The *Legionella pneumophila* metaeffector Lpg2505 (SusF) regulates SidI-mediated translation inhibition and GDP-dependent glycosyltransferase activity

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Abstract

Legionella pneumophila, the etiological agent of Legionnaires Disease, employs an arsenal of hundreds of Dot/Icm-translocated effector proteins to facilitate replication within eukaryotic phagocytes. Several effectors, called metaeffectors, function regulate the activity of other Dot/Icm-translocated effectors during infection. The metaeffector Lpg2505 is essential for *L. pneumophila* intracellular replication only when its cognate effector, SidI, is present. SidI is a cytotoxic effector that interacts with the host translation factor eEF1A and potently inhibits eukaryotic protein translation by an unknown mechanism. Here, we evaluated the impact of Lpg2505 on SidI-mediated phenotypes and investigated the mechanism of SidI function. We determined that Lpg2505 binds with nanomolar affinity to SidI and suppresses SidI-mediated inhibition of protein translation. SidI binding to eEF1A and SusF is not mutually exclusive and these proteins bind distinct regions of SidI. We also discovered that SidI possesses GDP-dependent glycosyltransferase activity and that this activity is regulated by Lpg2505. We have therefore renamed Lpg2505, SusF (suppressor of SidI function). This work reveals novel enzymatic activity for SidI and provides insight into how intracellular replication of *L. pneumophila* is regulated by a metaeffector.

Introduction

Legionella pneumophila is the etiological agent of Legionnaires' Disease, a severe inflammatory pneumonia that results from uncontrolled bacterial replication within alveolar macrophages. Upon phagocytosis, *L. pneumophila* avoids lysosomal degradation through

establishment of an endoplasmic reticulum-derived compartment called the *Legionella*-containing vacuole (LCV) (1). For biogenesis of the LCV and acquisition of nutrients from the host cell, *L. pneumophila* is dependent on a massive arsenal of over 300 individual effector proteins that are translocated directly into the host cell through a Dot/Icm Type IVB secretion system (T4BSS) (2). The cellular functions of the majority of effectors have yet to be elucidated, due in part to their functional redundancy within macrophages (3).

Metaeffectors have emerged as a common theme in *L. pneumophila* pathogenesis and are used by *L. pneumophila* to regulate effector function (4). The first metaeffector described was LubX, which temporally regulates function of its cognate effector SidH by hijacking host ubiquitination machinery to facilitate proteasomal degradation of SidH (5). At least 20 of the over 300 identified *L. pneumophila* effectors are metaeffectors (4) and two of these – SidJ and Lpg2505 – are members of a small group of effectors that are individually important for *L. pneumophila* intracellular replication within macrophages (6–8). SidJ is a glutamylase that covalently modifies and abrogates the function of the SidE family of effector ubiquitin ligases (9–11). Lpg2505 is a metaeffector of unknown function that suppresses toxicity of its cognate effector, SidI (Lpg2504), and is important for intracellular replication only when wild-type *sidI* is expressed (6). This was demonstrated by restoration of *L. pneumophila* Δ *lpg2505* intracellular replication upon either deletion of *sidI* or expression of a non-toxic *sidI* allele (R453P). It was further found that Lpg2505 was sufficient to suppress SidI-mediated toxicity towards the yeast *Saccharomyces cerevisiae* (6). Thus, SidI activity is deleterious to *L. pneumophila* in the absence of Lpg2505, a unique phenotype for a translocated effector.

SidI is one of seven cytotoxic *L. pneumophila* effector proteins that inhibit host cell protein translation (12). Like the majority of *L. pneumophila* effectors, *sidI* alone is dispensable for intracellular replication within macrophages, likely due to functional redundancy with other effectors (12). Other translation inhibiting effectors include Lgt1-3, SidL, LegK4, and Lpg1489 (12–16). Lgt1-3 are glycosyltransferases that inhibit translation by glucosylating eukaryotic elongation factor 1A (eEF1A) at Ser-53 (17–19). LegK4 is an effector kinase that phosphorylates heat-shock protein 70 (Hsp70), thereby reducing its ATPase activity and protein refolding activities (16). SidL and Lpg1489 have been experimentally demonstrated to inhibit host protein synthesis (13, 15), but their modes of action are unknown. Like Lgt1-3, SidI also interacts with eEF1A; however, this interaction is insufficient for translation inhibition (12). SidI also interacts with eEF1B γ and induces the host heat shock response (12). To date, the mechanism(s) by which SidI functions within the host cell to inhibit host protein synthesis have not been elucidated.

In this study we aimed to discern how Lpg2505 regulates SidI and gain insight into the molecular mechanism of SidI function. We discovered that Lpg2505 and SidI bind with nanomolar affinity and that Lpg2505 suppresses SidI-mediated translation inhibition *in vitro*. Furthermore, SidI interaction with Lpg2505 and eEF1A are not mutually exclusive and these two proteins bind with distinct regions of SidI. Finally, we discovered novel GDP-dependent glycosyltransferase activity for SidI, which is regulated by Lpg2505. For this reason, we have named Lpg2505 as *suppressor of SidI function* (SusF).

Results

SusF and SidI bind with nanomolar affinity.

Lpg2505 (SusF) is sufficient to suppress SidI-mediated cytotoxicity and promote *L. pneumophila* intracellular replication (6). To reveal a potential mechanism for SusF-mediated regulation of SidI, we evaluated whether SusF interacts with SidI. Since effectors function within host cells, we investigated whether SidI and SusF interact in the presence of host cell lysates. HEK 293 cells stably producing 3FLAG-eipotpe tagged SusF were generated and we initially attempted to ectopically express GFP-tagged *sidI* within these cells for co-immunoprecipitation. However, wild-type SidI could not be detected, likely due to potent translation inhibition. Thus, we generated recombinant GST-tagged SidI (GST-SidI) and evaluated its ability to interact with 3FLAG-SusF within HEK 293 lysates (see *Materials and Methods*). We found that GST-SidI, but not GST alone, retained 3FLAG-SusF on glutathione-coated beads (**Fig 1A**). Furthermore, GST-SidI - but not GST alone - was retained on Ni-NTA beads coated with recombinant His₆-SusF (**Fig 1B**). Thus, SusF interacts with SidI in the presence or absence of mammalian cell lysates.

To determine if SidI and SusF bind directly, we examined the ability of these proteins to associate with one another throughout the course of sequential column chromatography procedures. SidI and SusF were co-expressed in *Escherichia coli*, purified by Ni-NTA affinity chromatography (see *Materials and Methods*), and the eluted proteins were separated by analytical format size exclusion chromatography. We found that SidI and SusF co-eluted from the column as a species corresponding to a molecular weight of ~150 kDa, as judged by comparison to a panel of known protein standards (**Fig 1C**). Bands corresponding to both proteins were detected in samples of

column fractions that had been separated by SDS-PAGE and analyzed by Coomassie staining (**Fig S1**). Thus, SidI and SusF interact directly and appeared to form a stable complex.

We subsequently used surface plasmon resonance (SPR) to investigate the affinity and kinetics of the SusF-SidI interaction. We immobilized SusF on an SPR surface using random amine chemistry and injected recombinant, purified SidI at increasing concentrations. We employed a single-cycle approach due to difficulty with regenerating the SusF surface following exposure to SidI. We found that the reference-corrected sensorgram could be described fairly well by a Langmuir binding model ($K_D = 0.89$ nM and $\chi^2 = 3.26$) (**Fig. S2**), but was better fit to a two-state reaction model ($K_D = 3.1$ nM and $\chi^2 = 0.79$) (**Fig 1D**). A particularly noteworthy feature of the SusF-SidI interaction is its long half-life, with an estimate dissociation rate constant between $2\text{-}5 \times 10^{-4}$ s⁻¹. This observation explains, at least in part, the ability of SusF and SidI to remain associated with one another throughout the co-purification procedures described above. Taken together, these data indicate that SusF binds directly to SidI and forms a high-affinity complex with a K_D of ~ 1 nM.

SusF suppresses SidI-mediated translation inhibition.

Based on the high-affinity interaction between SidI and SusF, we hypothesized that SusF represses SidI-mediated translation inhibition. To test this hypothesis, we quantified translation of *Firefly* luciferase mRNA (Luc mRNA) *in vitro* (see *Materials and Methods*). This assay was used previously to demonstrate that ≥ 5 ng of recombinant SidI was sufficient to completely abolish translation (12). We confirmed that purified His₆-SidI significantly attenuates protein translation

and additionally revealed that purified SusF alone has no impact on translation (**Fig 2A**). We further determined that SusF significantly rescues translation in the presence of SidI ($P < 0.01$, **Fig 2B**). Thus, SusF is sufficient to suppress SidI-mediated translation inhibition *in vitro*.

Our SPR data demonstrates that the SidI-SusF interaction occurs rapidly. We therefore hypothesized that pre-formation of the SidI-SusF complex would not result in further attenuation of SidI-mediated translation inhibition. To determine whether or not pre-formation of the SidI-SusF complex would enhance SusF-mediated suppression of translation inhibition, we incubated SidI with SusF for 30 min prior to addition to the *in vitro* translation reaction. Pre-formation of the SidI-SusF complex did not further attenuate SidI-mediated translation inhibition (**Fig 2C**; see *Materials and Methods*). Furthermore, SusF was insufficient to reverse SidI-mediated translation inhibition since addition of SusF to the reaction after either 30 min or 60 min did not restore translation (**Fig 2C**). Finally, we evaluated whether suppression of SidI-mediated translation inhibition by SusF was dose dependent. SusF was added in concentrations ranging from equimolar up to a 15-fold molar excess relative to SidI. An equimolar amount of SusF was sufficient to suppress SidI-mediated translation inhibition and translation was not further enhanced by addition of up to a 15-fold molar excess of SusF (**Fig 2D**). Thus, equimolar amounts of SusF are sufficient to suppresses SidI-mediated translation inhibition.

