-C}$ expressed in cells highly overlapped with GM130, but did not disturb
20 Golgi structure as wild-type SdeA did (Fig. 1C). This data suggests that the C-
21 terminal part of SdeA is critical for its ER as well as its Golgi membrane
22 localization.

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SdeA induces Golgi fragmentation

To investigate whether Golgi localization of SdeA is critical for its ligase function, we co-expressed wild type SdeA or the truncated mutant SdeA$^{1\text{-}972}$ with its known Golgi associated substrate Rab33b (Qiu et al., 2016). Western blot analysis showed that the truncated form of SdeA could not ubiquitinate Rab33b even through it was able to ADP-ribosylate ubiquitin (Figure 2-figure supplement 1A). This data suggests that the C-terminus region of SdeA is critical not only for its localization but also for its ability to ubiquitinate Golgi proteins. During our localization studies, we observed that expression of wild-type SdeA, but not the CT-truncated mutants, results in dispersed GM130 staining. This implicates an effect of SdeA activity on the structural stacking organization of the Golgi apparatus. We then sought to investigate the possibility of PR-ubiquitination activity of SdeA is responsible for the Golgi fragmentation by comparing the effects between wild-type SdeA with SdeA catalytic mutants (Fig. 1A). The expression of PDE defective mutant (SdeA H277A) or mART defective mutant (SdeA EE/AA) revealed that these catalytically dead mutants have no significant impact on the structure of the Golgi (Fig. 2A, B). In addition, the effect of wild-type SdeA on the Golgi structure could be counteracted by co-expression of DupA, the specific deubiquitinase for PR-ubiquitination, but not its catalytically dead mutant DupA H67A (Fig. 2A, B). These findings suggest that the Golgi fragmentation observed in cells

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1 expressing SdeA is likely to be caused by the accumulation of its ubiquitinated
2 substrates. Similar observations were also made in HeLa cells stained with both
3 cis (GM130) and trans (TGN46) Golgi marker antibodies (Figure 2-figure
4 supplement 1B). These observations are in apparent agreement with previous
5 study (Jeong et al., 2015). In order to evaluate the physiological relevance of
6 SdeA in triggering Golgi fragmentation, we infected human lung carcinoma
7 A549 cells with either a wild-type *Legionella* strain, a mutant strain missing
8 genes encoding SidE family proteins (*ΔsidEs*) or a mutant that does not express
9 DupA and DupB (*ΔdupA/B*). As expected, we observed a scattering of the Golgi
10 apparatus in cells infected with wild-type but not Δ*sidEs Legionella* or control
11 cells. Infection by *Legionella* without DupA/B showed more dramatic
12 fragmentation of the Golgi, compared to the wild-type *Legionella* (Fig. 2C, D).
13 Taken all together, these data suggest that SdeA induces Golgi fragmentation
14 under both exogenous expression and physiological bacteria infection as a
15 consequence of PR-ubiquitinating host substrates.
16
17 ***In vitro* and *in vivo* validation of PR-ubiquitination of Golgi substrates by**
18 **SdeA**
19 Using the PR-deubiquitinase DupA as a bait, we pulled-down over 180 potential
20 host substrate proteins of SdeA upon *Legionella* infection. (Shin et al., 2020).
21 Among these identified proteins, a number of ER resident proteins, and proteins
22 related to Golgi components were highly enriched. Notably, Golgi tethering

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1 factors GRASP55 and GCP60 had the highest ratios among the putative Golgi
2 protein substrates (Fig. 3A). Since GRASP55 and GCP60 play roles in
3 membrane stacking of Golgi cisternae and interact with the Golgi resident
4 proteins (Shorter et al., 1999; Sohda et al., 2001), we hypothesized that SdeA
5 modifies and inactivates Golgi proteins related to structure maintenance,
6 thereby inducing Golgi fragmentation. GRASP65, which shares high sequence
7 similarity with GRASP55, is localized to the *cis* Golgi and is also found in
8 dispersed Golgi apparatus in cells expressing wild-type SdeA (Figure 3-figure
9 supplement 1A). *In vitro* ubiquitination assays were performed, incubating
10 purified GRASP55 or GRASP65 with SdeA and ubiquitin for 30 min, to monitor
11 potential PR-ubiquitination of the two Golgi proteins. We observed that SdeA is
12 able to modify both GRASP55 and GRASP65 *in vitro* (Fig. 3B). Furthermore,
13 cellular expression of wild-type SdeA, but not inactive PDE or mART mutants,
14 resulted in the appearance of ubiquitinated GRASP55 and GRASP65. This PR-
15 ubiquitination was lost when PR-ubiquitination specific deubiquitinase, DupA,
16 was co-expressed with wild-type SdeA (Fig. 3C, D). Similar observations were
17 made for GCP60, where purified GCP60 from cells incubated with wild-type
18 SdeA exhibited PR-ubiquitination (Figure 3-figure supplement 1B). Such
19 modification also appeared in cells when GCP60 was co-expressed with wild-
20 type SdeA but not upon co-expression of SdeA EE/AA mutant (Figure 3-figure
21 supplement 1C). Along our hypothesis that SdeA is actively targeted to the
22 Golgi, exogenous expression of CT-truncated SdeA mutants showed markedly

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1 reduced activity in modifying substrate GRASP55 (Figure 3-figure supplement
2 1D), similar to the effect observed on PR-ubiquitination of Rab33b, indicating
3 that Golgi localization of SdeA is important for substrate modification. Our
4 previous work indicated that M408 and L411 are two essential amino acids in
5 the substrate binding region of SdeA (Kalayil et al., 2018). To distinguish
6 whether SdeA targets GRASP55 protein specifically via its substrate
7 recognition region or if modification is an overexpression artifact and due to
8 high amounts of SdeA located at the Golgi, we performed an *in vitro*
9 ubiquitination assay by incubating purified GRASP55 with wild type SdeA and
10 SdeA ML/AA mutant, respectively. The Coomassie staining showed that the
11 SdeA ML/AA mutant did not ubiquitinate GRASP55 (Figure 3-figure supplement
12 1E). Similarly, GRASP55 ubiquitination is reduced in cells expressing SdeA
13 ML/AA mutant compared to cells expressing wild type SdeA (Figure 3-figure
14 supplement 1F) and the interaction with GRASP55 is much reduced for SdeA
15 mutant compared to wild type SdeA in Co-IP experiments (Figure 3-figure
16 supplement 1G). Together, these results suggest that the PR-ubiquitination of
17 Golgi tethering proteins GRASP55, GRASP65 and GCP60 by SdeA is a
18 selective and functional part of the hijacking strategy of Legionella.
19

20 **_Legionella_ infection causes PR-ubiquitination of GRASP55 and GRASP65**

21 To check whether these Golgi proteins are PR-ubiquitinated upon *Legionella*
22 infection, we immunoprecipitated GFP-tagged GRASP55 and GRASP65 from

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1 HEK293T cells infected with *Legionella* strains and analyzed them for PR-
2 ubiquitination. The results showed that both GRASP55 and GRASP65 were
3 ubiquitinated in a time-depending manner following *Legionella* infection (Fig.
4 4A, B). Moreover, the ubiquitination level was increased in cells infected with
5 the *Legionella ΔdupA/B* mutant strain, indicating that more PR-ubiquitinated
6 protein accumulated in the absence of the deubiquitinases (Fig. 4A, B).
7 *Legionella* infection-induced GRASP55 and GRASP65 PR-ubiquitination was
8 lost when cells were infected with a strain that lacks SidE family effectors
9 (*∆sidEs*) (Fig. 4C, D), thus, confirming that these effectors as essential for PR-
10 ubiquitination of host substrate proteins. Similar results were obtained for
11 GCP60 (Figure 4-figure supplement 1A). To further confirm that this detected
12 ubiquitination is exclusively PR-ubiquitination caused by SidE family effectors
13 directly, we incubated GRASP55 or GRASP65, isolated from infected HEK293T
14 cells, with purified DupA. As expected, DupA was able to remove the
15 ubiquitination of GRASP55 and GRASP65 induced by *Legionella* infection
16 (Figure 4-figure supplement 1B, C). These data suggest that SdeA PR-
17 ubiquitinates Golgi tethering proteins GRASP55 and GRASP65 during
18 *Legionella* infection, further supporting our hypothesis that this modification has
19 a directed function.
20
21 **SdeA ubiquitinates multiple serines of GRASP55 protein**
22 Previous studies provided insights how SdeA targets and bridges Arg42 of Ub

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1 to serine residues of substrate proteins via a phosphoribosyl linker (Bhogaraju
2 et al., 2016; Qiu et al., 2016). To gain insight into the mechanism of activity
3 regulation of GRASP proteins by PR-ubiquitination, we used mass
4 spectrometry to identify modified residues on GRASP55 following *in vitro*
5 ubiquitination by SdeA (Fig. 5A). Four modified serine residues were identified
6 in GRASP55 (S3, S408, S409, S449) (Fig. 5B, Figure 5-figure supplement 1).
7 To further confirm these ubiquitination sites, we replaced seven serine residues
8 (GRASP55 7S*) by either threonine (S3, S4, S449, S451) or alanine (S408,
9 S409, S441). We observed that ubiquitination of GRASP55 in cells co-
10 expressing SdeA was markedly decreased when the serines were replaced,
11 compared with that of the wild-type GRASP55 (Fig. 5C). Similarly, we confirmed
12 that GRASP55 bearing the seven mutated serine residues can not be
13 ubiquitinated when cells were infected with wild-type or *ΔdupA/B Legionella*
14 strains (Fig. 5D).
15

16 **PR-ubiquitination disrupts GRASP interactions**

17 Studies have shown that GRASP proteins function in the maintenance of Golgi
18 structure through self-interaction and interactions with Golgi matrix proteins
19 (Jarvela and Linstedt, 2012; Rabouille and Linstedt, 2016). Their activity can be
20 regulated by post-translational modifications, for example, phosphorylation of
21 serines within GRASP proteins was shown to result in Golgi fragmentation
22 (Feinstein and Linstedt, 2008). We hypothesized that PR-ubiquitination of

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1 serines in GRASP proteins may affect self-interactions that are necessary for
2 keeping the stacked structure of the Golgi apparatus intact. To test this, we
3 firstly PR-ubiquitinated purified GRASP55-GFP *in vitro* and then subsequently
4 incubated the modified GRASP55 with purified His-tagged GRASP55. Co-IP
5 analyses showed that PR-ubiquitinated GRASP55 exhibited reduced self-
6 interaction compared to unmodified GRASP55 (Fig. 6A). This effect could also
7 be seen in cells when the HA-tagged wild type GRASP55 or GRASP55 7S*
8 serine mutant were co-expressed with GFP-tagged GRASP55 7S* in the
9 presence of SdeA. The capacity of PR-ubiquitinated wild type HA-GRASP55 to
10 self-interact with GFP-GRASP55 7S*, was decreased in comparison to SdeA
11 resistant HA-GRASP55 7S* (Fig. 6B). To analyze the functional impact of this
12 observation on cells, we expressed wild-type GRASP55 or the GRASP55 7S*
13 serine mutant in *GRASP55/GRASP65* KO HeLa cells, and then monitored the
14 structural stability of the Golgi in cells co-expressing SdeA. As previously shown,
15 double knockout of *GRASP55* and *GRASP65* induced fragmentation of the
16 Golgi (Bekier et al., 2017) (Figure 6-figure supplement 1). This phenotype could
17 be rescued by ectopic expression of either wild type GRASP55 or GRASP55
18 7S* (Figure 6-figure supplement 1), suggesting that serine mutations do not
19 interfere with the function of GRASP55. Fragmentation re-occurred when SdeA
20 was concomitantly expressed with GRASP55 (Fig. 6C, D). However, the Golgi
21 apparatus appeared less scattered when GRASP55 7S* was expressed,
22 indicating that the higher resistance of GRASP55 serine mutant to SdeA

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1 ubiquitination activity results in increased structural stability of the Golgi in cells
2 expressing SdeA (Fig. 6C, D). This data indicates that SdeA-caused Golgi
3 fragmentation is supposedly the result of the modification of GRASP proteins,
4 disturbing the tether between Golgi cisternae.
5

6 **Golgi fragments are not directly recruited to *Legionella* containing**
7 **vacuole**

8 Intracellular pathogens tend to create a membrane surrounded niche for
9 maturation, proliferation, and escape from defense mechanisms such as
10 selective autophagy within the host cell. Along this line, Chlamydia infection
11 induces Golgi fragmentation in order to generate Golgi ministacks for bacterial
12 inclusions (Heuer et al., 2009). As for *Legionella*, *Legionella* containing
13 vacuoles (LCVs) recruit ER membranes, thus converting the phagosome into a
14 specific compartment that has features of ER (Kotewicz et al., 2017; Shin et al.,
15 2020; Xu and Luo, 2013). We hypothesized that *Legionella* infection induces
16 Golgi fragmentation in order to facilitate the fusion of vesicles from the Golgi
17 with LCV to enhance the formation of LCV and, ultimately, intracellular
18 replication. To test this hypothesis, we infected HEK293T cells overexpressing
19 GRASP55 or trans-Golgi marker GalT. The immunostaining showed that
20 exogenous GRASP55 was recruited to LCV, however, our study recognizes the
21 fact that exogenously overexpressed GRASP55 and GalT were shown to be
22 partially localized in ER, which can be remodeled and recruited to LCV during

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1 infection. The recruited GRASP55 could very well be derived from the ER, and
2 not the fragmented Golgi (Fig. 7A, B). To address whether *Legionella* indeed
3 recruits fragmented Golgi cargo, we infected A549 cells with *Legionella*, stained
4 cells with antibodies against endogenous cis-Golgi protein GM130 or trans-
5 Golgi protein TGN46 and used microscopy to determine whether these Golgi
6 markers are recruited to LCV upon infection. Immunostaining results suggested
7 that neither cis-Golgi marker nor trans-Golgi accumulated on LCV (Fig. 7C, D).
8 These data suggest that against our initial hypothesis *Legionella* does not
9 induce Golgi fragmentation simply to recruit Golgi-derived vesicles for the
10 creation of LCVs, but that there must be another functional reasoning behind.
11