SusF does not affect the interaction between SidI and eEF1A.

A previous report demonstrated that SidI interacts directly with the transcription factor eukaryotic elongation factor 1A (eEF1A) (12). We therefore investigated whether the interactions

between SidI and eEF1A or SusF are mutually exclusive. GST-SusF or GST alone were immobilized on glutathione beads and incubated with His₆-SidI that was pre-incubated with HEK 293T lysates. We found that eEF1A was retained on the beads only in the presence of SidI and that eEF1A did not impair interaction between SusF and SidI (**Fig 3A**). Subsequently, we asked whether increasing the concentration of SusF would influence the SidI-eEF1A interaction. We incubated GST-SidI with eEF1A (from HEK 293T lysates, see *Materials and Methods*) followed by increasing concentrations of purified recombinant SusF. Despite SusF concentration increasing 100-fold, GST-SidI still retained eEF1A on the beads (**Fig 3B**), suggesting that SidI interacts with SusF and eEF1A simultaneously. The same result was observed when SusF was incubated with GST-SidI prior to HEK 293T lysates (**Fig S3**). Thus, SidI binding to eEF1A and SusF is not mutually exclusive.

SusF and eEF1A interact with distinct regions of SidI.

Since both SusF and eEF1A are capable of binding SidI simultaneously, we hypothesized that these proteins interact with SidI at distinct sites. SidI has a molecular weight of ~ 110 kDa and consists of 942 amino acids. Since the structure of SidI has not been solved, we used the RaptorX webserver (20) to predict the domain structure of SidI. Based on the predicted domains (**Fig S4**), we generated SidI truncations consisting of amino acid residues 1-268 (SidI_N), 269-942 (SidI_C) and 269-874 (SidI_{CA68}) (**Fig 4A**). To determine which of these putative SidI domains is involved in interaction with SusF and eEF1A, we immobilized GST-tagged full-length SidI, SidI_N, SidI_C, SidI_{CA68}, or GST alone on glutathione beads followed by incubation with lysates from HEK 293

cells stably producing 3FLAG-SusF. Western blot analysis was used to detect 3FLAG-SusF and eEF1A bound to SidI truncation proteins (see *Materials and Methods*). We confirmed that GST-SidI, but not GST alone, was capable of retaining both SusF and eEF1A on the beads. 3FLAG-SusF was further retained on beads coated with GST-SidI_N and GST-SidI_C but not GST-SidI_{CA68}, whereas eEF1A was retained by GST-SidI_C and GST-SidI_{CA68} (**Fig 4B**). To control for the potential influence of 3FLAG-SusF on eEF1A binding to SidI, we repeated this experiment using HEK 293 cells that do not express *3FLAG-susF*. We observed the same pattern of interactions between eEF1A and the C-terminal region of SidI in the absence of 3FLAG-SusF (**Fig S5**). Based on these data, we conclude that SusF interacts with SidI at regions within amino acid residues 1-268 and 874-942 whereas eEF1A interaction with SidI is dependent on amino acid residues 269-874. Together, these data suggest SusF interacts with two regions of SidI that are distinct from the site of SidI interaction with eEF1A.

SidI is not dependent on SusF for translocation into host cells.

The majority of *L. pneumophila* effector proteins rely on a C-terminal translocation signal for Dot/Icm-mediated translocation into host cells (21, 22). Based on the observed interaction between SusF and the C-terminal 68 amino acid residues of SidI, we hypothesized that SusF may impact Dot/Icm-mediated translocation of SidI. To test this hypothesis, we quantified the export of SidI fused to *Bordetella pertussis* adenylate cyclase (CyaA) (21). When CyaA fusion proteins reach the cytosol of eukaryotic cells they catalyze formation of cAMP, which can be quantified using cAMP-specific ELISA. Based on potent toxicity associated with wild-type SidI, a non-toxic

sidI allele (R453P; SidI_{RP}) was used for these experiments (6, 12). Expression of CyaA-SusF and CyaA-SidI_{RP} was confirmed using Western blot analysis (**Fig 5A**). Subsequently, we found that cAMP production by host cells was significantly increased following infection with wild-type and Δ *susF*, but not Δ *dotA*, strains of *L. pneumophila* producing CyaA-SidI_{RP} (**Fig 5B**). Translocation of CyaA-RalF was used as a positive control for Dot/Icm-dependent translocation (21) (**Fig 5A, B**). Together, these data demonstrate that SidI is not dependent on SusF for translocation into host cells.

Binding to eEF1A is insufficient for SidI-mediated translation inhibition.

Subsequently, we investigated the ability of truncated SidI proteins to influence protein translation. At concentrations equivalent to SidI, neither SidI_N nor SidI_{CA68} were sufficient to inhibit protein translation *in vitro* (**Fig 6A**). Based on the interaction between SidI_{CA68} and eEF1A, we also quantified translation in the presence of increasing concentrations of His₆-SidI_{CA68}. We found that His₆-SidI_{CA68} significantly decreased translation at concentrations up to 25-fold greater than SidI (**Fig 6B**), suggesting that this truncation retains some activity; however, SidI_{CA68}-mediated translation inhibition is modest in comparison to an equal amount of SidI despite the ability to interact with eEF1A (**Fig 4B, Fig S5**). These data further confirm that interaction with eEF1A is insufficient for SidI-mediated translation inhibition (12).

SidI is a GDP-dependent glycosyltransferase.

206 Like many other bacterial effectors, the primary amino acid sequence of SidI does not have
 207 obviously conserved motifs. Therefore, to gain insight into the putative function of SidI, we used
 208 a variety of computational methods to predict the structure and function of SidI. Though very
 209 low primary sequence identity was present in the templates used, the HHPred webserver (23),
 210 the Phyre2 webserver (24), the Raptor-X webserver (20), and the I-TASSER webserver (25–27) all
 211 produced models of various lengths, but with the same fold for overlapping regions. A DALI
 212 search (28) for structural homologs against each of these models revealed that amino acid residues
 213 368–868 of SidI have predicted structural homology to multiple bacterial and eukaryotic
 214 glycosyltransferases with GT-B folds, including PimB, a GDP-mannose-dependent
 215 mannosyltransferase (**Table S1, Fig S6**). Orthogonally, the Ginzu domain parser on the Robetta
 216 server also predicted the presence of a glycosyltransferase domain (29). Based on the consensus
 217 between orthogonal computational approaches, we hypothesized that SidI possesses GDP-
 218 dependent glycosyltransferase activity and that GDP-mannose could be a substrate. To test this
 219 hypothesis, we utilized a functional luminescence-based assay to quantify cleavage of GDP-
 220 mannose (see *Materials and Methods*). As a control, we also evaluated the ability of SusF, which
 221 does not have predicted glycosyltransferase activity, to cleave GDP-mannose. We observed high
 222 levels of free GDP only following incubation of recombinant His₆-SidI with GDP-mannose,
 223 suggesting that SidI indeed possesses GDP-dependent glycosyltransferase activity (**Fig 7A**). We
 224 further investigated whether SidI glycosyltransferase activity was specific to GDP-sugars and
 225 evaluated its ability to cleave UDP-glucose, which is cleaved by Lgt1–3 (18, 30). SidI was capable
 226 of cleaving UDP-glucose, but far less efficiently than GDP-mannose (**Fig 7B**), suggesting that

GDP-mannose is the preferred substrate. Together, these data suggest that SidI functions specifically as a GDP-dependent glycosyltransferase.

The entire predicted GDP-dependent glycosyltransferase domain of SidI is encoded by amino acids 350-874, which is encompassed within SidI_{CA68} truncation (**Fig 4A**). To determine whether SidI_{CA68} alone is sufficient to cleave GDP-mannose, we incubated His₆-SidI_{CA68} with GDP-mannose and quantified generation of free GDP. Full-length SidI and SidI_N, which is not predicted to possess glycosyltransferase activity, were included as controls. Neither SidI_N nor SidI_{CA68} were sufficient to cleave GDP-mannose (**Fig 7C**), suggesting that SidI enzymatic activity is dependent on both the N- and C-terminal domains.

SidI enzymatic activity is dampened by SusF

Based on the ability of SusF to suppress SidI-mediated translation inhibition, we hypothesized that SusF could regulate SidI-mediated GDP-dependent glycosyltransferase activity. Thus, SidI-mediated GDP-mannose cleavage in the presence of equimolar amounts of SusF was quantified as above. We found that SusF was sufficient to significantly decrease – but not inhibit – SidI GDP-dependent glycosyltransferase activity (** $P < 0.01$, **Fig 7D**). We further evaluated whether addition of molar excess of SusF would further decrease SidI glycosyltransferase activity; however, addition of up to a 5-fold molar excess of SusF to SidI was not sufficient to significantly inhibit SidI activity compared to equimolar quantities (**Fig 7E**). Thus, SusF dampens SidI glycosyltransferase activity *in vitro*.

247

248 Discussion

249 *Legionella pneumophila* is an opportunistic, intracellular pathogen that exploits host cell
250 machinery to promote intracellular replication through the translocation of effector proteins. SidI
251 is one of seven cytotoxic effectors that inhibit eukaryotic protein translation (SidI, SidL, Lgt1-3,
252 LegK4, Lpg1489) (12, 13, 15, 18). Within this family of effectors, SidI is distinct as its regulation by
253 the metaeffector Lpg2505 (SusF) is essential for intracellular replication (6). Our study aimed to
254 explore the role of SusF in regulation of SidI function and elucidate potential mechanisms behind
255 SidI-mediated toxicity. Here we demonstrate that SusF binds directly to SidI with high affinity
256 and modulates both SidI-mediated translation inhibition and novel GDP-dependent
257 glycosyltransferase activity, which has not been previously observed for a bacterial effector. We
258 further demonstrate that SidI is able to simultaneously bind SusF and eEF1A, which interact with
259 distinct regions of SidI. This work is the first to define the enzymatic activity of SidI and the
260 contribution of SusF to SidI-mediated phenotypes.

261 *L. pneumophila* is reliant on the host cell-derived amino acids for intracellular replication
262 (31). It can therefore be speculated that *L. pneumophila* utilizes multiple effector proteins to halt
263 host protein synthesis at the elongation step in order to facilitate proteasomal degradation of
264 partially folded polypeptides. The translation inhibiting effectors characterized to date have
265 diverse functions. The effector Lgt1 was found to target the host elongation factor eEF1A and
266 homology searches led to the discovery of the orthologous effectors Lgt2-3(19). The Lgt effectors
267 function by glycosylation of eEF1A at Ser-53, which results in blockade of host protein synthesis

(32). The Lgts also glycosylate a eukaryotic release factor related protein (eRF3) and the Hsp70 subfamily B suppressor 1 (Hbs1) (33, 34). Based on the function of Lgt1-3, attempts were made to identify SidI-mediated post-translational modification of eEF1A; however, no modifications were discovered (12). Although SidI was previously assumed to lack glycosyltransferase activity (12, 35), we discovered that SidI, like the Lgts, is indeed able to cleave GDP-mannose, suggesting that SidI is a mannosyltransferase. However, future investigation is required to reveal the target of SidI's activity *in vivo* and the molecular mechanism by which SidI inhibits translation.

SidI induces the host stress response through formation of a complex between heat shock factor 1 (HSF1) and eEF1A(12). The formation of this complex in conjunction with a non-coding RNA promotes the binding of HSF1 to the heat shock element (HSE), inducing host cell *Hsp70* expression (12, 36). Notably, despite the upregulation of transcription, Hsp70 protein levels did not differ between infected and uninfected cells (12), likely due to robust translation inhibition by *L. pneumophila* effectors. A more recent study revealed that the eEF1A1 isoform facilitates expression of heat shock genes independently of its role in protein translation (37). Since SidI, but not Lgt1, induced expression of *Hsp70* in addition to eEF1A-mediated activation of the heat shock factor 1 (HSF1) transcription factor, it could be hypothesized that SidI modulates eEF1A to specifically amplify heat shock genes, which could enhance survival of *L. pneumophila* infected cells. Moreover, the effector LegK4 phosphorylates Hsp70, which results in loss of protein translation. It is tempting to envision a scenario whereby SidI and LegK4 function in concert to modulate host Hsp70 activity. The importance for modulation of heat shock proteins has also been demonstrated by previous observations that Hsp90 is essential for *L. pneumophila* replication

in *Acanthamoeba castellanii* (38). Further investigation is required to define the influence of SusF on SidI-mediated modulation of the heat shock response.

Regulation of effector function by metaeffectors is an essential component of the *L. pneumophila* virulence strategy. The first identified *L. pneumophila* metaeffector, LubX, functions as an E3 ubiquitin ligase that is translocated into the host cytosol at late stages of infection and catalyzes ubiquitination of the effector protein SidH, targeting it for proteasomal degradation (5). *L. pneumophila* Δ *lubX* mutants replicate similarly to wild-type bacteria, suggesting that LubX is not individually required for intracellular replication in host cells examined (5). Like SusF, the metaeffector SidJ directly contributes to intracellular replication by suppressing the toxicity of the SidE family of effectors (SdeA, SidE, SdeB and SdeC) (7, 39). However, unlike SusF, overexpression of SidJ is toxic to eukaryotic cells (39). The SidE family of effectors also contributes directly to intracellular replication through a novel mechanism of phosphoribosyl-ubiquitin conjugation to host substrates (40–42). Unlike the SidE effectors, loss of *sidI* or the *sidI-lpg2505* operon has no effect on *L. pneumophila* intracellular growth (6), suggesting that SidI functions redundantly within macrophages. However, in the absence of SusF, SidI is deleterious to *L. pneumophila* intracellular replication (6), a phenotype not observed for any other effector.

Several modes of metaeffector function have been described. A large-scale screen by Urbanus and colleagues led to the discovery of 17 novel *L. pneumophila* metaeffectors that function to suppress the toxicity of their cognate effectors (4). They uncovered several mechanisms by which metaeffectors regulate their cognate effectors through abrogation of effector enzymatic activity *in vitro* (LegL1, SidP, LupA). Similarly, we found that SusF dampens SidI activity *in vitro*;

however, residual SidI activity is retained, suggesting that SusF functions to fine-tune SidI activity. Although we have not identified the *bona fide* substrate of SidI, our observation that SidI interacts with both SusF and eEF1A simultaneously at distinct regions suggests that SidI may modify eEF1A or use interaction with eEF1A to gain access to a host substrate. Future investigation will reveal the role of SusF in SidI function and how this contributes to *L. pneumophila* intracellular replication.

Through structural homology prediction and biochemical analysis, we revealed that SidI likely possesses GDP-dependent glycosyltransferase activity. Although we have not directly demonstrated the transfer of mannose to a substrate protein, it is unlikely that SidI functions only as a nucleotide-sugar hydrolase in the context of infection. Moreover, detection of free nucleotide liberation from nucleotide-sugar donors is a pre-requisite for transfer of glycans to substrate molecules and is an established method to identify glycosyltransferase activity without knowing the acceptor substrate (43). Our use of a luciferase-based assay eliminates the requirement for radioactive nucleotide-sugars.

Several *L. pneumophila* effectors function as glycosyltransferases, including the translation inhibitors Lgt1-3; however, none have been shown to utilize GDP-conjugated sugars. In fact, bacterial GDP-dependent glycosyltransferases seem to be involved primarily in biogenesis of cell surface structures (44–46). SidI has predicted structural homology to mycobacterial phosphatidylinositol mannosides (PIMs), which are GT-B glycosyltransferases (45, 47). To our knowledge, no other translocated bacterial effector has been demonstrated to have GDP-dependent glycosyltransferase activity. The primary site of protein glycosylation in eukaryotic

cells is the Golgi apparatus; however, nucleotide sugars, including GDP-mannose, are synthesized in the cytoplasm of eukaryotic cells prior to transport into the Golgi (48, 49). Thus, SidI likely hijacks cytoplasmic GDP-mannose prior to its translocation into the Golgi. Based on the relatively low abundance of individual effectors in *L. pneumophila* infected cells it is unlikely that SidI function influences glycoprotein production by host cells. Thus, SidI is a GDP-dependent glycosyltransferase and this activity is likely critical for SidI-mediated translation inhibition and cytotoxicity. Further biochemical and structure-function analysis will reveal the detailed molecular mechanism by which SidI functions, its *bona fide* substrate in host cells and how SusF regulates its activity to promote *L. pneumophila* intracellular replication.

In this study, we have revealed a direct high-affinity interaction between SidI and its metaeffector SusF, defined a novel enzymatic activity for SidI and uncovered that SusF can modulate SidI function *in vitro*. Our work has provided a foundation for future biochemical and cell biological studies to reveal how SidI functions to modulate host cell processes. The severe virulence defect resulting from expression of *sidI* in the absence of *susF* underlies the importance of defining the molecular mechanism of SidI-SusF function. Moreover, the lack of precedent for a bacterial GDP-dependent glycosyltransferase effector protein suggests that SidI possesses novel enzymatic activity, which must be regulated by SusF to promote *L. pneumophila* intracellular replication. Our future work will focus on uncovering this mechanism in order to gain critical insight into translation inhibition and metaeffector function.

Materials and Methods

Bacterial strains, cell culture, growth conditions and reagents.

Escherichia coli strains used for cloning (Top10; Invitrogen) and protein expression [BL21 (DE3); a gift from Dr. Craig Roy, Yale University] were maintained in Luria-Bertani (LB) medium supplemented with antibiotics as appropriate for plasmid selection [50 µg mL⁻¹ kanamycin (GoldBio), 100 µg mL⁻¹ ampicillin (GoldBio) and 25 µg mL⁻¹ chloramphenicol (GoldBio)]. *Legionella pneumophila* Philadelphia-1 Lp01 (50) and $\Delta dotA$ (51) strains were cultured on supplemented charcoal-N (2-acetamido)-2-aminoethanesulfonic acid (ACES)-buffered yeast extract (CYE) and grown at 37°C as described previously (52, 53). CYE was supplemented with 10 µg mL⁻¹ chloramphenicol for plasmid maintenance as required. Protein expression in *E. coli* and *L. pneumophila* was induced with 1 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG) (GoldBio).