12 **Serine ubiquitination regulates secretory pathway in host cells**

13 In eukaryotic cells, the Golgi stack receives newly synthesized proteins from
14 the ER, proteins then undergo modifications before being sorted via the trans-
15 Golgi network. Vesicular stomatitis virus glycoprotein (VSVG) is a
16 transmembrane protein that has been widely used as a tool to monitor protein
17 trafficking through the secretory pathway (De Jong et al., 2006; Scidmore et al.,
18 1996). This reporter contains a thermoreversible mutation which causes its
19 misfolding and retention in the ER at 40 °C, while at the lower temperature of
20 32 °C, the protein folds correctly and is exported out of the ER to the plasma
21 membrane via the secretory pathway (Bergmann, 1989; Presley et al., 1997).
22 To access the functionality of the Golgi apparatus upon *Legionella* infection, we

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1 used a VSVG-GFP tracker protein to follow its transit through the Golgi, stained
2 with the Golgi marker GM130. Immunofluorescence analyses indicated that in
3 control A549 cells or cells infected with *Legionella* SidEs deletion strain, VSVG
4 reached its peak of accumulation in the Golgi after 20 min of incubation at 32 °C,
5 and the colocalization index then gradually decreased as the protein is
6 trafficked from the Golgi to secretory vesicles. This process was slower in cells
7 infected with wild type *Legionella* or *ΔdupA/B* mutant strain, where maximal
8 colocalization of VSVG with the GM130 occurred at a later time point and was
9 more prolonged, indicating lower efficiency of protein trafficking through the
10 secretory pathway (Fig. 8A, B). This was further confirmed by monitoring the
11 sensitivity of VSVG glycosylation to Endoglycosidase H (EndoH). EndoH is an
12 enzyme that removes mannose rich ER resident protein but not complex forms
13 of N-like oligosaccharides from glycoproteins that are present in Golgi or post
14 Golgi compartments. The transformation of a glycoprotein from EndoH
15 sensitive to EndoH resistant form has been widely used to monitor protein
16 trafficking through the Golgi (Burke et al., 1984; Ernst et al., 2018). To
17 specifically analyze the effect of PR-ubiquitination on VSVG trafficking through
18 the Golgi with EndoH cleavage, we infected HEK293T cells at 40 °C and
19 collected cells lysates at different time points after incubation at 32 °C, before
20 treating them with EndoH. Western blots showed that VSVG trafficking was
21 inhibited in cells infected with wild type *Legionella* or *Legionella* DupA/B
22 deletion strain, compared with control cells or cells infected with *Legionella*

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1 ΔsidE strain. In control cells or cells infected with *Legionella* SidE deletion strain,
2 the EndoH-resistant form of VSVG started to appear after 15 min incubation at
3 32 °C, and gradually accumulated over time, until almost all VSVG became
4 EndoH-resistant form after 120 min. In cells infected with wild type *Legionella*,
5 the EndoH resistant-form of VSVG increased rather slowly, only around ~50%
6 protein were converted to EndoH-resistant form at the same time point (Fig. 8C,
7 D). These data further demonstrate that PR-ubiquitination caused by SidE
8 effectors decelerates VSVG trafficking through the Golgi. This is further
9 confirmed with a VSVG assay in cells expressing SdeA (Figure 8-figure
10 supplement 1A, B). However, SdeA expression did not change the final
11 glycosylation of LAMP1 in cells, as no significant band shift was detected on
12 blot (Figure 8-figure supplement 1 C). This suggests that activity of SdeA slows
13 down trafficking through the Golgi but without completely inhibiting the function
14 of the Golgi.
15 As part of immune response, macrophage cells secret cytokines upon bacterial
16 infection. Since ER-to-Golgi route trafficking plays an important role in
17 conventional trafficking of most of the cytokines, and maintenance of Golgi
18 structure is critical for secretion of some cytokines, such as TNFα (Micaroni et
19 al., 2013), we decided to examine the effect of PR-ubiquitination on cytokine
20 secretion of macrophage cells upon *Legionella* infection. THP-1 cells were
21 treated with Phorbol 12-myristate 13-acxetate (PMA) to induce differentiation to
22 macrophage cells, then cells were infected with wild type or *Legionella* strains.

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1 Media were collected and filtered for ELISA. The ELISA results show that cells
2 infected with *Legionella* lacking SidE family effectors released more TNFα than
3 cells infected with wild-type or *ΔdupA/B Legionella* strains (Figure 8-figure
4 supplement 1D). Interleukin (IL)-1β is one of the cytokines secreted
5 independently of the conventional ER-Golgi trafficking. To address whether the
6 regulation of TNFα secretion by SdeA effector is specific, we then measured IL-
7 1β released with ELISA. Similar to TNFα, secretion of IL-1β by THP-1 cells
8 infected with *Legionella* lacking SidE family effectors was elevated, compared
9 to cells infected with wild-type or *ΔdupA/B Legionella* strains (Figure 8-figure
10 supplement 1E) indicating that both conventional and unconventional secretion
11 processes can be affected. As GRASP55 is involved in IL-1β unconventional
12 secretion (Chiritoiu et al., 2019), SidE effectors may regulate IL-1β release as
13 well, but possibly by a different avenue than TNFα secretion. Taken together,
14 these data demonstrate that Golgi disruption caused by SidE effectors impairs
15 protein secretory pathways.
16
17 **Discussion**
18 To date, considerable effort has been focused on investigating the mechanism
19 and substrates of novel PR-ubiquitination catalyzed by SidE family of *Legionella*
20 effectors. However, the functional consequences of PR-ubiquitination in the
21 regulation of cellular processes has been poorly understood. In this study, we
22 investigated the modification of Golgi proteins catalyzed by SidE effectors and

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1 explored the consequences of PR-ubiquitination in regulating Golgi morphology
2 and secretory pathway.

3 The Golgi protein GRASP55 was identified as one of the most enriched
4 candidates among all PR-Ub modified substrate proteins using mass-
5 spectrometry (Shin et al., 2020). By conducting *in vitro* reactions and MS-based
6 analysis, we identified several serine residues in GRASP55 potentially modified
7 by SdeA. Validation of the identified as well as adjacent serine by mutagenesis
8 markedly suppressed the PR-ubiquitination of GRASP55 both in cells
9 expressing SdeA or infected with *Legionella*. Notably, mutation of these serines
10 did not completely abolish the ubiquitination signal from purified GRASP55,
11 suggesting that alternative residues in GRASP55 could also be modified by
12 SdeA. This finding is consistent to other known substrates like Rab33b, in which
13 S154 has been identified as a ubiquitination site for SdeA, yet S154A mutation
14 does not abrogate ubiquitination (Bhogaraju et al., 2016). SdeA appears to
15 modify substrate serine sites independent of specific structural motifs and
16 serines in the flexible regions are prone to modification as shown for Rab
17 proteins (Wang et al., 2018).

18 Immunoblotting analyses of GRASP55 purified from cells either expressing
19 SdeA or infected with *Legionella* revealed that PR-ubiquitinated GRASP55 is
20 detected as high molecular weight smear under long exposure. This is likely
21 due to the multi-ubiquitination event taking place on several serines of
22 GRASP55, besides the identified preferred serines by SdeA. This hypothesis is

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1 supported by the observation that incubation with deubiquitinase DupA
2 eliminated the high molecular bands from ubiquitinated GRASP proteins
3 (Figure 4-figure supplement 1).
4 GRASP proteins contain a conserved N-terminal GRASP domain that is used
5 to localize the proteins to the Golgi as well as to tether other GRASP proteins
6 through trans-dimerization, which is regulated through phosphorylation of the
7 C-terminal serine and proline rich (SPR) domain by mitotic kinases (Feinstein
8 and Linstedt, 2008; Wang et al., 2005). Several serines in this C-terminal region
9 of GRASP55 including S408, S409, S441, S449 identified to be PR-
10 ubiquitinated in this study, have also been reported to be phosphorylated in
11 previous studies (Bian et al., 2014; Kim et al., 2016). Phosphorylation mimics
12 at these sites disrupt the homodimerization of GRASP, possibly through protein
13 conformational changes (Kim et al., 2016; Truschel et al., 2012). We show that
14 PR-ubiquitination of GRASP55 also affects its homodimerization. We noticed
15 that self-interaction was diminished when GRASP55 was PR-ubiquitinated by
16 SdeA, both in *vitro* and *in vivo*. This disruption of GRASP protein
17 homodimerization by PR-ubiquitination may lead to unlinking of Golgi ribbon
18 and unstacking of Golgi cisternae. Thus, these data provide new insights into
19 the mechanism by which PR-ubiquitination by *Legionella* induces Golgi
20 fragmentation.
21 Many pathogens have been characterized to require host organelles for their
22 own intracellular survival and proliferation. As for Legionella, numerous host

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1 proteins have been detected on the LCVs. Of note, PI4P decoration on LCV,
2 which functions to recruit bacterial effectors during infection, was shown to be
3 derived directly from the Golgi body of host cells (Weber et al., 2018). This
4 propelled us to address the question whether *Legionella* recruits Golgi
5 component from fragmented Golgi. Our results indicate that *Legionella* does
6 not directly recruit Golgi membrane pools containing endogenous GRASP55 or
7 GRASP65, as these substrate proteins were not detected on the LCVs. Our
8 data suggest that *Legionella* effectors fragment the Golgi but are not involved
9 in the recruitment of Golgi components. This is in agreement with earlier studies,
10 in which LCVs were purified from infected host cells and analyzed using
11 proteomics approach, but Golgi proteins were rarely identified (Schmölders et
12 al., 2017; Urwyler et al., 2009). During our ongoing study and preparation of
13 this manuscript, Wan and colleagues reported as well Golgi fragmentation upon
14 Legionella infection and the PR-ubiquitination of GRASP55. In their study it
15 showed that GRASP55 was recruited to LCV upon *Legionella* infection (Wan et
16 al., 2019). However, it should be noted that, unlike the endogenous GRASP55
17 protein that mainly localizes to the Golgi apparatus, overexpressed GRASP55
18 in their study was detected as largely localized to ER that could be recruited to
19 LCV. The recruited GRASP55 could very well be derived from the ER, but not
20 the fragmented Golgi. Recently, a study reported that PI(4)P-containing
21 vesicles derived from Golgi are involved in mitochondria division (Nagashima
22 et al., 2020). Given the fact that mitochondrial dynamics is tightly modulated

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1 during *Legionella* infection (Escoll et al., 2017), it is possible that *Legionella*
2 SdeA affects mitochondria fission to facilitate bacterial replication. Further
3 efforts will be needed to address the effect of PR-ubiquitination mediated Golgi
4 fragmentation on mitochondria.

5 The Golgi apparatus plays a central role in the secretory pathway. Using VSVG
6 as a marker, we were able to dissect the effect of SidE mediated PR-
7 ubiquitination on protein trafficking. Our findings provide insights into the
8 functional roles of Golgi substrate PR-ubiquitination and subsequent Golgi
9 fragmentation which impacts Golgi-associated protein secretory pathway. We
10 have shown that PR-ubiquitination decelerates VSVG trafficking through the
11 Golgi using microscopy and EndoH digestion assay. Moreover, we have found
12 that secretion of cytokine TNFα was increased for THP-1 cells infected with
13 *Legionella* lacking SidE family effectors, compared with cells infected with wild
14 type *Legionella*. The opposite effect was observed in infection with *Legionella*
15 missing DupA/B. This finding is consistent with previous study showing that
16 SdeA expression inhibits secretion of secreted embryonic alkaline phosphatase
17 reporter (SEAP) (Qiu et al., 2016).

18 Unconventional secretion of IL-1β has been shown as dependent on GRASP55
19 and GRASP65 (Chiritoiu et al., 2019). Based on the findings from a study using
20 *in vivo* GRASP55/66 depletion, Grond *et al*. proposed that, instead of stacking
21 the Golgi cisternae core, GRASP proteins function in linking of the rims of Golgi
22 cisternae, which might be the source of membranes required for unconventional

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1 secretion (Grond, R., et al., 2020). It is possible that PR-ubiquitination prohibits
2 GRASP protein oligomerization, leads to unlinking of Golgi ribbon and
3 vesiculation of Golgi cisternae rims, thus resulting in dispersed Golgi and
4 inhibition of unconventional secretion.
5 Notably, multiple *Legionella* effectors have been suggested to regulate
6 secretory pathways by yet unclear mechanisms (Machner and Isberg, 2006;
7 Nagai et al., 2002). Identification of effectors involved in the regulation of the
8 host secretory pathways will help us better understand both the bacterial
9 pathogen and host cellular processes involved in infection, and thus further
10 studies are needed.
11 Taken together, our study demonstrates that SdeA targets the Golgi and
12 ubiquitinates Golgi tethering proteins GRASP55 and GRASP65, resulting in
13 Golgi fragmentation and inhibition of secretory pathway. By revealing the
14 biological consequences of PR-ubiquitination on Golgi proteins, our study
15 provides a Golgi manipulation strategy, which *Legionella* utilizes to benefit
16 bacterial infection and replication in host cells. It will be interesting to study
17 whether PR-ubiquitination confers additional versatile mechanisms to facilitate
18 bacterial infection by verifying more substrates of SidE effectors in future.
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1 **Materials and Methods**

2

3 **Antibodies and reagents**

4 All reagents were from Sigma, Roche or Roth. The following antibodies were
5 used: antibodies against HA (C29F4), GFP (sc-9996), GRASP65 (sc-374423),
6 from Santa Cruz; ubiquitin (P4BD) and ubiquitin (ab7254) from Cell signaling
7 and Abcam respectively; mCherry (ab125096), Tubulin (ab6046), Calnexin
8 (ab22595), *Legionella* (ab20943) from abcam; GAPDH (D16H11) from Cell
9 signaling; GM130 (610823) from BD; GRASP55 (10598-1-AP) from proteintech,
10 TGN46 (ab50595) from Biorad. Monoclonal Anti-HA−Agarose antibody (HA-7)
11 was purchased from Sigma.

12

13 **Cloning and mutagenesis**

14 For protein expression in mammalian cells, GFP or mCherry tagged DupA, wild
15 type EGFP-SdeA and catalytically defective mutants SdeA H277A and SdeA
16 EEAA were generated as described previously (Sagar Bhogaraju et al., 2016).
17 SdeA plasmids were digested with BamHI and XhoI, then inserted into
18 mCherry-C1 vectors digested with BamHI and XhoI to generate N terminally
19 mCherry tagged wild type and mutated SdeA. Deletion of SdeA was designed
20 according to the known structure and sequence prediction analyses. Truncated
21 deletions $SdeA^{1\text{-}972}$ and $SdeA^{909\text{-}C}$ were amplified from full-length SdeA cDNA
22 and digested with BamHI and XhoI. The digested DNA fragments were inserted

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1 into pEGFP-C1 vectors digested with BamHI and XhoI. GFP or HA tagged
2 GRASP55 and GRASP65-GFP were generated by PCR from GRASP55 or
3 GRASP65 cDNA and digested with XhoI and BamHI or HindIII and KpnI
4 respectively, then inserted into pEGFP-N1 or pHA-N1 vector. For generation of
5 the GRASP55 7S* mutant, identified serines and adjacent serines S3, S4, S449,
6 S451 were mutated to threonine to minimally effect the physio-chemical
7 properties of these amino acids, in addition, S408, S409, S441 were mutated
8 to alanine by site-directed mutagenesis. For protein expression in *E. coli*, SdeA
9 was amplified from SdeA cDNA and digested with BamHI and XhoI. The
10 digested DNA fragments were inserted into pGEX-6p-1 vector digested with
11 BamHI and XhoI. GRASP55 and GRASP65 cDNA were amplified from
12 mammalian vector and digested with NdeI and BamHI and cloned into pET15b
13 and pGEX-6p-1 vector respectively. Serine to threonine or alanine mutations
14 were generated by site-directed mutagenesis.
15
16 **Cell lines culture and Transfection**
17 HEK293T, A549, COS7 cells were purchased from ATCC. Cells were cultured
18 in high glucose Dulbecco's Modified Eagles Medium (DMEM) supplemented
19 with 10% fetal bovine serum (FBS), 100 U/mL penicilin and 100 mg/mL
20 streptomycin at 37 °C, 5% CO_2 in a humidified incubator. Transfection was
21 performed using polyethyleneimine (PEI) reagent or Genejuice (Merck).
22

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1 **_Legionella_ culture and infection**

2 *Legionella* strains were obtained from Dr. Zhao-Qing Luo lab (Purdue
3 University). Cells were streaked and cultured at 37°C on N-(2-acetamido)-2-
4 aminoethanesulfonic acid (ACES)-buffered charcoal-yeast extract (BCYE) agar
5 plates for 3 days, followed by inoculation and growth for 20 h in 3 mL CYE liquid
6 media. Post-exponential *Legionella* with OD_{600} between 3.6-3.8 were used to
7 infect A549 or HEK293T cells. HEK293T cells were transfected with FCγRII and
8 GRASP55-GFP or GRASP65-GFP for 24 hrs. Indicated *Legionella* strains were
9 opsonized with antibody against *Legionella* (1: 500) at 37 °C for 30 min before
10 infection. The HEK293T cells were infected with different *Legionella* strains at
11 an MOI of 2 (for confocal imaging), or 10 (for Western blot) for the indicated
12 time.
13

14 **SdeA mediated PR-ubiquitination reaction**

15 SdeA mediated PR-Serine ubiquitination *in vitro* reaction was done as
16 previously described (Kalayil et al., 2018). Briefly, 5 μM GRASP proteins were
17 incubated with 1 μM of SdeA and 25 of μM ubiquitin in the presence of 200 μM
18 of NAD^+ in 40 μL of reaction buffer (50 mM NaCl and 50 mM Tris, pH 7.5) for 1
19 hour at 37 °C. Deubiquitination assay were performed by incubating PR-
20 ubiquitinated proteins with 1 μg of GST-DupA at 37 °C for 1 h in reaction buffer
21 (150 mM NaCl, 50 mM Tris-HCl pH 7.5). The reaction products were analyzed
22 by SDS-PAGE with Coomassie staining or western blotting using antibodies

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against GST (cell signaling technology), His (Roche), GRASP55 (Proteintech), GRASP65 (Sino biotech.), Ub (Abcam, or Cell signaling technology). To assess the PR-ubiquitination of GRASP55 and GRASP65 in cells, plasmids for expression of GRASPs-GFP, GFP-SdeA or mCherry-SdeA, were co-transfected into HEK293T cells, cells were then cultured at 37 °C for 24 h. Whole cell lysates were subjected to immunoprecipitation with GFP-trap beads and the products or the whole cell lysates were separated with SDS-PAGE and blotted with antibodies against GFP or GRASP proteins.

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) at 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

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1 (Sigma Aldrich), mixed with 10 µL GFP-trap or HA antibody conjugated agarose,
2 and incubated for 4 h in cold room with end to end rotation. Beads were washed
3 3 times in IP buffer containing 500 mM NaCl. Proteins were eluted by
4 resuspending with 2X SDS sample buffer followed by boiling for 5 min at 95 °C.
5 Samples were then submitted to western blotting analysis.
6
7 **Protein expression and purification**
8 GRASP55 and GRASP65 cDNA were cloned into p15b and pGEX-6p-1 vector
9 respectively. Full length SdeA was cloned into pGEX-6P-1 vector. E.coli
10 competent cells (NEB T7 express) were transformed with plasmid, colonies
11 were inoculated and cultured in LB medium overnight at 37 °C, The next day 5
12 mL culture was transferred to 1 L flask for further culture at 37 °C until the OD_{600}
13 reaches to 0.6-0.8. Protein expression was induced by adding 0.5 mM IPTG
14 and cells were further cultured overnight at 18 °C. The cells were harvested
15 and the cell pellet was resuspended in lysis buffer (300 mM NaCl, 50 mM Tris-
16 HCl pH 7.5) followed with sonication and centrifuged at 13,000 rpm to clarify
17 the supernatant. Clarified lysates were then incubated with TALON beads or
18 glutathione-S-Sepharose pre-equilibrated with washing buffer. Once eluted,
19 proteins were further concentrated with filters and then purified by anion
20 exchange chromatography on HitrapQ (GE Healthcare) and collected fractions
21 were further loaded onto size exclusion column (Superdex 75 16/60, GE
22 Healthcare). Proteins were concentrated and used for *in vitro* reaction.

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1

2 **Identification of PR-ubiquitination serine sites on GRASP55**

3 His-GRASP55 were purified from *E. coli* and PR-ubiquitinated by SdeA *in vitro*.
4 Samples were prepared as previously described (Bhogaraju et al., 2016; Kalayil
5 et al., 2018). Briefly, added urea buffer containing 8 M urea, 0.1 M Tris, pH 7.5
6 to the reaction mixture to a final volume of 200 μL, the reactions were then
7 transferred to 30 kDa filter (Amicon Ultra, 0.5 mL, Merck) and washed 3 times
8 with 200 μL of urea buffer by centrifugation to remove free ubiquitin. Proteins
9 were washed 2 times with 50 mM ABC, pH 7.5, then digested with trypsin in 50
10 mM ABC pH 7.5 at trypsin to protein ratio 1:50 for 6 h and subsequently
11 desalted by C18 and analyzed by LC MS/MS.

12

13 **Data quantification**

14 Data shown in Figure 2B, 2D, 6D, 8B, 8D and Figure 8-figure supplement 1D,
15 E were analyzed with GraphPad Prism 5.0. Three independent experiments
16 were performed, p values were determined using unpaired two-tailed t test, ***,
17 **, * and ns represent $p<0.0001$, $p<0.01$, $p<0.05$ and not significant respectively.
18 For Figure 2B and 2D, more than 70 SdeA transfected or *Legionella* infected
19 cells were examined from 3 replicates of each condition. Values of percentage
20 of cells with fragmented Golgi were input into GraphPad Prism, and analyzed.
21 For Figure 6D, Golgi areas of more than 60 cells from 3 replicates of each
22 condition were measured with ImageJ software. For Figure 8B, data represents

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1 30 cells taken from 3 independent experiments. For Figure 8D, gray values of
2 VSVG bands shown as Figure 8C from 3 replicates were measured with ImageJ.
3
4 **VSVG trafficking assay**
5 HEK293T or A549 cells were co-transfected with VSVG-GFP and FcγRII or
6 transfected with VSVG-GFP respectively and cultured at 37 °C for 24 h to
7 express the proteins before being transferred to 40 °C. After 16 h incubation at
8 40 °C, cycloheximide was added into medium to inhibit further protein synthesis,
9 after 2 h treatment cells were infected with *Legionella* for another 2 h then
10 washed 3 times with PBS and cultured with fresh medium at 32 °C to remove
11 the bacteria outside host cells, and then moved to 32 °C for different time points
12 to release VSVG from ER. A549 cells were fixed and VSVG trafficking was
13 acquired with confocal microscopy after immunofluorescence staining. DAPI
14 marks nucleus and cytosolic bacteria. For calculating Manders coefficient in
15 FIJI, ROIs of 30 μm^2 are chosen from the perinuclear region containing the
16 Golgi marked by GM130. Manders coefficient is calculated using Coloc2 plugin
17 in FIJI and denotes fraction of VSVG-GFP pixels that is positive for GM130. For
18 EndoH cleavage assay, HEK293T cells were lysed with lysis buffer containing
19 1% SDS, 50mM Tris, pH 8.0. Benzonase was added to reduce the viscosity
20 caused by released DNA. Cell lysates were mixed with denaturing buffer then
21 boiled for 10 min at 95 °C. Denatured proteins were incubated with EndoH for
22 3 h at 37 °C to cleave the EndoH sensitive form of glycosylation, final products

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1 were separated with SDS-PAGE and the EndoH-caused band shift was
2 analyzed by blotting GFP.
3
4 **ELISA**

5 To investigate the effect of PR-ubiquitination on secretion pathway, we
6 analyzed the secretion of pro-inflammatory cytokines in PMA-treated THP-1
7 cells upon infection with *L. pneumophila*. Cytokine secretion analyses were
8 performed with ELISA kits ordered from R&D system (IL-1β: DY201-05; TNFα:
9 DY210-05) according to the manufacturer's instructions.
10
11 **Immunofluorescence**

12 HEK293T, COS7 or A549 cells were seeded on a coverslip in 12-well plates
13 and cultured in CO_2 incubator. Next day cells were transfected with plasmids
14 encoding SdeA. The immunostaining was performed following the protocol
15 previously described (Bhogaraju et al., 2016). Briefly, cells were washed once
16 with PBS, pH 7.4, and fixed with 4% paraformaldehyde (PFA) in PBS for 10 min
17 at room temperature. Cells were washed again with PBS 2 times, then
18 permeabilized with 0.1% saponin in PBS for 10 min, and blocked with blocking
19 buffer containing 0.1% saponin and 2% BSA in PBS for 1 h at room temperature.
20 Cells were stained with antibodies diluted in blocking buffer overnight at 4 °C
21 and washed with PBS three times next day. Cells were further incubated with
22 Alexa Flour dyes-conjugated secondary antibodies for 1 h at room temperature

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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.

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14

15 **Author contributions**

16 Y.L. and I.D. designed the study and experiments. R.M. performed the VSVG
17 trafficking experiment. F.B., T.C. and I.M. performed mass spectrometry
18 experiments and data-analysis. Y.L. performed biochemical, cell biological and
19 bacterial infection experiments and data-analysis. Y.L. and I.D. wrote the
20 manuscript and all authors commented on it.

21

22

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Figure legends

Figure 1. SdeA partially localizes to the Golgi

(**A**) Schematic diagrams of full-length wild-type SdeA, SdeA catalytic mutant SdeA H277A or SdeA EE/AA, truncated SdeA$^{1-972}$ and SdeA^{909-C}. (**B**) Confocal images showing the co-localization of SdeA (green) with ER protein Calnexin (red). COS7 cells were transfected with plasmids encoding GFP-tagged wild type SdeA or truncated mutant. Cells were cultured for 24 hours after transfection, then fixed, permeabilized, and stained with Calnexin antibody and visualized using confocal microscope. (**C**) Confocal images showing the co-localization of SdeA (green) with Golgi protein GM130 (red). Cells were cultured for 24 hours after transfection, then fixed, permeabilized, and ultimately stained with GM130 antibody and visualized using confocal microscope.

Figure 2. *Legionella* effector SdeA mediates Golgi fragmentation in cells.

(**A**) Confocal images showing Golgi (red) fragmentation caused by exogenously expressed SdeA (green). GFP-tagged SdeA wild-type or catalytically defective mutants were expressed or co-expressed with DupA in COS7 cells. Cells were cultured for 24 hours after transfection then fixed with 4% PFA. (**B**) Quantification of the percentage of cells with dispersed Golgi in (**A**). Data are shown as means ± SEM of more than 60 cells taken from three independent experiments. ***P<0.001. (**C**) Confocal images showing Golgi fragmentation caused by *Legionella*. A549 cells were infected with wild-type or mutant *Legionella* as indicated. Cells were washed 3 times with PBS after 2 hours infection to remove non-phagocytosed bacteria, then fixed with 4% PFA and stained with indicated antibodies. (**D**) Quantification of the percentage of cells with dispersed Golgi in (**C**) Data are shown as means ± SEM of more than 70 cells taken from three independent experiments. Data were analyzed with unpaired t test, ***P<0.001, *P<0.01, *P<0.05.