All mammalian cells were grown at 37°C/5% CO₂ for up to 30 passages. HEK 293 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM; Gibco) supplemented with 10% heat-inactivated fetal bovine serum (HIFBS; Gibco). CHO FcγRII cells (54) (a gift from Dr. Craig Roy) were cultured in MEMα (Gibco) supplemented with 10% HIFBS.

Unless otherwise specified, all chemicals were obtained from MilliporeSigma (St Louis, MO). Oligonucleotide primers used in this study are listed in **Table 1**.

Molecular cloning, plasmid construction and generation of *Legionella* strains.

Legionella pneumophila Lp01 gDNA was isolated using the Illustra genomicPREP DNA isolation spin kit (GE Healthcare) and was used as a template for cloning *sidI* and *susF* into the indicated plasmid vectors. For recombinant protein production, *sidI* was amplified using primer pairs SidIBamHI-F/SidINotI-F and cloned as a BamHI/NotI fragment into pGEX-6P-1 (GE Healthcare) and pT7HMT (55). *susF* was amplified using primer pairs SusFBglII-F/SusFNotI-R and cloned as a BglII/NotI fragment into BamHI/NotI-digested pGEX-6P-1 and pcDNA4T/O-3xFLAG (56). For cloning into pT7HMT, *susF* was amplified using SusFSall-F/SusFNotI-R primer pairs and cloned as a Sall/NotI fragment. For generation of SidI truncations, the regions of interest were amplified with primer pairs SidIBamHI-F/SidINNotI-R (SidI_N), SidICBamHI-F/SidINotI-R (SidI_C), or SidICBamHI-F/SidICΔ68NotI-R (SidI_{CA68}) and cloned as BamHI/NotI fragments into pT7HMT or pGEX-6P-1. For generation of *pcyaA::sidI_{RP}*, *sidI_{R453P}* was amplified from pSR47S::*sidI_{R453P}* (6) with SidIBamHI-F2/SidIPstI-R and cloned as a BamHI/PstI fragment into *pcyaA* (21). DNA sequences were confirmed by Sanger sequencing (GENEWIZ, South Plainfield, NJ).

L. pneumophila Lp01 wild-type and Δ*dotA* producing CyaA-SidI constructs were generated by electroporation of *pcyaA::sidI_{RP}* into competent strains using a BioRad Gene Pulser at 2.4 kV, 200 Ω, and 0.25 μF and plated on CYE supplemented with 10 μg mL⁻¹ chloramphenicol. CyaA-SidI production was confirmed by Western blot as described below. *L. pneumophila* strains harboring *pcyaA::ralF* (21) were a gift from Dr. Craig Roy (Yale University).

Transfection and selection of stable tissue culture cells

HEK 293 cells were transfected with pcDNA4T/O-3FLAG::*susF* using FuGENE HD (Roche) transfection reagent according to manufacturers' instructions. At 48 h post-transfection, cells 500 $\mu\text{g mL}^{-1}$ zeocin (Invitrogen) was added to culture medium and this selection was maintained for 10 days. Subsequently, cells were maintained in 200 $\mu\text{g mL}^{-1}$ zeocin and production of 3FLAG-SusF was confirmed by Western blotting, as described.

Recombinant protein expression and purification

Overnight *E. coli* BL21 (DE3) cultures were sub-cultured at 1:100 for 3 h in LB supplemented with the appropriate antibiotics followed by induction of protein expression with 1 mM IPTG, and induced at 16°C overnight. Bacterial cultures were centrifuged at 4,200 r.c.f. for 5 min at 4°C and washed with ice-cold PBS followed by incubation in bacterial lysis buffer [50 mM Tris pH 8, 100 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 200 $\mu\text{g mL}^{-1}$ lysozyme, 2 mM dithiothreitol (DTT), 10 $\mu\text{g mL}^{-1}$ DNase, and complete protease inhibitor] for 30 min on ice. Bacteria were sonicated on ice followed by centrifugation at 17,000 r.c.f. for 30 min at 4°C. Clarified bacterial lysates were incubated with either Ni-NTA beads plus 15 mM imidazole (His-tag) or Glutathione Agarose beads (GST-tag) for 1-2 h at 4°C with rotation. For His-tagged proteins, Ni-NTA agarose beads were transferred to 10 mL polyprep chromatography columns (BioRad) and washed with 20 mL of ice-cold wash buffer (50 mM Tris pH 8, 100 mM NaCl, 1 mM EDTA, 40 mM imidazole) followed by elution in 7 mL of elution buffer (50 mM Tris pH 8, 100 mM NaCl, 1 mM EDTA, 200 mM imidazole). For GST-tagged proteins, glutathione agarose beads were transferred to a 10 mL polyprep chromatography column and washed with 20 mL wash

buffer I (PBS, 0.05% Triton X-100), followed by wash buffer II (PBS, 0.05% Triton X-100, 0.5 M NaCl) and eluted in GST elution buffer (50 mM Tris pH 9.5, 10 mM glutathione). Protein eluates were visualized by SDS-PAGE and Coomassie brilliant blue staining. Elution fractions containing protein were combined, dialyzed into PBS and quantified by Bradford Assay (Thermo Scientific).

In some cases, the poly-histidine tag was removed from recombinant proteins by site-specific proteolysis using Tobacco Etch Virus protease. Following digestion as previously described (55), the sample was reappplied to a Ni-NTA beads and the unbound fraction containing the target protein was collected. Samples were further purified by gel filtration chromatography using either a Superdex 75 (26/60) or Superdex 200 (26/60) column attached to an AKTA-format FPLC (GE Healthcare) and PBS as a running buffer. Elution fractions containing protein were analyzed by SDS-PAGE and Coomassie brilliant blue staining, as described above.

Affinity chromatography for protein-protein interaction

Overnight *E. coli* cultures grown in LB plus appropriate antibiotic were sub-cultured 1:100 into 20 mL LB and grown at 37°C for 3 h followed by induction with 1 mM IPTG for 4 h. Cultures were centrifuged for 10 min at 1500 r.c.f., washed with ice-cold PBS and pelleted in 1 mL aliquots prior to storage at -20°C for <72 hours until use. Pellets were re-suspended in 1 mL of cold lysis buffer (50 mM Tris pH 8, 100 mM NaCl, 1 mM EDTA) supplemented with 200 µg mL⁻¹ lysozyme, and complete protease inhibitor and incubated on ice for 30 min. Two millimolar DTT was added to lysates before sonicating on ice. Lysates were then clarified by centrifugation at 12,600 r.c.f. for 15 min at 4°C. Supernatants were collected in fresh, pre-chilled microcentrifuge tubes.

Supernatants were added to either pre-equilibrated Ni-NTA magnetic beads or magnetic glutathione agarose beads following manufacturer's protocol (Pierce) for binding His- or GST-tagged fusion proteins, respectively. After the initial batch binding, beads were washed 2x with wash buffer [Ni-NTA: 50 mM Na₃PO₄, 300 mM NaCl, 15 mM imidazole, 0.05% Tween-20, pH 8; Glutathione: 125 mM Tris-Cl, 150 mM NaCl, 1mM DTT, 1 mM EDTA, pH 7.4] prior to addition of recombinant protein or subsequent cell lysate and batch binding for 1 h at 4°C with rotation. Beads were washed 2x with wash buffer before adding the final cell lysate or recombinant protein and incubating for 1 h at 4°C with rotation. Following binding, beads were washed 2x with wash buffer and transferred to fresh, pre-chilled 1.5 mL microcentrifuge tubes and resuspended in 25 µL of 3x Laemmli sample buffer and boiled for 10 min. Samples were analyzed by SDS-PAGE and Coomassie brilliant blue or Western Blot, as indicated.

For experiments to examine eEF1A binding, lysates were derived from either HEK293T or HEK293 3FLAG-SusF cells grown to ≥70% confluence on tissue culture (TC) treated dishes. Cells were washed 1x with ice-cold PBS and lysed in 1 mL of mammalian lysis buffer [1% NP-40 (v/v), 150 mM NaCl, 20 mM Tris-Cl pH 7.5, 10 mM Na₄P₂O₇, 50 mM NaF, and complete protease inhibitor]. Lysates were collected in pre-chilled, 1.5 mL centrifuge tubes and centrifuged at 11,000 r.c.f. for 20 min at 4°C. Supernatants were collected in pre-chilled, 1.5 mL centrifuge tubes and stored on ice until use.

CyaA effector translocation assay

Translocation of CyaA fusion proteins was performed as described (21). Briefly, CHO FcγrII cells (a gift from Dr. Craig Roy) were seeded into 24-well plates at 1x10⁵ cells per well 24 h prior to infection. Cells were infected at a multiplicity of infection (MOI) of 30 with *L. pneumophila* Lp01 wild-type or $\Delta dotA$ that had been cultured on CYE supplemented with 10 μg mL⁻¹ chloramphenicol and 1 mM IPTG followed by opsonization by incubation for 30 min with an α-*L. pneumophila* antibody (Invitrogen, PA17227; 1:1000) at RT. Cell culture media was supplemented with 1 mM IPTG to ensure expression of CyaA-SidI fusion protein. Infected cells were incubated for 1-2 hours at 37°C/5% CO₂ followed by aspiration of culture media and washing 3 times with ice-cold PBS. Cells were lysed for 30 minutes in 200 μL of ice-cold lysis buffer (50 mM HCl, 0.1% Triton-X100) with rocking at 4°C. Samples were either stored at -80°C until use or immediately mixed with 12 μL 0.5M NaOH in 95% ethanol and centrifuged for 5 min at 11,000 r.c.f. Supernatants were dried in a Speed Vac and stored at -80°C until use. cAMP was quantified using a cAMP Direct Biotrak EIA (non-acetylation) kit (GE Healthcare). Briefly, dried samples were resuspended in 250 μL Assay Buffer and ELISA was performed following manufacturer's protocol. Absorbance at 655 nm was read in a Victor 2 microplate reader (PerkinElmer).