Figure 2-figure supplement 1

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1 (**A**) Western blot analysis of modification of Golgi protein substrate by wild-type GFP-tagged
2 SdeA or SdeA$^{1-972}$ missing membrane targeting region. HEK293T cells were transfected with
3 full-length or truncated SdeA, cells were lysed and blotted after 24 hours transfection. (**B**)
4 Confocal images showing SdeA expression fragments the Golgi in HeLa cells. GFP-tagged
5 SdeA wild type or catalytically defective mutants were expressed in HeLa cells. Cells were
6 cultured for 24 hours after transfection then fixed with 4% PFA, after permeabilization, cells
7 were stained with antibodies against cis-Golgi and trans-Golgi markers GM130 and TGN46.
8
9 **Figure 3. SdeA ubiquitinates Golgi tethering factor GRASP proteins**
10 (**A**) Potential ER and Golgi protein substrates of SdeA identified by mass spectrometry. Values
11 indicate intensity ratios between proteins enriched from samples infected with different
12 *Legionella* strains (*ΔdupA/B* over *ΔsidE*). Among the substrate candidates, Golgi tethering
13 factor GRASP55 (red) is one of the highly ubiquitinated proteins. (**B**) GRASP55 and GRASP65
14 ubiquitination by SdeA *in vitro*. Purified GRASP55 or GRASP65 were incubated with SdeA in
15 the presence of NAD^+ and ubiquitin. Reaction products were separated with SDS-PAGE and
16 then stained with Coomassie blue or blotted with antibodies against ubiquitin, GRASP55 or
17 GRASP65. (**C**) Modification of GRASP55 by exogenous SdeA in cells. HEK293T cells were
18 transfected with wild type SdeA or indicated SdeA mutants, after 24 hours cells were collected
19 and lysed, the total cell lysates were separated with SDS-PAGE and blotted with antibody
20 against GRASP55. (**D**) Modification of GRASP65 by exogenous SdeA in cells. HEK293T cells
21 were transfected with wild type SdeA or indicated SdeA mutants, after 24 hours cells were
22 collected and lysed, GFP-tagged GRASP65 proteins were isolated from cell lysate and
23 separated with SDS-PAGE followed by blotting with antibody against GFP.
24
25 **Figure 3-figure supplement 1. SdeA ubiquitinates Golgi tethering proteins**
26 (**A**) Confocal images showing Golgi localization of endogenous GRASP55 and GRASP65. (**B**)
27 GCP60 ubiquitination by SdeA *in vitro*. Purified Myc-tagged GCP60 was incubated with SdeA
28 in the present of NAD^+ and ubiquitin. Reaction products were blotted with antibodies against
29 ubiquitin or Myc. (**C**) Modification of GCP60 by exogenous SdeA in cells. HEK293T cells were
30 co-transfected with GCP60 and wild type SdeA or SdeA mutant, after 24 hours cells were

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1 collected and lysed, followed with Myc-IP. IP products were washed and separated with SDS-
2 PAGE and blotted with antibody. (**D**) Modification of Golgi substrate GRASP55 by wild-type
3 SdeA or SdeA$^{1\text{-}972}$ missing membrane targeting region. (**E**) *In vitro* reaction of wild-type or
4 ML/AA mutant with purified GRASP55. Reaction products were separated with SDS-PAGE and
5 then stained with Coomassie blue. Ubiquitinated GRASP55 bands were indicated. (**F**) Analysis
6 of the effect of SdeA ML/AA mutant on PR-ubiquitination of GRASP55 in cells. (**G**) Assay of
7 protein interaction between SdeA and GRASP55. HEK293T cells were transfected with GFP-
8 tagged wild-type SdeA or SdeA ML/AA mutant, after 24 hours cells were lysed and GFP fusion
9 proteins were isolated with GFP-trap beads. Then the pulled-down proteins were separated
10 with SDS-PAGE and blotted with antibody against GRASP55.

11

12 **Figure 4. *Legionella* infection causes ubiquitination of GRASP proteins, which**
13 **is dependent on SidE effectors.**

14 (**A**) Ubiquitination assay of GRASP55-GFP purified from HEK293T cells infected with
15 *Legionella* strains. HEK293T cells were seeded in 6-well plate and co-transfected with plasmids
16 encoding C-terminally GFP tagged GRASP55 and FcγRII then were infected for indicated times
17 with *Legionella* bacteria opsonized by *Legionella* antibody. Cells were lysed with IP lysis buffer
18 and purified GRASP55 proteins were separated by SDS-PAGE followed by blotting using anti-
19 GFP and anti-ubiquitin antibodies. (**B**) Ubiquitination assay of GRASP65-GFP purified from
20 HEK293T cells infected by *Legionella* wild type *or* Δ*dupA/B* mutant. (**C**) Ubiquitination assay of
21 GRASP55-GFP purified from HEK293T cells infected with *Legionella* wild-type, Δ*sidEs or*
22 Δ*dupA/B* strains. (**D**) Ubiquitination assay of GRASP65-GFP purified from HEK293T cells
23 infected with *Legionella* wild-type, Δ*sidEs or* Δ*dupA/B* strains.

24

25 **Figure 4-figure supplement 1. *Legionella* infection causes ubiquitination of**
26 **Golgi proteins, which is dependent of SidE family proteins**

27 (**A**) Ubiquitination assay of GCP60-Myc purified from HEK293T cells infected with
28 *Legionella* strains. (**B**) Cleavage assay of PR-ubiquitination of GRASP55 with DupA. (**C**)
29 Cleavage assay of PR-ubiquitination of GRASP65 with DupA.

30

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1 **Figure 5. Identification of GRASP55 ubiquitination with mass spectrometry**

2 (**A**) *In vitro* reaction of GRASP55 ubiquitination by SdeA for mass spectrometry analyses. 20 μg
3 purified GRASP55 was incubated with SdeA and ubiquitin in the present of NAD. 10% reaction
4 products were separated with SDS-PAGE and then stained with Coomassie blue or blotted with
5 with antibodies against ubiquitin, GRASP55 to check the ubiquitination, the rest samples were
6 subjected to mass spectrometry analyses. (**B**) Spectrum of GRASP S408-ubiquitin cross-linked
7 peptide. (**C**) Validation of of ubiquitination sites in GRASP55. C-terminally HA-tagged wild-
8 type and GRASP55 mutant were co-expressed with SdeA in HEK293T cells. After 24 h the
9 cells were lysed for HA immunoprecipitation. Purified GRASP55-HA proteins were separated
10 with SDS-PAGE followed by blotting using anti-HA and anti-ubiquitin antibodies. (**D**)
11 Ubiquitination assay of wild type GRASP55 and mutant in cells infected with *Legionella*.

12

13 **Figure 5-figure supplement 1. High resolution ETD spectrum of ubiquitin cross**

14 (**A**) High resolution ETD spectrum of ubiquitin cross linked Serine 3 of GRASP55. (**B**) High
15 resolution ETD spectrum of ubiquitin cross linked Serine 408 of GRASP55. (**C**) High
16 resolution ETD spectrum of ubiquitin cross linked Serine 409 of GRASP55. (**D**) High
17 resolution ETD spectrum of ubiquitin cross linked Serine 449 of GRASP55.

18

19

20 **Figure 6. Serine ubiquitination impairs GRASP55 function**

21 (**A**) Assay of the effect of PR-ubiquitination on GRASP55 dimerization *in vitro*. GRASP55-GFP
22 purified from HEK293T cells were modified *in vitro* using SdeA and ubiquitin in the presence of
23 NAD^+, ubiquitinated GRASP55-GFP was then incubated with purified His-tagged GRASP55.
24 Interaction between differently tagged GRASP55 proteins were analyzed with co-
25 immunoprecipitation followed with western blotting. (**B**) Assay of the effect of PR-ubiquitination
26 on GRASP55 dimerization *in vivo*. HA-tagged GRASP55 and GFP-tagged GRASP55 serine
27 mutant were co-expressed with SdeA in HEK293T cells. Protein interaction between differently
28 tagged GRASP55 were analyzed with co-IP and western blotting. (**C**) Confocal images showing
29 that GRASP55 mutant is resistant to Golgi fragmentation caused by SdeA expression. Golgi
30 areas of more than 60 cells from 3 replicates of each condition were measured with ImageJ

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1 software. (**D**) Data are shown as means ± SEM of more than 70 cells taken from three
2 independent experiments. Data were analyzed with unpaired t test, ***P<0.001.
3
4 **Figure 6-figure supplement 1**
5 (**A**) Confocal images showing exogenously expressed wild-type GRASP55-HA and mutant
6 rescue Golgi fragmentation caused by GRASP55/GRASP65 knockout. (**B**) Western blotting of
7 cell lysates from wild-type and G55/G65 knockout HeLa cell lines. Knockout of G55 and G65
8 were validated with antibodies against G55, G65 respectively.
9
10 **Figure 7. *Legionella* does not recruit fragmented Golgi**
11 (**A**) Confocal images showing *Legionella* recruits overexpressed Golgi protein GRASP55.
12 HEK293T cells transfected with plasmids encoding FCγRII and GFP-tagged GRASP55 were
13 infected with indicated *Legionella* strains. Cells were washed 3 times with PBS after 2 hours
14 infection to remove un-endocytosed bacteria, then fixed with 4% PFA and stained with antibody
15 against *Legionella.* (**B**) Confocal images showing *Legionella* recruits overexpressed Golgi
16 marker GalT. (**C**) Confocal images showing *Legionella* does not recruit endogenous cis-Golgi
17 protein GM130 or trans-Golgi protein TGN46. A549 cells were infected with Legionella
18 expressing dsRed and stained with antibodies against GM130 and TGN46.(**D**) Confocal images
19 showing *Legionella* does not recruit endogenous cis-Golgi protein GRASP65 or trans-Golgi
20 protein GRASP55. A549 cells were infected with Legionella expressing dsRed and stained with
21 antibodies against GRASP65 and GRASP55.
22
23 **Figure 8. SdeA induced serine ubiquitination inhibits VSVG trafficking through**
24 **Golgi membranes.**
25 (**A**) Confocal images showing the effect of SidE family effectors on VSVG trafficking during
26 *Legionella* infection. (**B**) Quantitative analysis of the effect of SidE family effectors on VSVG
27 trafficking during *Legionella* infection. Co-localization between VSVG and GM130 was shown
28 as Manders coefficient. Data represents 30 cells taken from 3 independent experiments. White
29 boxes indicate insets which are split into red, green, blue channels and displayed on the right
30 side of the image. Center lines show the medians; box limits indicate the 25th and 75th

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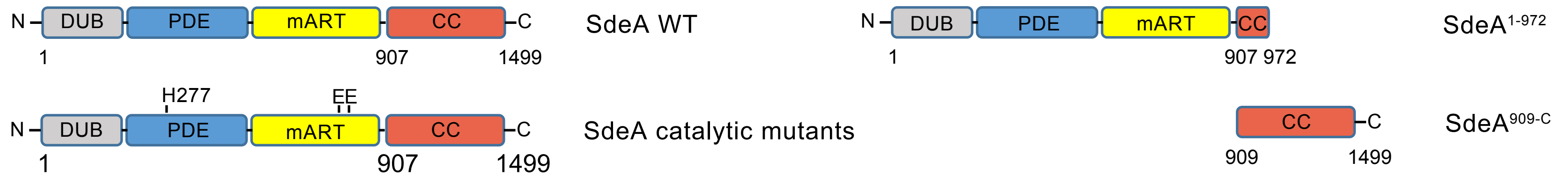
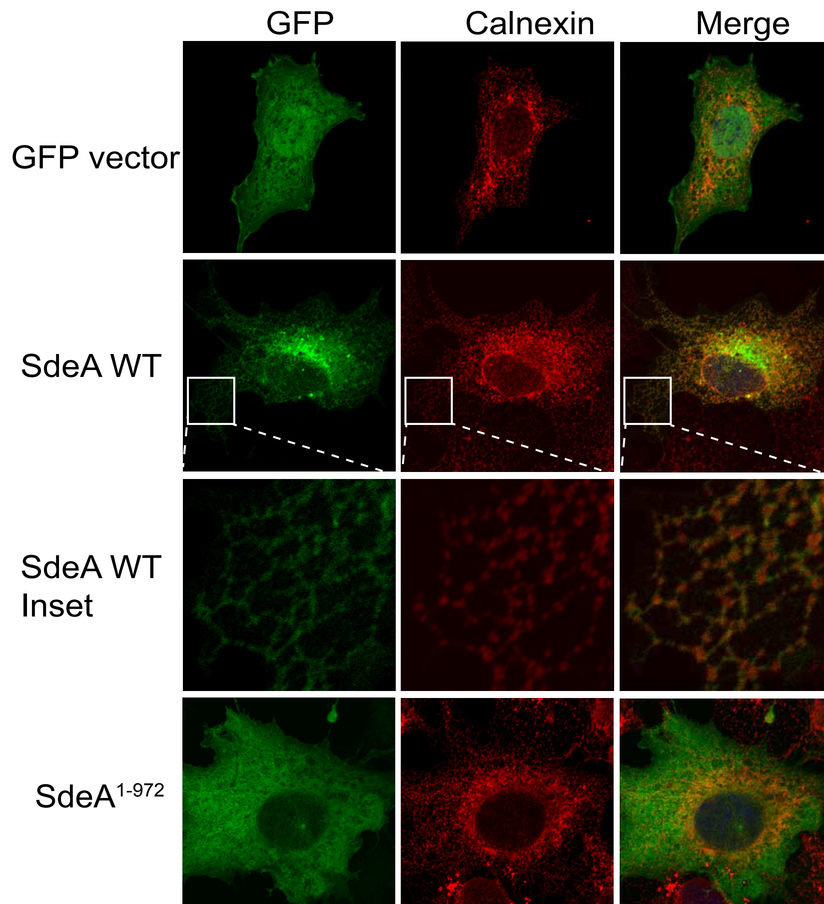
1 percentiles as determined by R software; whiskers extend 1.5 times the interquartile range from
2 the 25th and 75th percentiles, outliers are represented by dots; data points are plotted as circles.
3 (**C**) Western blotting analysis of the effect of SidE family effectors on VSVG trafficking during
4 *Legionella* infection using EndoH. Upper bands indicate the EndoH resistant form and lower
5 bands indicate the EndoH sensitive form of VSVG. (**D**) Quantification of (**C**) to indicate the effect
6 of *Legionella* infection on the conversion of EndoH sensitive form to resistant form of VSVG
7 upon 32 °C incubation. Data were analyzed with unpaired t test, ***P<0.001, *P<0.01, *P<0.05.
8

9 **Figure 8-figure supplement 1. SdeA expression in cells impairs protein**
10 **trafficking**

11 (**A**) Analysis of VSVG trafficking in HEK293T cells expressing SdeA. Cells grown in 40 °C were
12 moved to 32 °C for indicated time points. (**B**) Analysis of VSVG trafficking in HEK293T cells
13 expressing SdeA using EndoH. Cells grown in 40 °C were moved to 32 °C for indicated time
14 points. Cell lysates were probed with GFP antibody. (**C**) Analysis of the effect of SdeA
15 expression on electrophoretic mobility of the Golgi protein LAMP1. Lysates from cells
16 expressing SdeA WT or SdeA EEAA mutant were probed with LAMP1 antibody. (**D**) ELISA
17 assay of TNFα secreted from THP-1 cells infected with *Legionella* strains. Data are shown as
18 means ± SEM of cytokine values of three independent experiments. Data were analyzed with
19 unpaired t test, *P<0.01, *P<0.05. (**E**) ELISA assay of IL-1β secreted from THP-1 cells infected
20 with *Legionella* strains. Data are shown as means ± SEM of cytokine values of three
21 independent experiments. Data were analyzed with unpaired t test, ***P<0.001, *P<0.01,
22 *P<0.05.

Figure 1. SdeA partially localizes to the Golgi





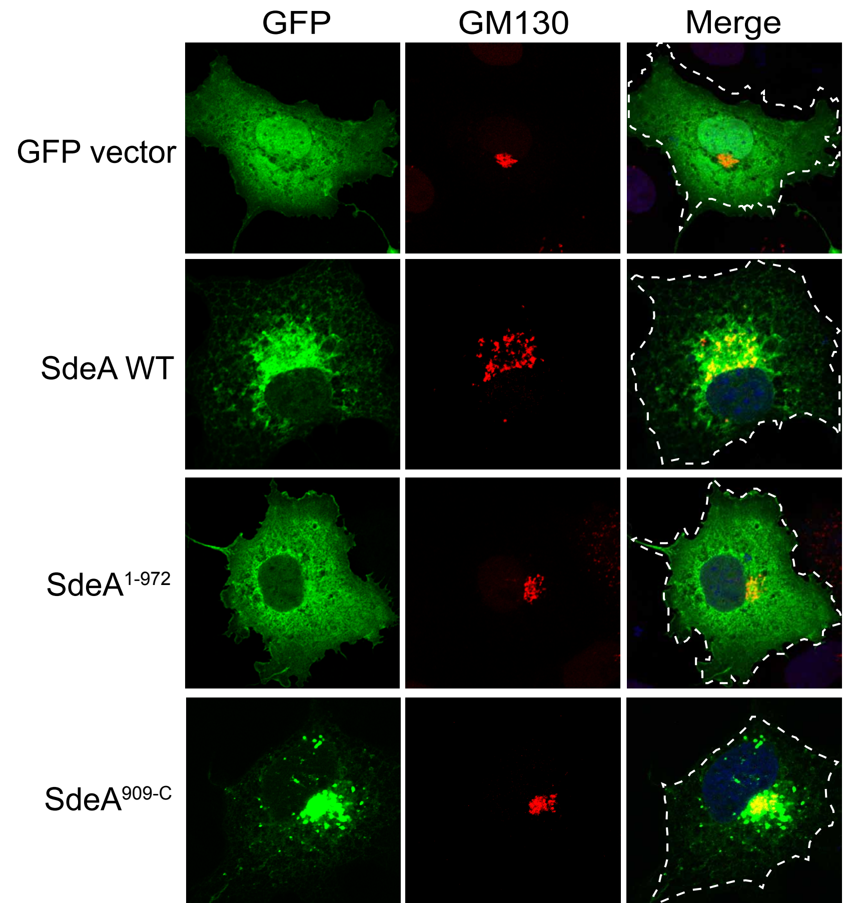


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Figure 2. *Legionella* effector SdeA mediates Golgi fragmentation

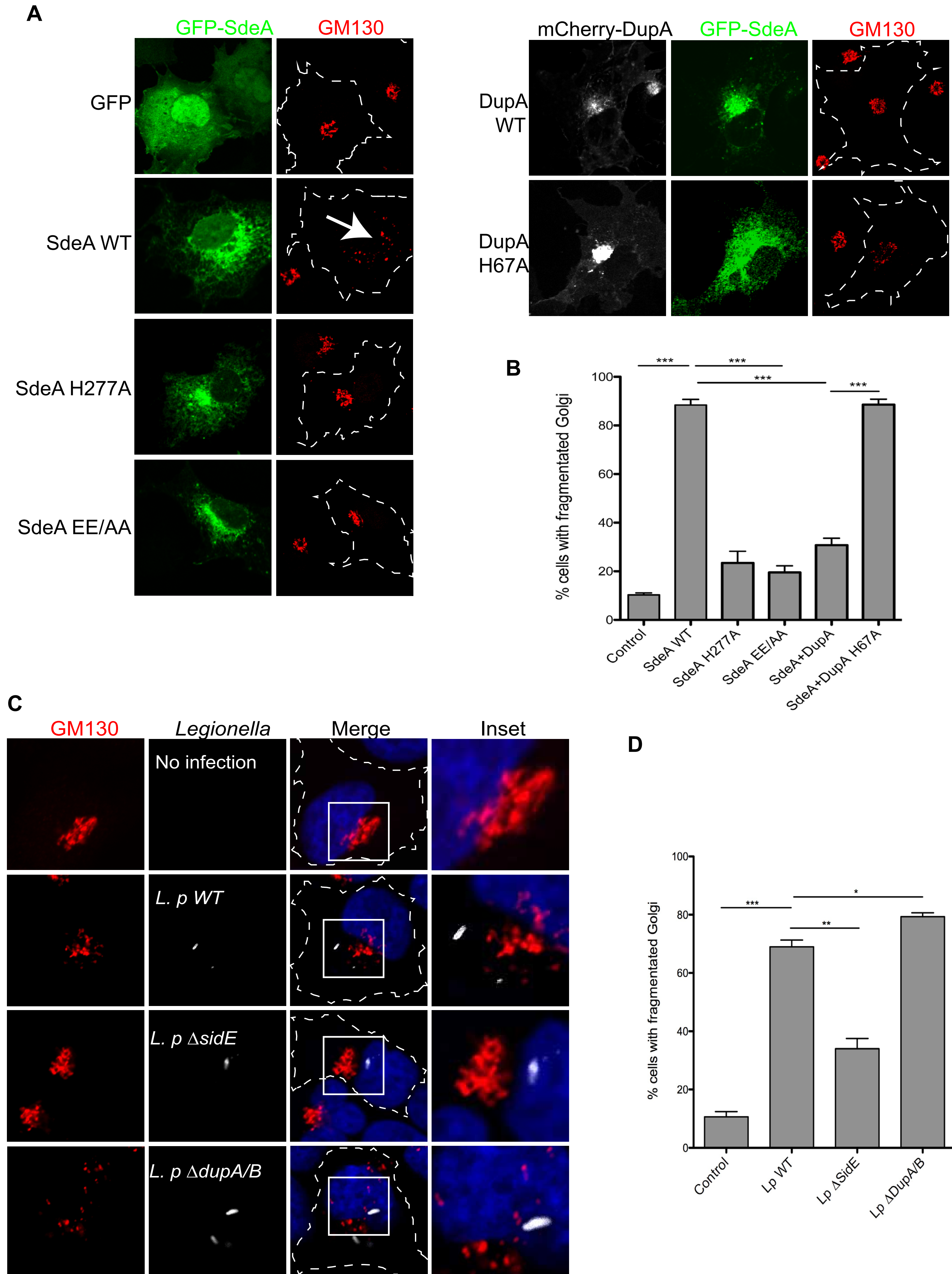
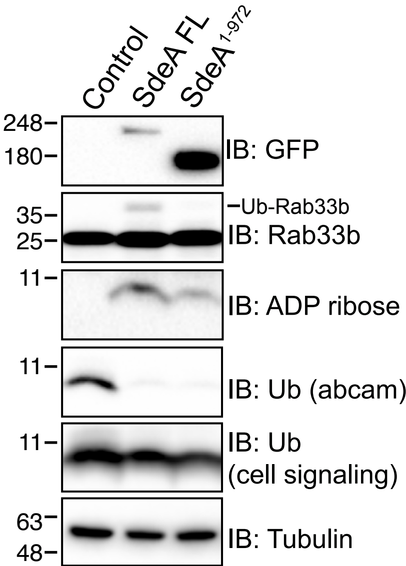


Figure 2-figure supplement 1. *Legionella* effector SdeA mediates Golgi fragmentation

A



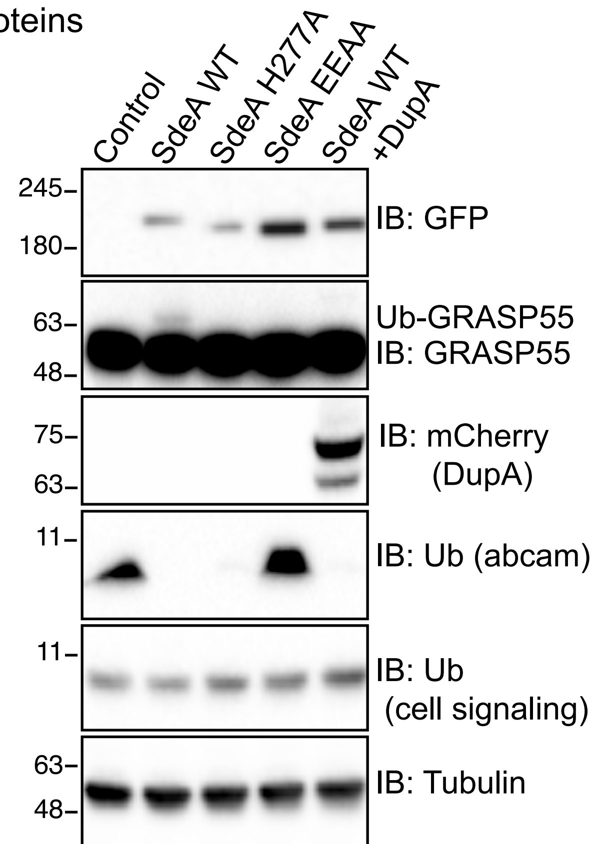
B

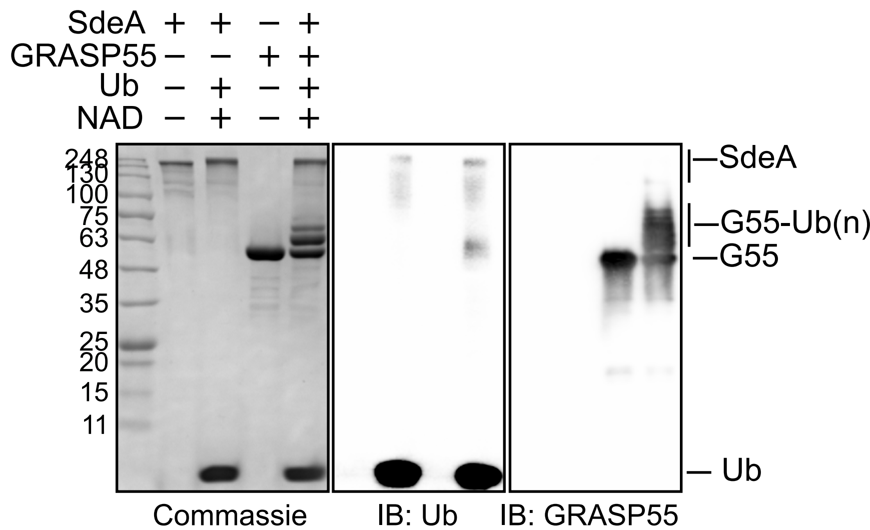
TGN46
GM130
Merge
Inset
EGFP
EGFP-SdeA
EGFP-SdeA
EE/AA

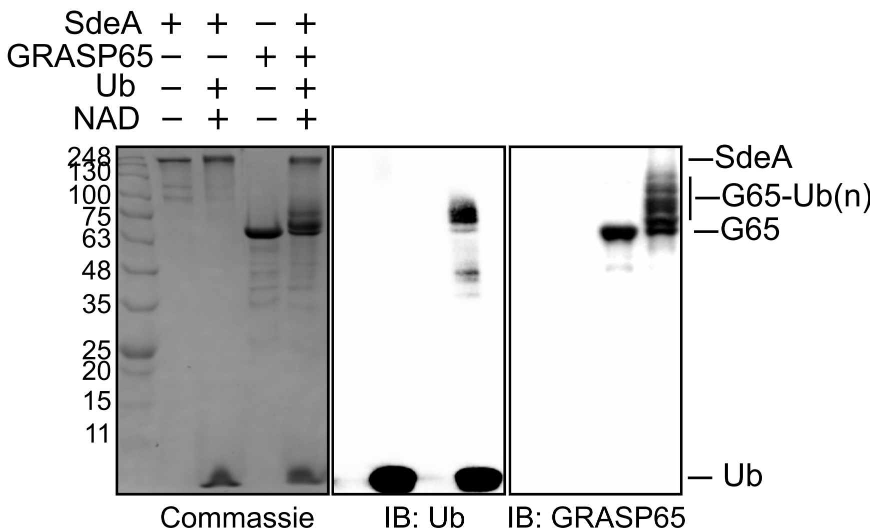
Figure 3. SdeA ubiquitinates Golgi tethering factor GRASP proteins