In vitro protein translation assay

Translation assays were performed using the Promega Flexi® Rabbit Reticulocyte Lysate System (RLL; L4540) following manufacturer's protocol. Briefly, a master mix of RLL was generated and aliquoted into individual tubes. Four nanograms of purified His₆- SidI (see above) were added as

indicated. Purified recombinant (untagged) SusF was added at the indicated concentrations. Proteins were equilibrated to room temperature before use. All reactions were brought to 50 μ L with ultra-pure water. Reactions were mixed by pipetting and briefly centrifuged before incubation at 30°C for 90 min. Translation of *Firefly* luciferase mRNA (Promega) was quantified using a Victor 2 microplate reader (PerkinElmer).

Glycosyltransferase activity assay

Glycosyltransferase activity was evaluated using GDP- or UDP-Glo™ Glycosyltransferase Assay kits (Promega) with GDP-mannose (VA1095) or UDP-glucose (V6991), respectively, following manufacturers' recommendations. Briefly, 5 μ g of purified His₆-SidI and/or molar equivalent (or excess as indicated) SusF were added to 100 μ L of 50mM Tris pH 7.4 with 10 μ M of GDP-mannose or UDP-glucose. Ten μ M GDP or UDP were used as controls as indicated. Reactions were carried out for an hour at 37°C and quantification of free nucleotide (GDP or UDP) was achieved by addition of GDP- or UDP-Glo™ nucleotide detection reagent following manufacturers' instructions and analyzed via luminescence using a Victor 2 microplate reader (PerkinElmer).

SDS-PAGE and Western Blot

Boiled protein samples were loaded onto either 4-20% gradient SDS-PAGE gels (BioRad), 12% or 15% SDS-PAGE gels. Following electrophoresis, proteins were visualized with Coomassie brilliant blue or transferred to PVDF membranes using a BioRad wet transfer cell. Membranes

were incubated with blocking buffer [5% nonfat milk powder dissolved in tris-buffered saline-0.1% Tween 20 (TBST)]. Primary antibodies [α -eEF1A (#2551S Cell Signaling Technology), α -Flag-M2 (Sigma), or α -CyaA[3D1] (#EG800 Kerafast)] were used 1:1000 in blocking buffer and detected with HRP-conjugated secondary antibodies (1:5000; ThermoFisher). Membranes were washed, incubated with ECL substrate (GE Amersham) and imaged by chemiluminescence using an Azure Biosystems c300 Darkroom Replacer.

Surface Plasmon Resonance

Direct binding of His₆-SidI to SusF was assessed by SPR using a Biacore T-200 instrument (GE Healthcare) at 25 °C, according to the general methods previously described (57). Briefly, all experiments were carried out in a running buffer of HBS-T (20 mM HEPES (pH 7.4), 140 mM NaCl, and 0.005% (v/v) Tween-20 and a flow-rate of 30 μ l/min (57). SusF (50 μ g mL⁻¹ in 10 mM acetate, pH 4.5) was immobilized to a final density of 1063 RU on one flow cell of a CMD-200M surface (XanTec Bioanalytics, GmbH; Dusseldorf, Germany) using standard NHS/EDC coupling. A reference surface was prepared in a similar manner by ethanolamine quenching of NHS/EDC-activated flow cell. Experimental sensorgrams of His₆-SidI binding to immobilized SusF were obtained in reference-corrected, single-cycle mode using sequential concentrations of 0.8, 4, 20, 100, and 500 nM His₆-SidI. Each association phase consisted of 2 min sample injection, followed by a 1.5 min dissociation phase, except for the final injection which incorporated a 60 min dissociation phase for more accurate determination of the dissociation rate. Kinetic analysis was

performed using Biacore T-200 Evaluation Software v3.1 (GE Healthcare). Sensorgrams were analyzed using both Langmuir and Two-State Reaction binding models and a local value of R_{max} .

Analytical Gel Filtration Chromatography

Samples of recombinant proteins were characterized by analytical-scale gel-filtration chromatography as a mean of assessing their apparent molecular weight. All samples and standards (500 μ l total volume) were separated on a Superdex 200 10/300 column (GE Healthcare) attached to an AKTA-format FPLC system using a flow-rate of 0.5 ml/min and PBS as a running buffer. Fractions of 1 ml were collected for subsequent analysis by SDS-PAGE and Coomassie brilliant blue, as described above.

Molecular Modeling

Homology models were created using four different servers, using the full sequence of SidI as inputs and default parameters for each server. Using HHPred (23), a number of templates were identified for residues 376 to 868, and the top 25 templates were forwarded for modeling. Using Phyre2 (24), a smaller region, residues 585 to 763, was chosen for modeling. Raptor-X (20) and I-TASSER (25–27) both produce full-length models. Raptor-X predicted residues 350 to 870 to be a domain. I-TASSER presented five models of low confidence, one of which contained a glycosyltransferase domain with a GT-B fold, which was selected as the working model.

Statistical Analysis

Statistical analysis was performed with GraphPad Prism software using Students' t-test, as indicated, with a 95% confidence interval. For all experiments, data are expressed as mean $\pm$ standard deviation (s.d.) of samples in triplicates.

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Tables

Table 1. Oligonucleotide primers used in this study

Name	Sequence (5' → 3') ^a
SusFSall-F	attg tcgaca atgataaaaggaaaacttatgcc
SusFBglII-F	tgg agatc tatgataaaaggaaaacttatgc
SusFPstI-R	gg ctgcag ttataaaataattggtcgag
SidIBamHI-F2	attg gatc cttatgactaaaatatacttattaactgc
SidIBamHI-F	attg gatc catgactaaaatatacttattaactgc
SidINotI-R	attg cggccg ctcaaaataccagtatcgattctttaag
SidICBamHI-F	attg gatc catgaatttttatgattttgatgg
SidICA68NotI-R	attg cggccg ctcatatattcttaataaatgatc

SidINNotI-R attg**cg**g**cc**gctcatctgaaacttttatcgtgctc

SidIPstI-R att**ctg****cag**tcaaaataccagtatcgattc

^a-restriction endonuclease cleavage sites are shown in bold

Figure Legends

Figure 1. SusF and SidI interact directly with nanomolar affinity. **(A)** Lysates from HEK 293 cells stably expressing 3FLAG-SusF were incubated with glutathione beads coated with either GST or GST-SidI followed by Coomassie staining for total protein and Western blotting for SusF (α -FLAG). Arrowheads indicate GST and GST-SidI proteins. **(B)** Lysates from *E. coli* overexpressing either GST or GST-SidI were incubated with Ni-NTA beads coated with His₆-SusF followed by SDS-PAGE and Coomassie staining for proteins retained on the beads. Left panel: whole cell lysates from uninduced and induced cultures of *E. coli* expressing GST and GST-SidI proteins; Right panel: proteins retained on Ni-NTA beads (see *Materials and Methods*). **(C)** Chromatograms resulting from analytical scale gel-filtration separation of either SusF alone (green trace) or SusF bound to SidI (red trace). A chromatogram of known size standards is provided for reference (blue trace). **(D)** Binding of His₆-SidI to immobilized SusF was assessed by SPR. The reference corrected sensorgram from a single-cycle experiment is shown in black, while the outcome of fitting to a two-state binding model is shown in red. The interaction is described by an apparent K_D of 3.1 nM, consisting of two individual steps where $k_{on,1} = 2.7 \times 10^4 \text{ M}^{-1}\text{s}^{-1}$ and $k_{off,1} = 5.1 \times 10^{-4} \text{ s}^{-1}$ and $k_{on,2} = 2.3 \times 10^{-3} \text{ s}^{-1}$ and $k_{off,2} = 4.5 \times 10^{-4} \text{ s}^{-1}$, respectively.

Figure 2. SusF modulates SidI-mediated translation inhibition *in vitro*. Translation of luciferase mRNA was quantified using a rabbit reticulocyte lysate kit. Translation of luciferase in the presence of **(A)** 4 ng His₆-SidI or 20 ng SusF; **(B)** His₆-SidI alone or 4 ng (37 fmol) His₆-SidI and an equimolar amount of SusF (37 fmol); **(C)** His₆-SidI alone or His₆-SidI and SusF were added to *in vitro* translation reactions at the indicated times (-30 indicates pre-incubation of His₆-SidI and SusF for 30 min prior to translation reaction); **(D)** Quantification of *In vitro* translation of Luc mRNA in the presence of His₆-SidI alone or His₆-SidI and SusF at increasing molar ratios (1:1, 2:1 and 5:1 molar ratios of SusF:His₆-SidI). Data are representative of at least two independent experiments and shown as mean $\pm$ s.d. of samples in triplicates. Asterisks denote statistical significance by *t*-test (**P*<0.05, ***P*<0.01; n.s., not significant).