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







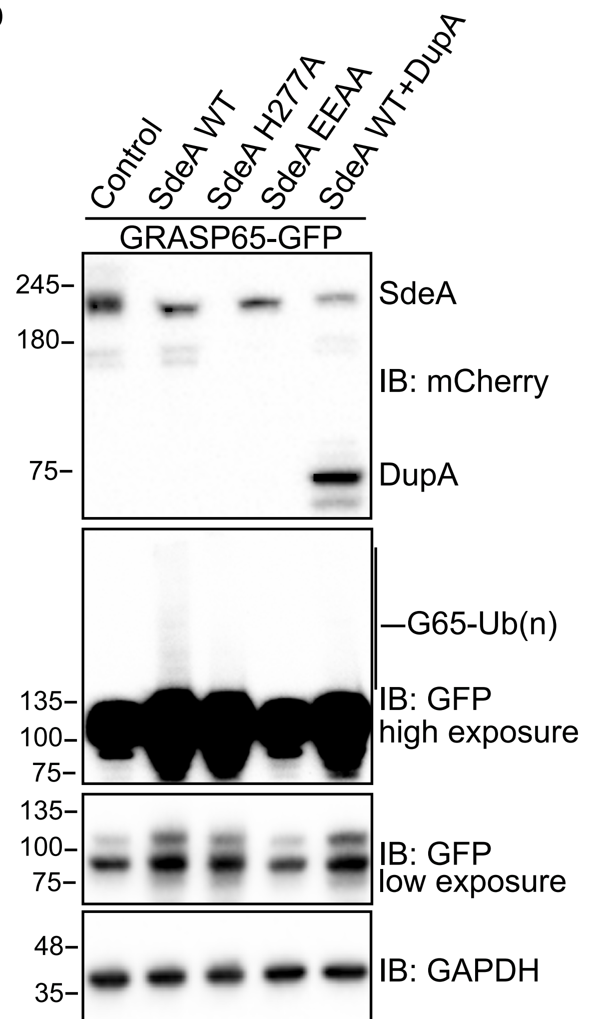


Figure 3-figure supplement 1. SdeA ubiquitinates Golgi proteins

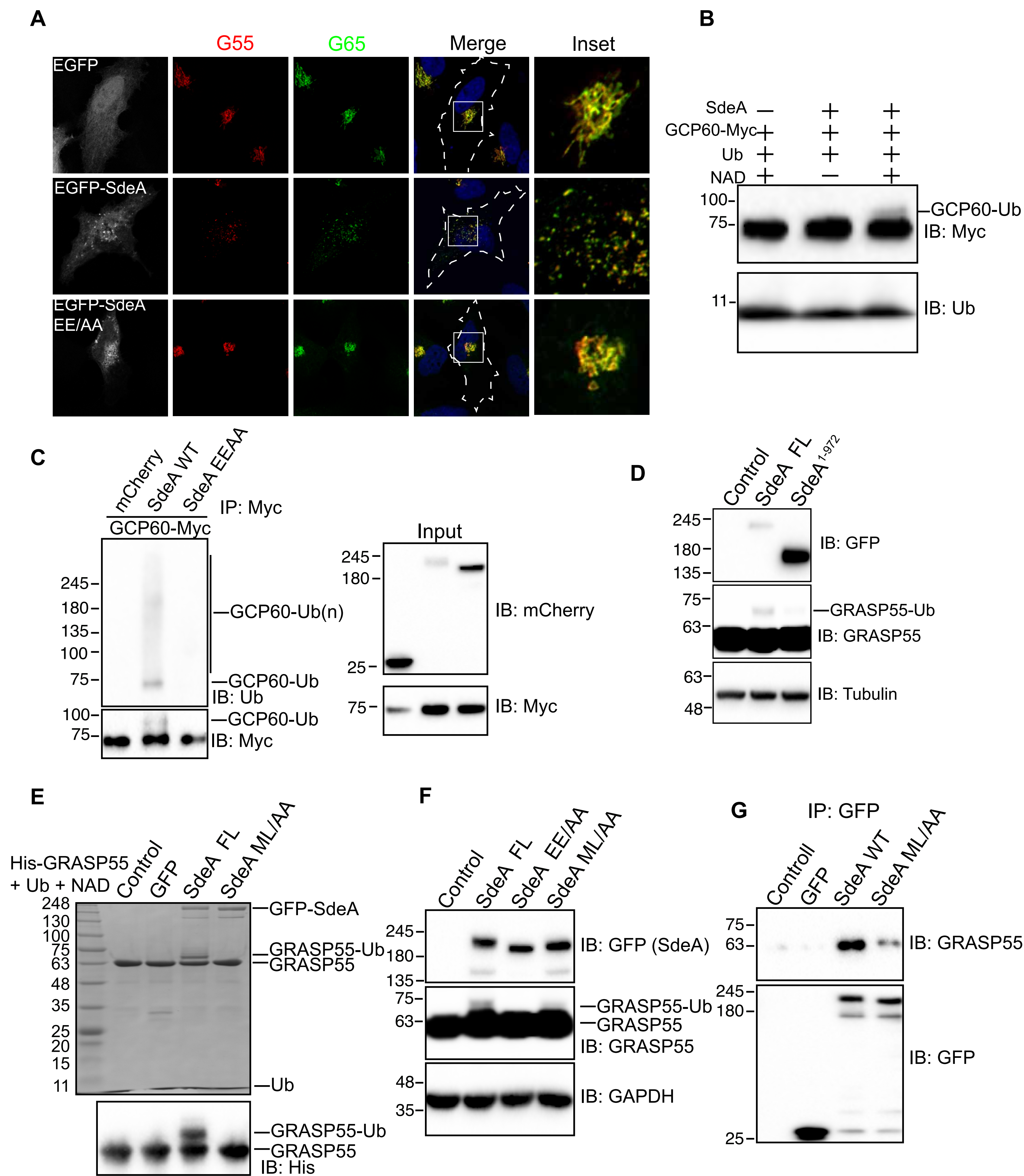


Figure 4. *Legionella* infection causes SidEs-catalyzed ubiquitination of GRASP proteins

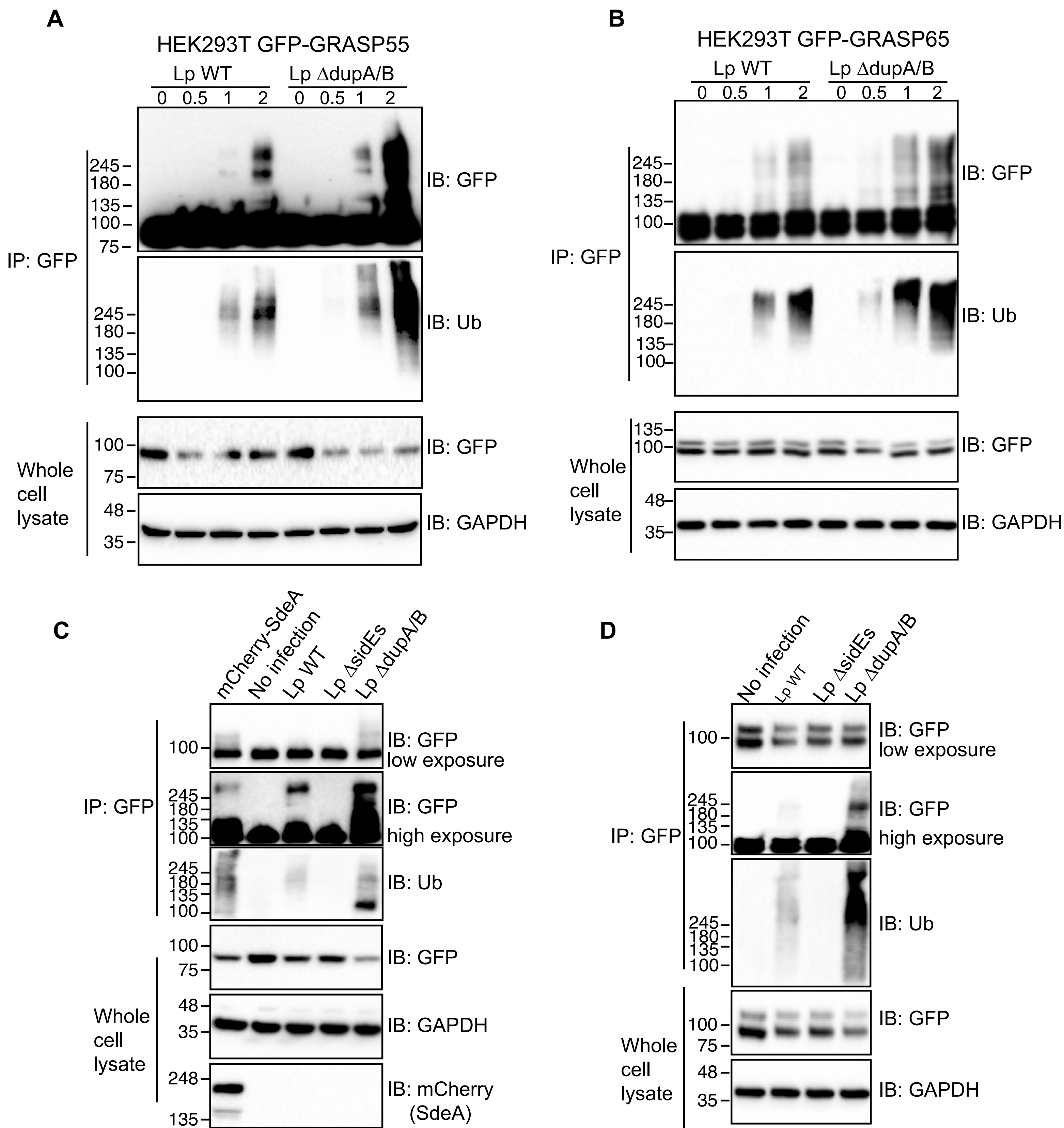


Figure 4-figure supplement 1. *Legionella* infection causes SidEs-catalyzed ubiquitination of GRASP proteins

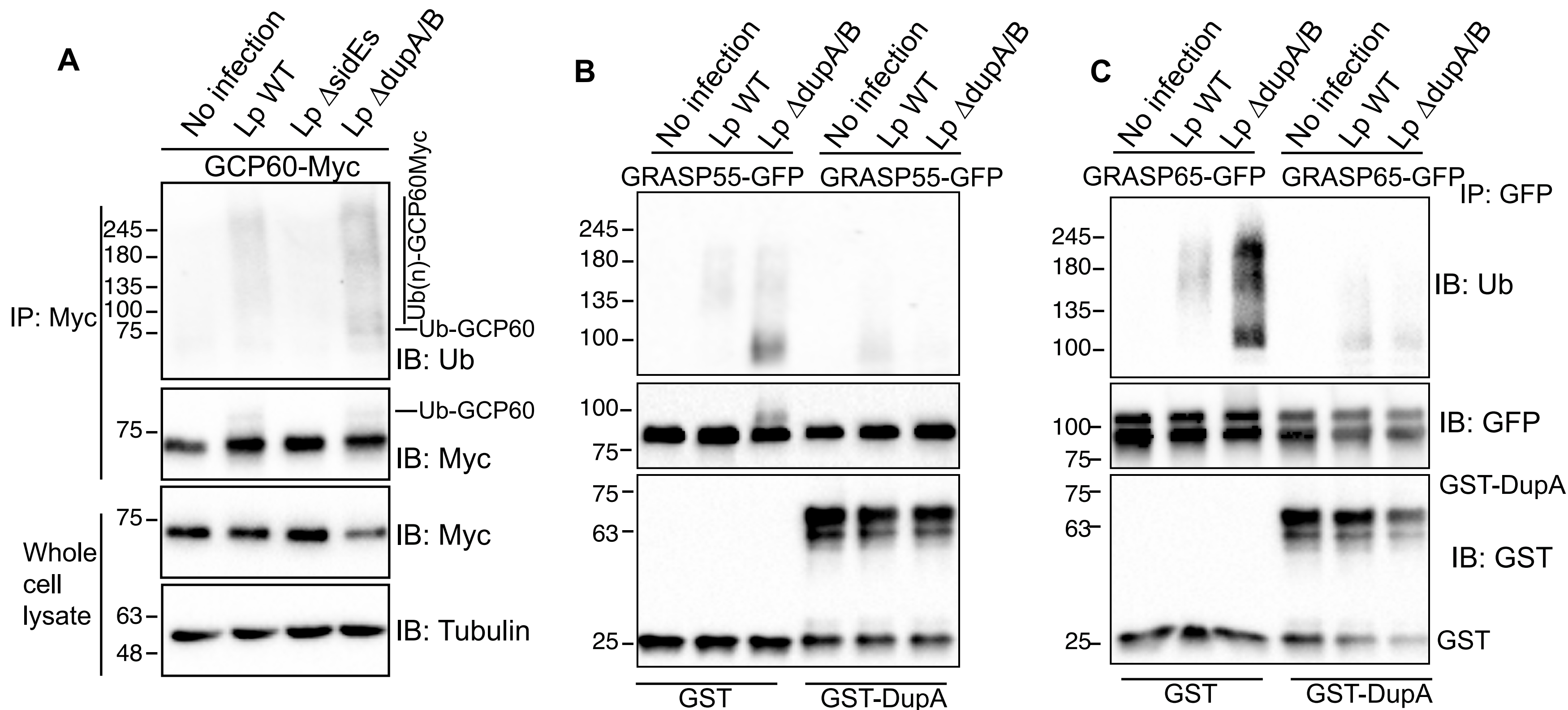
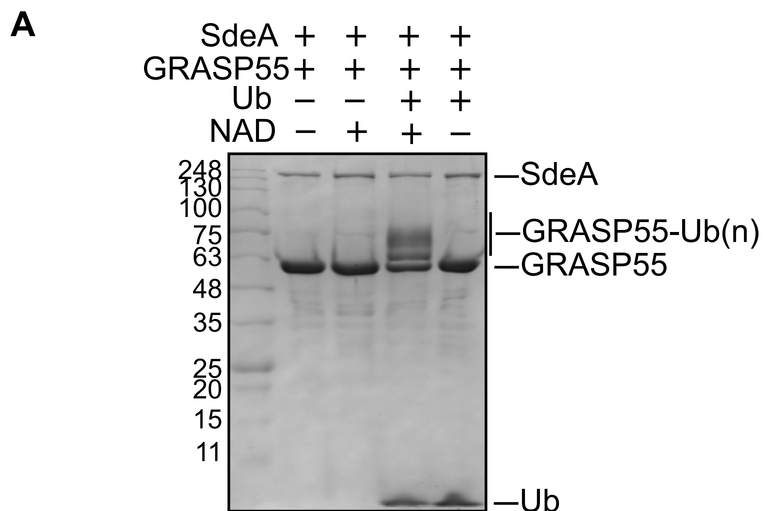
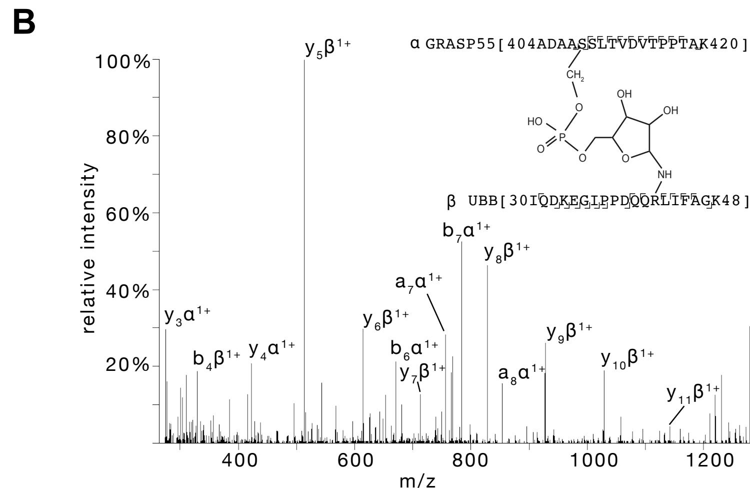
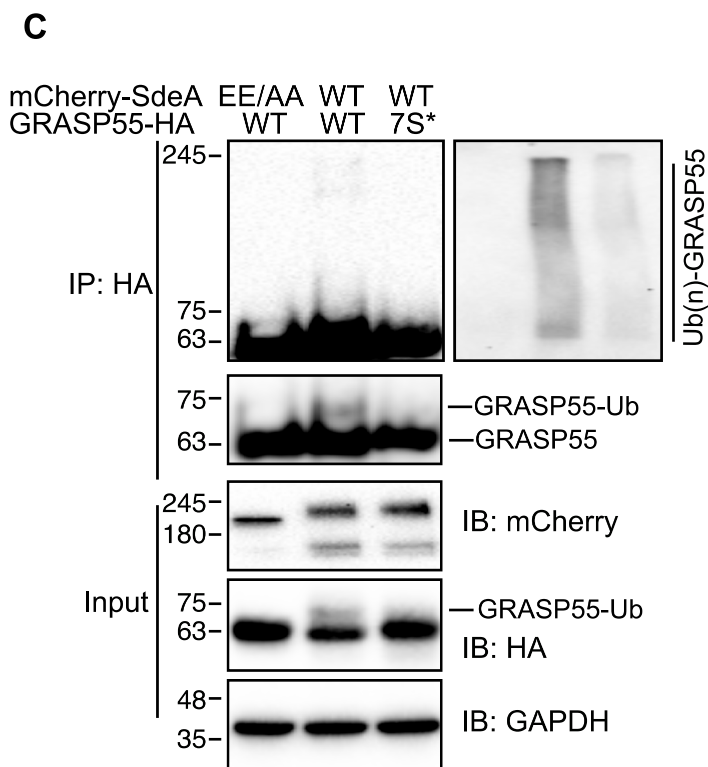


Figure 5. Identification of GRASP55 ubiquitination sites







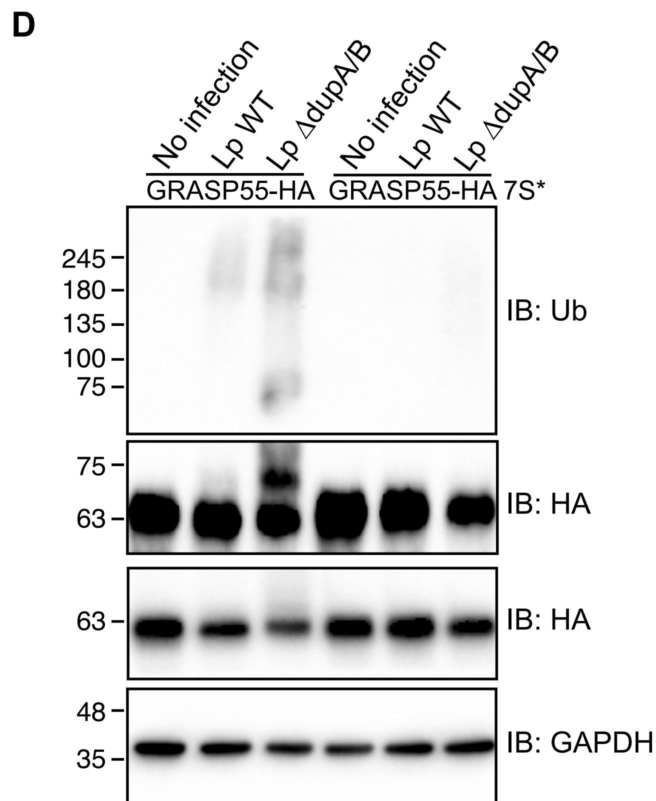
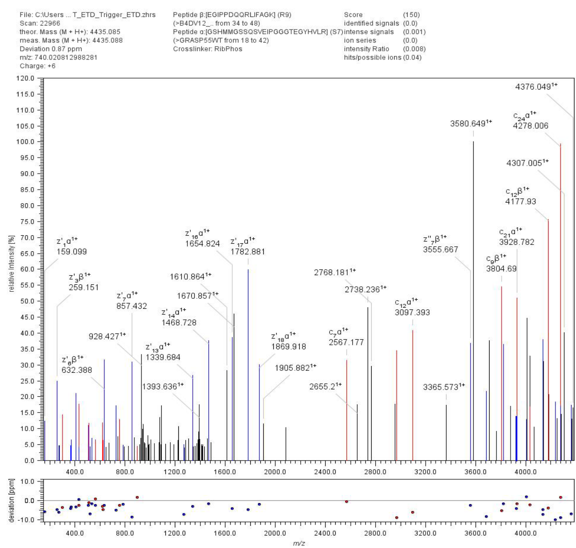


Figure 5-figure supplement 1. Identification of GRASP55 ubiquitination sites

A



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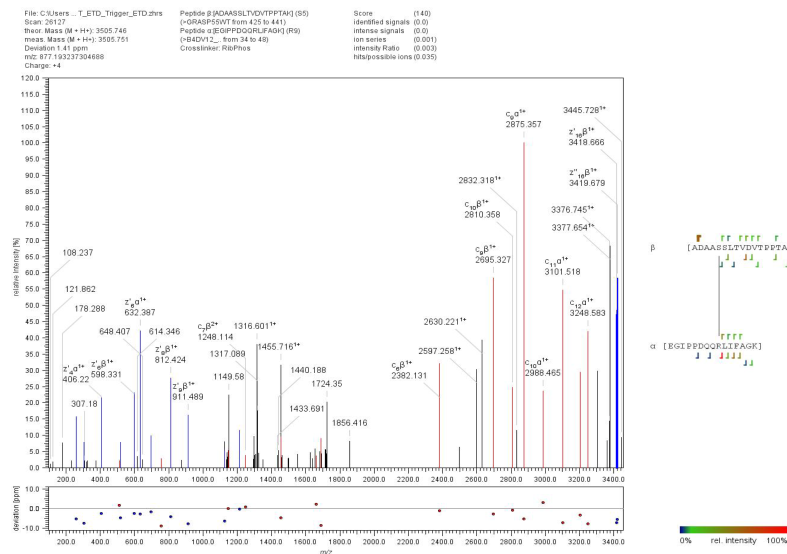

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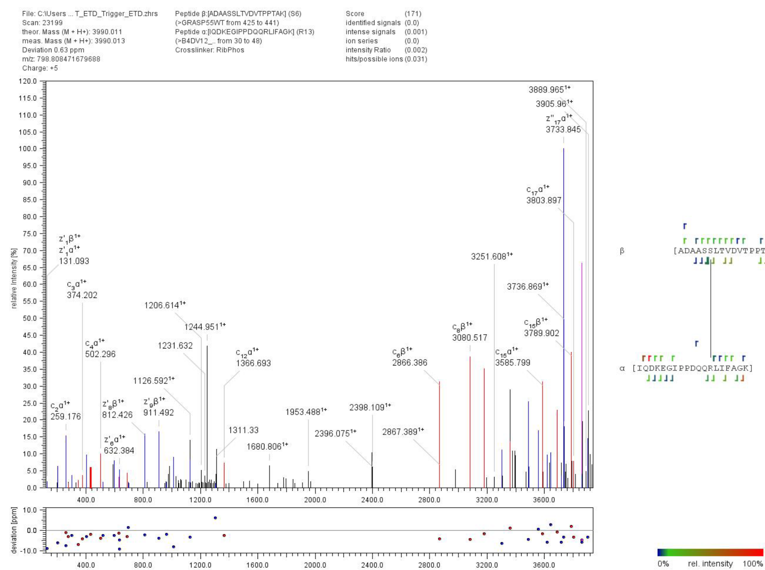

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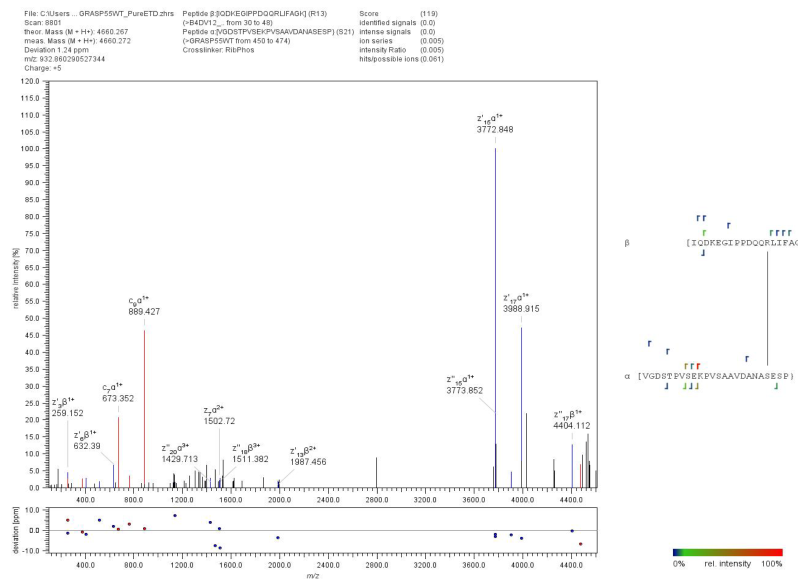


Figure 6. Serine ubiquitination impairs GRASP55 function

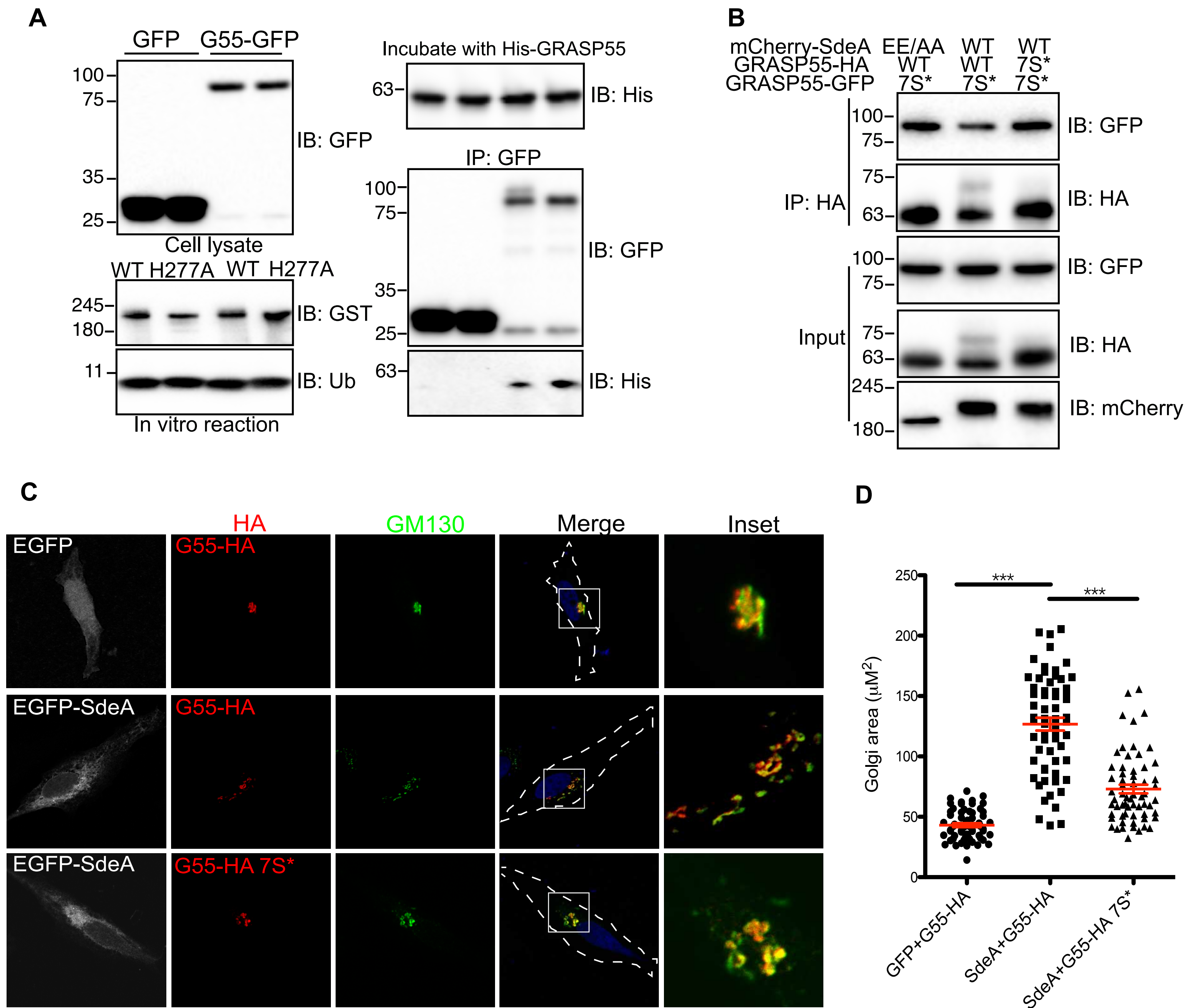


Figure 6-figure supplement 1. GRASP55 mutant rescues Golgi fragmentation of GRASP55/65 KO cells