Figure 3. SusF does not impair interaction between SidI and eEF1A. **(A)** GST-SusF or GST alone were immobilized on magnetic glutathione agarose beads followed by incubation with 100 μ g purified His₆-SidI alone or that had been preincubated with lysates from HEK 293T cells as indicated (Lysates). Proteins were separated by SDS-PAGE and visualized by Coomassie staining or Western blot as indicated. **(B)** Lysates from *E. coli* expressing GST-SidI or GST alone were incubated with magnetic glutathione agarose beads and lysates from HEK 293T followed by 10, to 1000 μ g of purified recombinant SusF (shown as increasing amounts in supernatants from beads). Proteins remaining on the beads were separated by SDS-PAGE and visualized by Coomassie stain or Western blot as indicated. GST-SidI (~130 kDa) and SusF (~27 kDa) are indicated with arrowheads. Data are representative of at least two independent experiments.

794

795 **Figure 4. SusF and eEF1A interact with distinct regions of SidI. (A)** Schematic representation of
796 SidI truncation proteins. **(B)** Lysates from *E. coli* expressing GST-SidI constructs were incubated
797 with glutathione agarose beads followed by washing and incubation with lysates from HEK 293
798 cells stably expressing 3FLAG-SusF as indicated. Proteins were separated by SDS-PAGE and
799 visualized by Coomassie stain (GST-SidI) or Western blot. Arrowheads indicate fusion proteins.
800 Data are representative of three independent experiments.

801

802 **Figure 5. SusF is not required for Dot/Icm translocation of SidI into host cells. (A)** Western blot
803 showing production of CyaA-RalF (~76 kDa) and CyaA-SidI_{RP} (~150 kDa) by the indicated *L.*
804 *pneumophila* strains. **(B)** Quantification of cAMP extracted from CHO FcγRII cells infected with
805 the indicated *L. pneumophila* strains. Asterisks denote statistical significance by *t*-test (***P*<0.01).
806 Data are representative of two independent experiments (n.s., not significant).

807

808 **Figure 6. Full-length SidI is required for translation inhibition.** Translation of luciferase mRNA
809 quantified using a rabbit reticulocyte lysate kit. **(A)** Translation of luciferase mRNA in the
810 presence of **(A)** 4 ng His₆-SidI, His₆-SidI_N, His₆-SidI_C, or His₆-SidI_{CA68} or **(B)** 4 ng of His₆-SidI alone
811 or increasing amounts of His₆-SidI_{CA68} (4 ng, 50 ng, 100 ng). Asterisks denote statistical significance
812 by *t*-test (***P*<0.01). Data are representative of at least two independent experiments.

813

Figure 7. SidI possesses glycosyltransferase activity. (A) Quantification of GDP liberated from GDP-mannose in the presence or absence of 5 µg purified His₆-SidI. (B) Quantification of UDP liberated from UDP-glucose in the presence or absence of 5 µg purified His₆-SidI. Quantification of free GDP from GDP-mannose in the presence or absence of (C) 5 µg His₆-SidI full-length or truncated proteins, (D) 5 µg His₆-SidI and/or molar equivalent of SusF, or (E) increasing molar amount of SusF (1:1, 2:1, or 5:1 molar ratio of SusF:His₆-SidI). Asterisks denote statistical significance by *t*-test (***P*<0.01 and **P*<0.05). Data are representative of at least two independent experiments.

Supplemental Information

Supplemental Figure Legends

Figure S1. Co-elution of SusF and SidI by gel filtration chromatography. A sample of recombinant SidI bound to SusF was separated by analytical scale gel filtration chromatography as presented in Figure 1C. Ten µl of each column fraction were analyzed by SDS-PAGE followed by Coomassie staining to assess the protein content within each fraction. Fractions corresponding to the peak of approximately 150 kDa apparent molecular weight in the sample of SidI bound to SusT (Fig. 1C, red trace) contained both proteins, consistent with formation of a 1:1 complex between these two molecules.

Figure S2. Binding of His₆-SidI to immobilized SusF was assessed by SPR. The reference corrected sensorgram from a single-cycle experiment is shown in black, while the outcome of fitting to a Langmuir binding model is shown in red. Using this model, the interaction is described by an apparent K_D of 0.89 nM where $k_{on} = 2.2 \times 10^5 \text{ M}^{-1}\text{s}^{-1}$ and $k_{off} = 1.9 \times 10^{-4} \text{ s}^{-1}$, respectively.

Figure S3. eEF1A interacts with the SidI-SusF complex. Lysates from *E. coli* expressing GST-SidI or GST alone were incubated with magnetic glutathione agarose beads and washed followed by addition of 10, 25, 50, 100, 200, 400, 800 or 1000 μg of purified recombinant SusF (shown as increasing amounts in supernatants from beads). Beads were subsequently incubated with lysates from HEK 293T cells. Proteins remaining on the beads were separated by SDS-PAGE and visualized by Coomassie stain or Western blot

Figure S4. Molecular model of SidI. Ribbon cartoons of SidI modeled using the RaptorX webserver. Putative SidI domains are shown in red (residues 1-268), magenta (residues 269-874) and cyan (residues 874-942).

Figure S5. Interaction between eEF1A and SidI truncations in the absence of SusF. Lysates from *E. coli* expressing GST-SidI constructs were incubated with glutathione agarose beads followed by washing and incubation with lysates from HEK 293 cells. Proteins were separated by SDS-

853 PAGE and visualized by Coomassie staining (GST-SidI) or Western blotting (eEF1A). Data are
854 representative of two independent experiments.

855

856 **Figure S6. SidI is modeled as a glycosyltransferase with a GT-B fold.** The model of the putative
857 glycosyltransferase domain is presented in magenta ribbons superimposed to that of PimB (PDB:
858 3OKA) represented as salmon ribbons, which was identified by DALI as the most similar
859 structure in the PDB. The structure of PimB was solved in the presence of GDP (salmon spheres).

860

Figure 1

Figure 2

Figure 3

Figure 4

A

B

Figure 5

Figure 6

Figure 7

Figure S1

Figure S2

Figure S3

Figure S4

Figure S5

Figure S6

Supplemental Tables

Table S1. Proteins with homology to SidI (HHPred).

No.	PDB		Prob	E-value	P-value	Score	SS	Cols	Query HMM	Template HMM
1	30KP_A	GDP-mannose-dependent a	99.8	8.2E-20	1.7E-24	181.2	33.6	338	376-868	22-376 (394)
2	4N9W_A	GDP-mannose-dependent a	99.8	3.7E-19	7.5E-24	175.7	36.7	342	370-868	17-364 (390)
3	5D01_B	N-acetyl-alpha-D-glucos	99.8	2.3E-19	4.7E-24	175.8	34.1	347	370-868	15-372 (379)
4	6D9T_A	Glycosyl transferase (E	99.8	1.3E-18	2.6E-23	173.7	33.4	348	370-868	30-387 (400)
5	2JJM_J	GLYCOSYL TRANSFERASE, G	99.8	1.1E-18	2.3E-23	174.8	32.9	346	372-868	27-382 (394)
6	3C48_A	Predicted glycosyltrans	99.8	3.6E-18	7.2E-23	172.6	35.2	352	370-868	40-422 (438)
7	3FRO_B	GlgA glycogen synthase	99.8	5.5E-18	1.1E-22	169.0	34.6	347	370-868	16-427 (439)
8	2R60_A	Glycosyl transferase, g	99.8	1.1E-17	2.3E-22	172.5	37.8	351	370-868	31-456 (499)
9	30Y2_B	Glycosyltransferase B73	99.7	1.3E-18	2.6E-23	176.1	28.3	339	376-868	17-387 (413)
10	6EJI_B	WlaC protein; Glycosylt	99.7	2E-17	4.1E-22	162.9	33.8	333	373-868	15-354 (373)
11	6GNE_A	Probable starch synthas	99.7	8.5E-18	1.7E-22	176.0	31.4	352	369-868	23-494 (508)
12	2QZS_A	PROTEIN; glycosyl-trans	99.7	1.6E-17	3.2E-22	171.3	28.9	347	371-869	16-474 (485)
13	2X6Q_A	TREHALOSE-SYNTHASE TRET	99.7	7.6E-17	1.5E-21	161.9	31.8	386	328-868	8-411 (416)
14	4XYW_A	O-antigen biosynthesis	99.7	1.4E-16	2.8E-21	155.5	31.5	315	376-868	17-336 (338)
15	4XS0_A	Glycosyltransferase (E.	99.7	4.4E-17	8.9E-22	162.6	28.5	291	429-868	81-381 (388)
16	1RZV_A	Glycogen synthase 1 (E.	99.7	2.3E-16	4.6E-21	162.3	33.7	344	371-868	16-472 (485)
17	1V4V_B	UDP-N-Acetylglucosamine	99.7	5.1E-17	1E-21	160.9	25.2	341	370-869	13-366 (376)
18	2IW1_A	LIPOPOLYSACCHARIDE CORE	99.7	4.5E-16	9.2E-21	150.8	31.0	344	372-868	14-368 (374)
19	5ENZ_A	UDP-GlcNAc 2-epimerase	99.6	1.3E-16	2.6E-21	159.1	26.6	344	367-869	6-366 (385)
20	1XV5_A	DNA alpha-glucosyltrans	99.6	8.9E-17	1.8E-21	163.7	25.6	348	370-868	13-399 (401)
21	2X0D_B	WSAF; GT4 FAMILY, TRANS	99.6	9E-16	1.8E-20	155.5	31.7	335	369-868	60-413 (413)
22	5N7Z_A	Lipopolysaccharide 1,6-	99.6	1.5E-15	3.1E-20	147.4	31.5	339	372-868	14-357 (359)
23	5E9T_C	Glycosyltransferase Gtf	99.6	1E-15	2E-20	163.6	32.1	312	388-868	169-497 (503)
24	1VGV_D	UDP-N-acetylglucosamine	99.6	1.9E-16	3.8E-21	156.8	24.3	340	367-868	5-369 (384)
25	3T7D_A	Putative glycosyltransf	99.6	5.8E-16	1.2E-20	169.6	30.2	297	430-868	136-471 (497)
26	4FKZ_B	UDP-N-acetylglucosamine	99.6	2.8E-16	5.7E-21	157.8	24.4	334	377-869	18-368 (388)
27	5DLD_A	UDP-N-Acetylglucosamine	99.6	4.3E-16	8.6E-21	158.4	25.6	338	369-868	16-378 (413)
28	3VUE_A	Granule-bound starch sy	99.6	3.1E-15	6.2E-20	162.0	32.3	350	372-868	26-508 (536)
29	5U0F_B	Alpha,alpha-trehalose-p	99.6	3.9E-16	7.9E-21	167.1	24.3	281	443-868	131-461 (481)