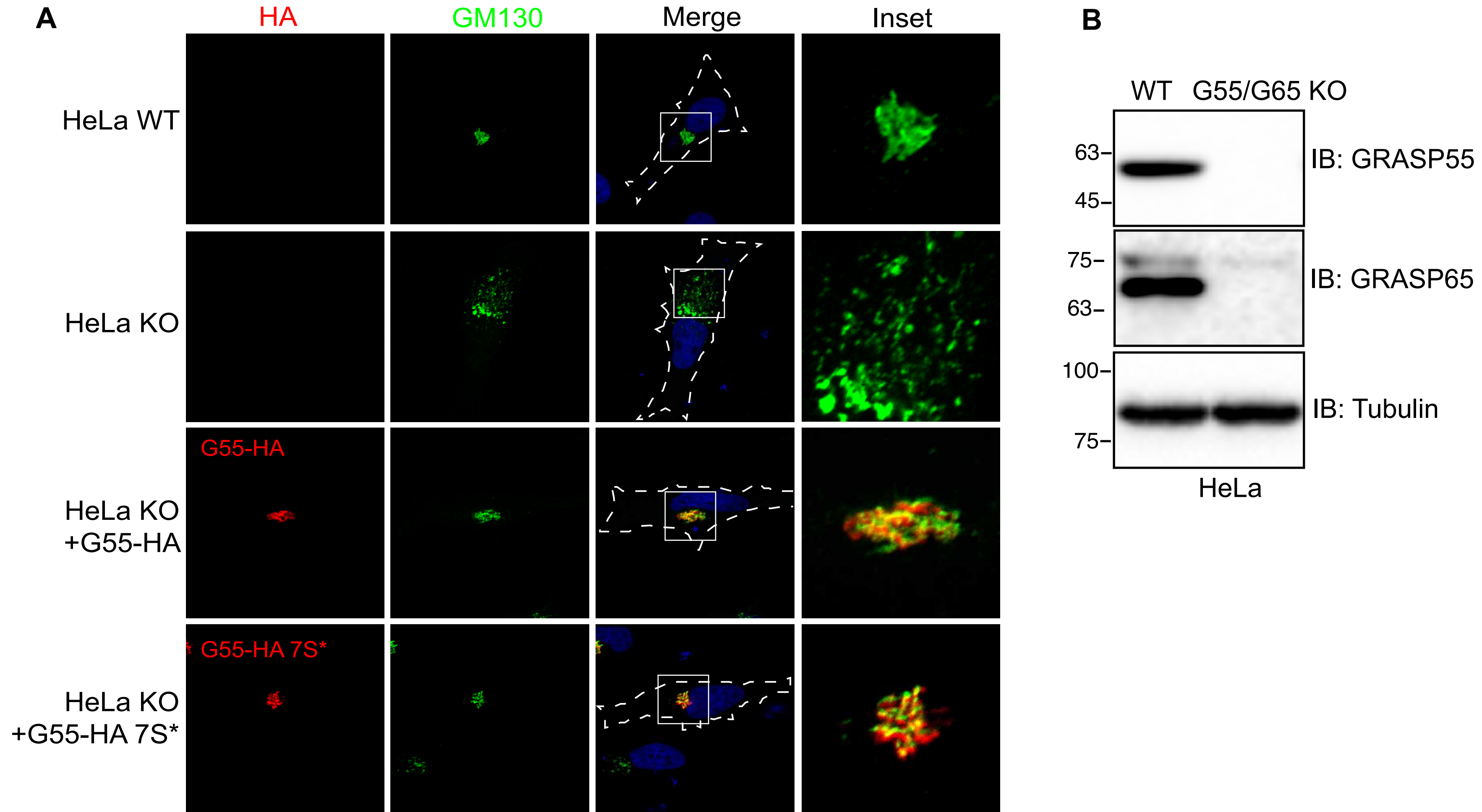


Figure 7. *Legionella* does not recruit fragmented Golgi

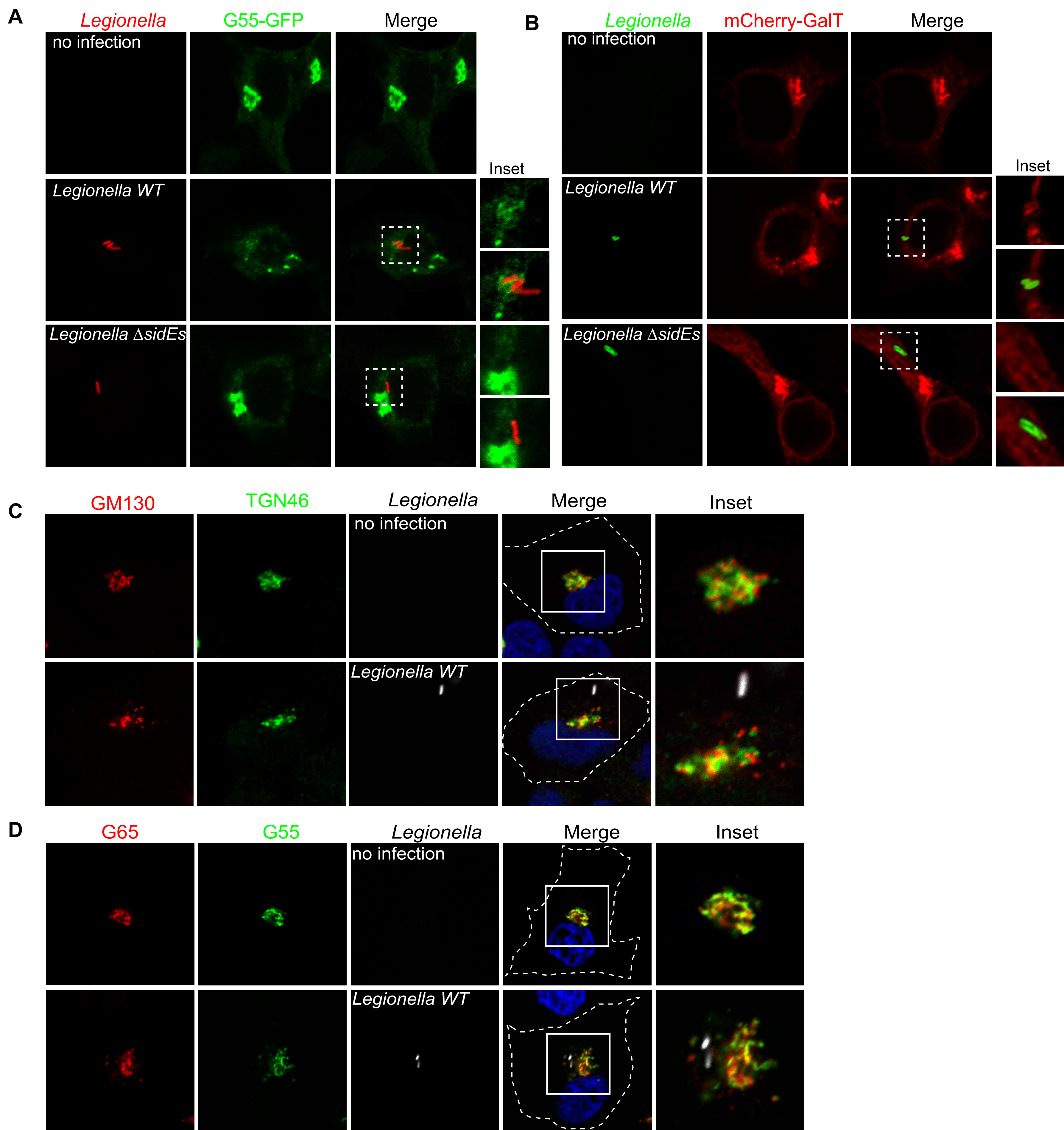
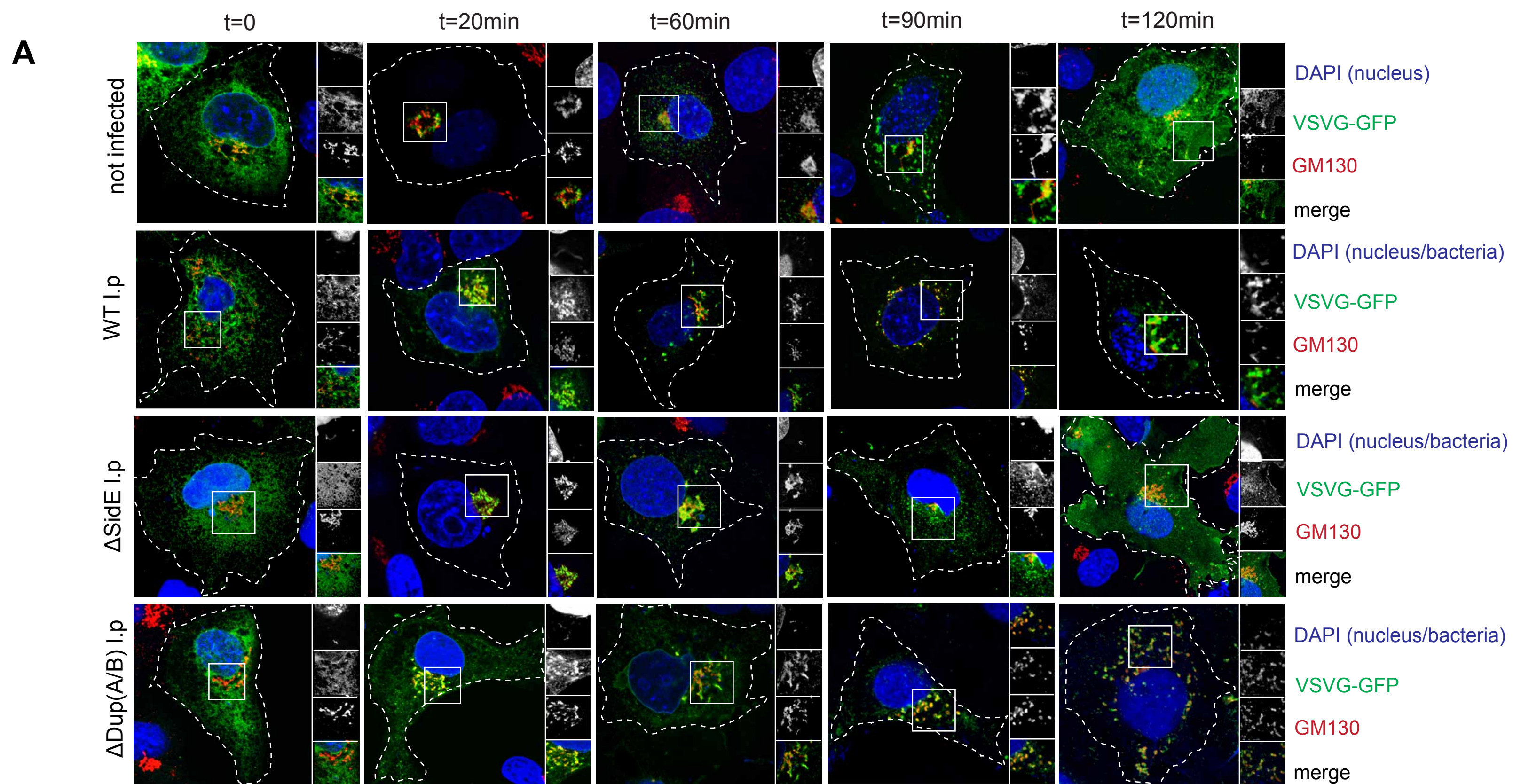
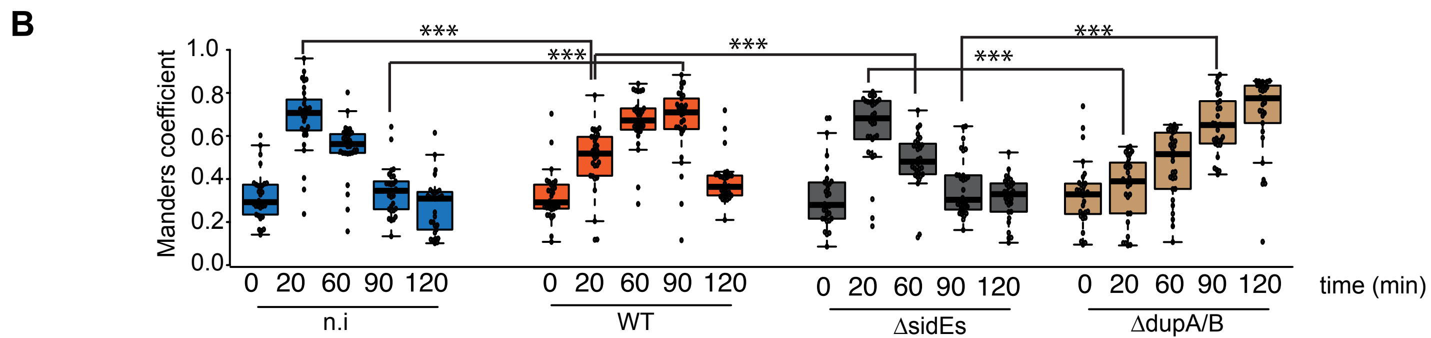
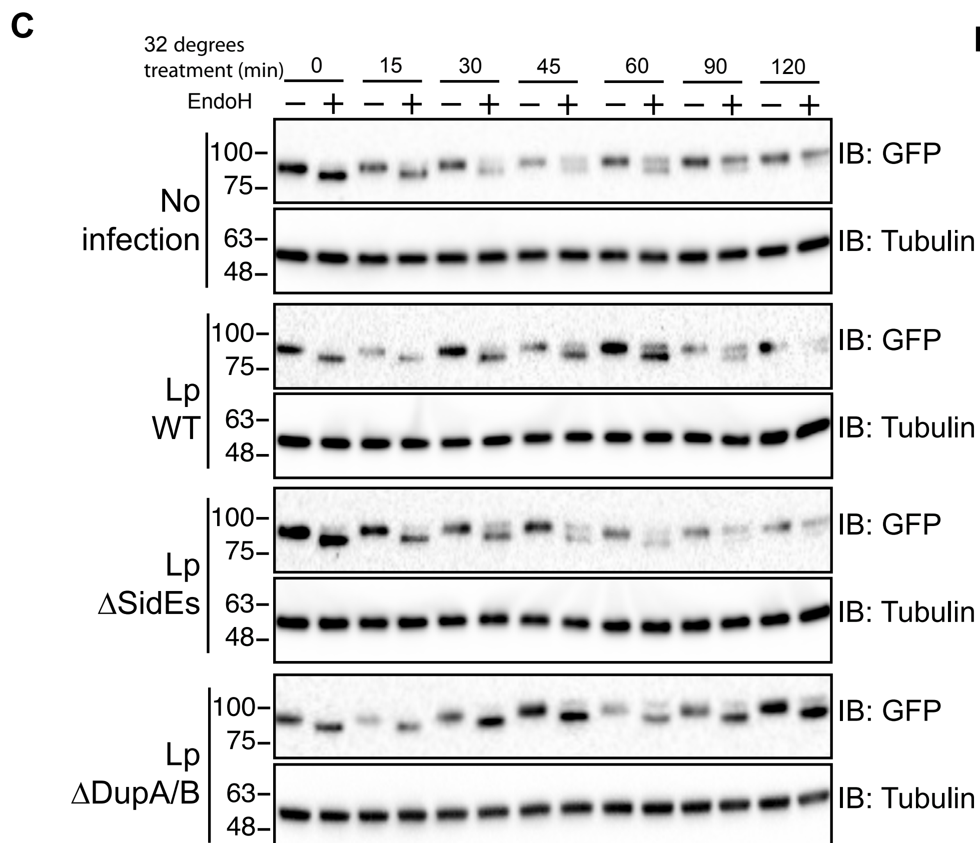


Figure 8. SdeA-induced serine ubiquitination inhibits trafficking pathway







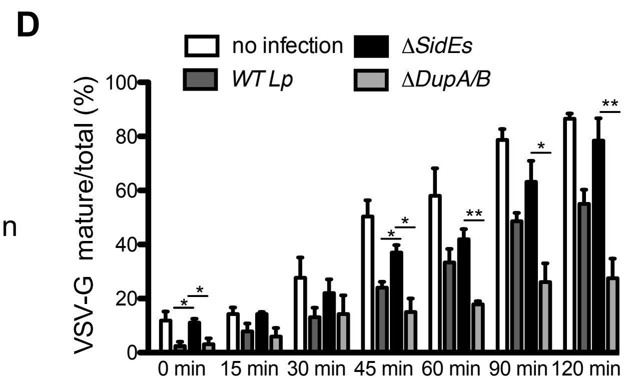


Figure 8-figure supplement 1. SdeA-induced serine ubiquitination inhibits trafficking pathway

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