896	30	3DZC_B	UDP-N-acetylglucosamine	99.6	5.8E-16	1.2E-20	156.0	23.7	331	378-866	41-396 (396)
897	31	6GNF_A	Glycogen synthase (E.C.	99.6	5.4E-15	1.1E-19	160.0	31.7	345	369-868	35-536 (544)
898	32	3S28_H	Sucrose synthase 1 (E.C	99.6	3.6E-15	7.3E-20	176.5	32.3	341	382-868	320-766 (816)
899	33	6NGG_A	Granule-bound starch sy	99.6	5.7E-15	1.2E-19	165.6	32.2	347	368-868	52-556 (612)
900	34	3OT5_B	UDP-N-acetylglucosamine	99.6	1.1E-15	2.2E-20	154.9	23.3	333	378-869	43-393 (403)
901	35	4HWG_A	UDP-N-acetylglucosamine	99.6	7.2E-16	1.5E-20	155.2	21.7	342	369-869	16-375 (385)
902	36	4X7R_B	TarM; Glycosyltransfera	99.6	7.7E-15	1.6E-19	162.3	31.8	283	429-869	204-492 (493)
903	37	5LQD_D	Alpha,alpha-trehalose-p	99.6	4.7E-15	9.6E-20	159.7	29.0	287	443-868	146-462 (465)
904	38	4HLN_A	Starch synthase I (E.C.	99.6	1.1E-14	2.2E-19	163.4	33.0	343	369-868	138-622 (633)
905	39	4PQG_A	Glycosyltransferase Gtf	99.6	4.5E-15	9.1E-20	160.2	28.8	285	427-868	203-505 (511)
906	40	5JIO_A	Alpha,alpha-trehalose-p	99.5	5.3E-15	1.1E-19	156.1	25.3	307	443-868	136-474 (487)
907	41	4L22_A	Phosphorylase (E.C.2.4.	99.5	2.8E-15	5.8E-20	176.9	25.4	298	442-868	269-754 (758)
908	42	5HVO_C	trehalose-6-phosphate p	99.5	4.5E-15	9E-20	160.5	24.7	285	443-868	141-474 (479)
909	43	3S2U_A	UDP-N-acetylglucosamine	99.5	3.4E-14	7E-19	138.8	27.3	316	372-868	13-355 (365)
910	44	4W6Q_A	Glc syltransferase C; G	99.5	4.2E-14	8.4E-19	139.3	27.5	276	417-868	38-330 (333)
911	45	4NES_A	UDP-N-acetylglucosamine	99.5	3.5E-15	7.2E-20	147.7	20.0	332	367-868	5-361 (374)
912	46	4RBN_B	Sucrose synthase:Glycos	99.5	7.8E-14	1.6E-18	165.4	31.1	299	430-868	378-754 (794)
913	47	4AMG_A	SNOGD; TRANSFERASE, POL	99.5	3.9E-14	7.9E-19	140.7	23.7	322	372-869	33-400 (400)
914	48	3RHZ_B	Nucleotide sugar synthe	99.5	9.8E-14	2E-18	136.6	26.1	279	414-868	44-334 (339)
915	49	3IA7_A	CalG4; Glycosyltransfe	99.5	3.1E-14	6.3E-19	139.0	22.3	343	372-869	15-398 (402)
916	50	5W8X_A	Lipid-A-disaccharide sy	99.5	8.6E-14	1.7E-18	137.4	25.3	324	370-868	15-377 (382)
917	51	2P6P_B	Glycosyl transferase; C	99.4	2.5E-13	5E-18	134.3	27.3	319	372-868	11-378 (384)
918	52	2IUY_A	GLYCOSYLTRANSFERASE; GL	99.4	1.5E-13	3.1E-18	136.3	25.7	296	370-868	28-332 (342)
919	53	5VAF_B	Accessory Sec system pr	99.4	6.3E-14	1.3E-18	152.9	24.9	282	429-869	214-520 (533)
920	54	5V0T_B	Alpha,alpha-trehalose-p	99.4	7.6E-14	1.5E-18	152.3	25.2	303	429-868	116-465 (494)
921	55	1UQT_B	ALPHA, ALPHA-TREHALOSE-	99.4	1.1E-13	2.3E-18	150.2	26.2	300	430-868	111-451 (482)
922	56	4RIF_B	Glycosyl transferase ho	99.4	3.8E-14	7.8E-19	140.7	20.1	321	370-868	9-373 (379)
923	57	5DXF_A	trehalose-6-phosphate p	99.4	2.9E-13	5.8E-18	150.5	29.1	313	430-868	185-523 (534)
924	58	1F0K_A	E. COLI MURG (E.C. 2.4.	99.4	1.8E-12	3.6E-17	127.8	30.3	311	370-868	15-354 (364)
925	59	3TSA_A	NDP-rhamnosyltransferas	99.4	2.1E-13	4.3E-18	134.2	23.5	320	372-868	12-387 (391)
926	60	5HVL_B	trehalose-6-phosphate p	99.4	2.4E-13	4.8E-18	146.1	25.8	286	443-868	134-467 (478)
927	61	6INF_A	UDP-glycosyltransferase	99.4	5.4E-13	1.1E-17	133.6	26.2	332	373-867	24-454 (466)
928	62	3RSC_B	CalG2; TDP, enediyne, S	99.4	6.8E-13	1.4E-17	131.3	26.5	320	372-868	31-412 (415)
929	63	3NB0_C	Glycogen [starch] synth	99.4	1.8E-13	3.6E-18	158.6	25.4	287	429-868	164-630 (725)
930	64	5IA5_A	Glycosyl transferases g	99.4	2.2E-13	4.4E-18	126.1	20.9	181	585-868	27-209 (218)
931	65	5ZFK_A	UDP-glucose:tetrahydrob	99.4	9.6E-13	1.9E-17	127.7	26.2	325	372-868	21-348 (354)

932	66	2HY7_A Crystal Structure of Gu	99.4	3.9E-13	8E-18	137.4	24.7	258	429-867	103-373 (406)
933	67	2XCI_C 3-DEOXY-D-MANNO-2-OCTUL	99.4	5.3E-13	1.1E-17	138.5	25.6	312	376-868	53-374 (374)
934	68	5ZLR_A NeuC protein; NeuC, UDP	99.4	5.2E-13	1.1E-17	132.2	24.0	315	376-868	18-364 (380)
935	69	30TI_B CalG3; Calicheamicin, T	99.3	6.1E-13	1.2E-17	131.7	21.7	323	370-868	29-396 (398)
936	70	5DU2_A CalS8; glycosyltransfer	99.3	1.1E-12	2.3E-17	129.9	23.1	322	372-868	37-415 (419)
937	71	1L5W_A MALTODEXTRIN PHOSPHORYL	99.3	2.1E-13	4.2E-18	162.7	21.2	346	429-868	277-791 (796)
938	72	4FZR_A SsfS6; Structural Genom	99.3	4.4E-13	8.9E-18	132.7	19.6	319	372-866	26-397 (398)
939	73	30TG_A CalG1; Calicheamicin, T	99.3	3.3E-12	6.6E-17	129.3	24.4	324	370-868	29-407 (412)
940	74	4QLB_D Probable glycogen [star	99.3	2.6E-12	5.4E-17	150.1	26.4	297	429-868	178-632 (674)
941	75	3HBJ_A Flavonoid 3-O-glucosylt	99.3	1.8E-11	3.6E-16	124.1	28.6	328	378-868	30-452 (454)
942	76	2IYA_B OLEANDOMYCIN GLYCOSYLTR	99.2	7.4E-12	1.5E-16	122.5	23.6	322	371-868	22-420 (424)
943	77	4X1T_A Monogalactosyldiacylgly	99.2	3.8E-11	7.7E-16	120.7	27.5	324	372-868	17-384 (408)
944	78	2ACV_B triterpene UDP-glucosyl	99.2	3.7E-11	7.5E-16	122.1	27.2	337	372-868	20-461 (463)
945	79	2YJN_A GLYCOSYLTRANSFERASE, DT	99.2	2.5E-12	5.1E-17	129.2	18.4	323	372-869	31-435 (441)
946	80	2VSY_B XCC0866; TRANSFERASE, G	99.2	1.9E-11	3.9E-16	135.3	26.9	326	371-868	217-556 (568)
947	81	3QHP_A Type 1 capsular polysac	99.2	6.1E-12	1.2E-16	110.8	17.6	163	586-863	1-166 (166)
948	82	3WAD_B Glycosyltransferase; GL	99.2	1.6E-11	3.2E-16	121.0	20.8	316	371-868	10-413 (419)
949	83	4ZHT_B Bifunctional UDP-N-acet	99.2	2.4E-11	4.9E-16	124.2	22.7	268	429-868	92-374 (411)
950	84	2C4M_A GLYCOGEN PHOSPHORYLASE	99.1	2.4E-11	4.9E-16	145.7	25.8	301	442-868	287-785 (796)
951	85	5DJS_B Tetratricopeptide TPR_2	99.1	1.1E-10	2.2E-15	128.0	26.9	330	370-868	173-512 (529)
952	86	2GJ4_A Glycogen phosphorylase,	99.1	2.6E-10	5.3E-15	137.7	30.3	295	442-868	318-813 (824)
953	87	5GL5_B Sterol 3-beta-glucosylt	99.1	2.6E-10	5.3E-15	117.1	25.5	358	373-868	51-459 (498)
954	88	5E9T_D Glycosyltransferase Gtf	99.0	6.5E-11	1.3E-15	129.3	21.3	230	475-869	215-446 (447)
955	89	2BFW_A GLGA GLYCOGEN SYNTHASE	99.0	1.1E-10	2.2E-15	106.4	18.7	161	585-856	34-200 (200)
956	90	4LDP_B NDP-forosamyltransferas	99.0	9.3E-11	1.9E-15	118.1	19.7	323	372-868	27-440 (455)
957	91	3Q3E_A HMW1C-like glycosyltran	99.0	2.3E-10	4.7E-15	132.2	24.2	279	425-868	330-624 (631)
958	92	4BQE_B ALPHA-GLUCAN PHOSPHORYL	99.0	5.5E-10	1.1E-14	136.0	28.0	315	442-868	364-865 (874)
959	93	1YGP_A YEAST GLYCOGEN PHOSPHOR	99.0	5.2E-10	1.1E-14	136.0	27.6	304	439-868	354-872 (879)
960	94	3H4T_A Glycosyltransferase Gtf	98.9	2.7E-10	5.5E-15	112.9	17.6	320	373-868	12-381 (404)
961	95	4REL_A UDP-glucose:anthocyanid	98.9	3.2E-09	6.4E-14	108.3	24.5	332	378-868	21-443 (446)
962	96	4BFC_A 3-DEOXY-D-MANNO-OCTULOS	98.8	9E-10	1.8E-14	105.4	18.2	180	586-869	40-232 (235)
963	97	2F9F_A first mannosyl transfer	98.8	9.1E-10	1.8E-14	100.5	17.2	157	582-849	18-175 (177)
964	98	5V2J_A UDP-glycosyltransferase	98.8	4.5E-09	9.1E-14	106.2	23.7	330	376-868	20-446 (449)
965	99	5LR8_A Hv_Ph01 (E.C.2.4.1.1);	98.8	2.8E-09	5.6E-14	130.9	25.4	241	494-868	527-917 (938)
966	100	6FJ3_A Parathyroid hormone/par	98.8	1.5E-09	3.1E-14	121.6	20.9	174	585-868	353-532 (602)
967	101	4M83_A Oleandomycin glycosyltr	98.8	2.1E-08	4.3E-13	103.3	25.6	318	378-868	24-398 (415)

968	102	1IIR_A	glycosyltransferase Gtf	98.7	2.8E-09	5.7E-14	105.8	16.5	313	373-868	12-398 (415)
969	103	1RRV_A	GLYCOSYLTRANSFERASE GTF	98.7	1.5E-08	3.1E-13	101.6	20.7	316	373-869	12-400 (416)
970	104	6IJ7_A	Rhamnosyltransferase pr	98.6	1.4E-07	2.8E-12	95.8	26.8	281	429-868	102-433 (435)
971	105	6BK0_A	UDP-glycosyltransferase	98.6	1.4E-07	2.8E-12	96.1	25.7	323	378-868	32-465 (467)
972	106	2VCH_A	HYDROQUINONE GLUCOSYLTR	98.3	7.9E-06	1.6E-10	84.3	28.7	340	373-868	18-467 (480)
973	107	3HBM_A	UDP-sugar hydrolase; ud	98.2	4.3E-07	8.7E-12	89.2	17.0	254	377-777	23-281 (282)
974	108	2PQ6_A	UDP-glucuronosyl/UDP-gl	98.0	1.6E-05	3.2E-10	81.4	22.3	333	378-868	25-477 (482)
975	109	2C1X_A	UDP-GLUCOSE FLAVONOID 3	97.6	0.00018	3.7E-09	74.3	21.4	284	429-868	95-450 (456)
976	110	5WQC_A	Orexin receptor type 2,	97.1	0.00055	1.1E-08	76.6	18.4	169	585-863	291-464 (560)
977	111	206L_B	UDP-glucuronosyltransfe	96.5	0.0036	7.2E-08	56.3	12.4	144	584-850	19-168 (170)
978	112	3L7I_A	Teichoic acid biosynthe	96.4	0.0077	1.6E-07	70.8	16.8	350	355-833	317-725 (729)
979	113	5U09_A	Cannabinoid receptor 1,	96.0	0.044	8.8E-07	61.4	18.2	177	585-870	243-424 (508)
980	114	5ZIC_A	Alpha-1,6-mannosylglyco	95.9	0.014	2.8E-07	69.0	13.7	157	585-873	226-407 (523)
981	115	1PSW_A	ADP-HEPTOSE LPS HEPTOSY	95.3	0.13	2.6E-06	52.7	14.8	166	585-831	179-348 (348)
982	116	5GVV_A	Glycosyl transferase fa	95.3	0.023	4.6E-07	62.2	9.9	111	606-778	289-401 (406)
983	117	2GT1_A	Lipopolysaccharide hept	94.9	0.16	3.3E-06	51.5	13.4	145	585-831	177-325 (326)
984	118	3TOV_B	Glycosyl transferase fa	94.4	0.24	4.8E-06	50.6	12.3	113	585-760	184-299 (349)
985	119	6JTD_A	C-glycosyltransferase;	92.6	1.7	3.5E-05	46.2	14.0	166	585-868	273-476 (483)
986	120	5NLM_B	indoxyl UDP-glucosyltra	90.1	8.9	0.00018	40.9	15.2	167	585-868	273-474 (478)
987	121	6MGB_A	Capsular polysaccharide	87.7	4.1	8.4E-05	43.9	10.4	102	584-746	153-262 (326)
988	122	5IJO_I	Nuclear pore complex pr	85.2	31	0.00064	41.9	16.5	192	129-377	322-515 (522)
989	123	6HLP_A	Substance-P receptor,Su	84.5	21	0.00043	40.1	13.9	177	585-870	256-437 (520)
990	124	6MGC_A	Capsule polysaccharide	83.7	5.7	0.00012	43.7	8.9	101	585-746	147-255 (361)
991	125	1JIX_A	DNA BETA-GLUCOSYLTRANSF	76.1	8.9	0.00018	43.9	6.9	108	696-855	229-345 (351)
992	126	6MGD_A	Capsular polysaccharide	66.3	30	0.00061	37.7	7.9	99	585-746	151-259 (332)
993	127	4RAP_I	Glycosyltransferase Tib	57.6	2.3E+02	0.0047	31.7	12.8	169	585-828	215-389 (406)
994	128	5IJO_G	Nuclear pore complex pr	57.6	1.2E+02	0.0024	37.8	11.3	178	121-308	241-431 (599)
995	129	5FA1_A	Cell division protein Z	56.1	57	0.0012	37.3	8.0	91	585-746	219-316 (410)
996	130	2JZC_A	UDP-N-acetylglucosamine	53.1	2.4E+02	0.0049	30.3	11.5	120	568-748	10-160 (224)
997	131	4UXV_A	SEPTATION RING FORMATIO	50.5	5.5E+02	0.011	29.9	14.6	150	138-328	361-515 (545)
998	132	2KS6_A	UDP-N-acetylglucosamine	49.7	1.8E+02	0.0036	30.6	9.6	108	582-750	1-139 (201)
999	133	5XEI_A	Chromosome partition pr	43.5	7.5E+02	0.015	28.8	21.8	316	136-481	161-482 (540)
1000	134	5ZIB_A	Alpha-1,6-mannosylglyco	42.1	54	0.0011	40.7	5.1	49	697-748	374-423 (626)
1001	135	4P5E_B	2'-deoxynucleoside 5'-p	36.7	3.1E+02	0.0063	25.7	8.2	80	625-746	1-99 (152)
1002	136	5NPS_A	UDP-N-acetylglucosamine	35.2	1.7E+02	0.0034	37.3	7.7	105	590-749	519-625 (718)
1003	137	6QAI_B	deoxyribosyltransferase	34.6	5.5E+02	0.011	24.5	10.1	83	624-746	3-99 (156)

[illegible]