

A general mechanism for initiating the bacterial general stress response

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This **important** study combines genetic analysis, biochemistry, and structural modeling to reveal new insights into how changes in protein-protein structure activate signal transduction as part of the bacterial general stress response. The data, which was collected using validated and standard methods, and its interpretations are **convincing**; however, to fully meet the title's promise, additional experimental evidence is needed to strengthen the proposed model and its potential application to other systems. This manuscript will be of broad interest to microbiologists, structural biologists, and cell biologists.

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Abstract The General Stress Response promotes survival of bacteria in adverse conditions, but how sensor proteins transduce species-specific signals to initiate the response is not known. The serine/threonine phosphatase RsbU initiates the General Stress Response in *Bacillus subtilis* upon binding a partner protein (RsbT) that is released from sequestration by environmental stresses. We report that RsbT activates RsbU by inducing otherwise flexible linkers of RsbU to form a short coiled-coil that dimerizes and activates the phosphatase domains. Importantly, we present evidence that related coiled-coil linkers and phosphatase dimers transduce signals from diverse sensor domains to control the General Stress Response and other signaling across bacterial phyla. This coiled-coil linker transduction mechanism additionally suggests a resolution to the mystery of how shared sensory domains control serine/threonine phosphatases, diguanylate cyclases and histidine kinases. We propose that this provides bacteria with a modularly exchangeable toolkit for the evolution of diverse signaling pathways.

Introduction

The General Stress Response (GSR) allows bacteria to survive in hostile and changing environments (Bergkessel et al., 2016; Harms et al., 2016; Gottesman, 2019; Guldemann et al., 2016; Hecker et al., 2007). A defining feature of the GSR is that it controls transcription of a large and diverse set of genes, dramatically reshaping the physiology of the organism (Gottesman, 2019; Guerreiro et al., 2020; Boekhoud et al., 2020; Boylan et al., 1993). Another defining feature is that the GSR is induced by a variety of stress conditions that are frequently associated with each other, including

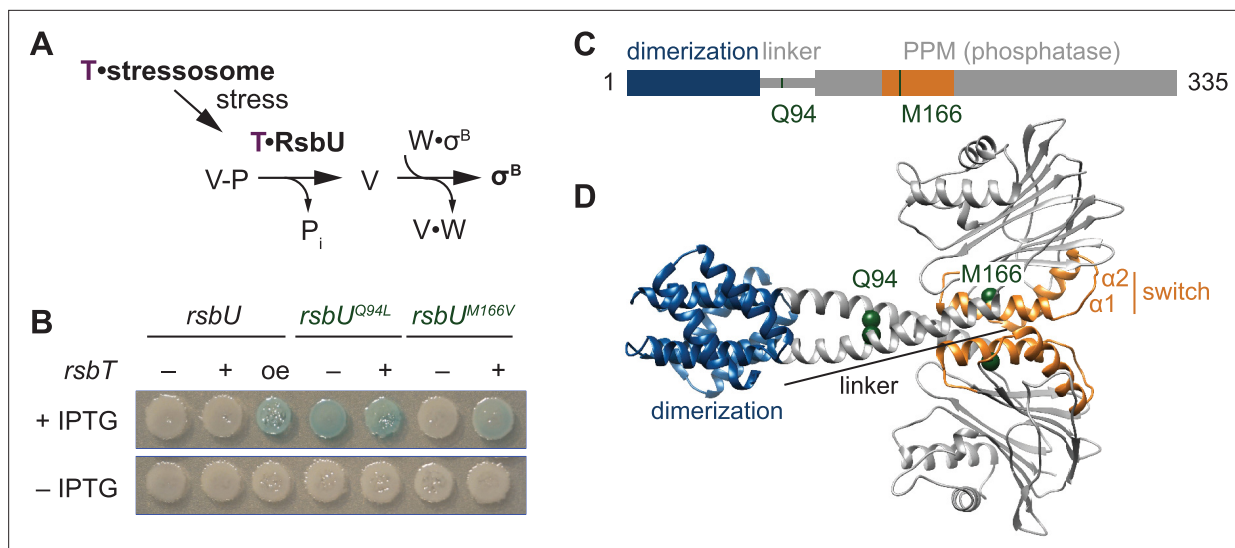


Figure 1. The linker controls RsbU activation through a conserved switch element. **(A)** Reaction scheme of the RsbU-dependent pathway of the environmental stress response. RsbU is activated by its partner protein RsbT (T, purple) to dephosphorylate its substrate protein RsbV-P (V-P). This initiates transcription by displacing σ^B from inhibition by the anti-sigma factor RsbW (W). **(B)** Amino acid substitutions in RsbU (Q94L and M166V) cause enhanced σ^B activity in *B. subtilis*. Strains carrying a *lacZ* reporter of σ^B activity (*ctc-lacZ*) were plated on X-gal indicator plates in the presence (top) or absence (bottom) of IPTG to induce expression of the *rsbU* constructs. Strains were deleted for *rsbT* (-) or not (+). A strain overexpressing *rsbT* is indicated by oe. Plates were imaged after 24 h of growth at 37 °C, which was shorter than the time used to visualize *lacZ* expression from the *rsbU^{M166V}* strain previously (Ho and Bradshaw, 2021). **(C)** Domain diagram of RsbU. The N-terminal domain of RsbU (blue; amino acids 1–81) is joined to the α -helical linker domain (grey; 82–112), which connects it to the PPM phosphatase domain (grey box; 121–335). The regulatory switch element of the PPM phosphatase domain is colored orange (156–201). The location of RsbT-bypass mutations are highlighted in green. **(D)** AlphaFold2 model of RsbU dimer. Domains are colored as in (C) and the C α atoms of residues Q94 and M166 are shown as spheres.

The online version of this article includes the following figure supplement(s) for figure 1:

Figure supplement 1. PAE plots of AlphaFold2 structure predictions.

Figure supplement 2. AlphaFold2 structure predictions with mapped pLDDT scores.

Figure supplement 3. Q94L stabilizes the packing interface of the coiled-coil of the linker.

stationary phase, nutrient depletion, cellular damage, and (for pathogens) host defense mechanisms (Gottesman, 2019). This multiplicity of initiating conditions allows cells to anticipate and prepare for accumulating adversity, suggesting why the GSR is present in every bacterial species that has so far been analyzed. The GSR additionally contributes to pathogenesis through expression of genes controlling virulence, biofilm formation, and quorum sensing (Kint et al., 2017; Battesti et al., 2015; Bartolini et al., 2019). Inactivation of the GSR, in turn, promotes the transition to long-term slow growth and antibiotic resistance, which are particularly important for persistent infections (Tuchscher and Löffler, 2016; Herbert et al., 2010; Bergkessel et al., 2016). Thus, deciphering the mechanisms that govern GSR activity is critical to understanding how bacteria interact with and adapt to their environments.

A major barrier to understanding initiation of the GSR is that we lack a unique, generalizable mechanistic model for how the activating signaling proteins have evolved to respond to species-specific signals. In a widespread mechanism for initiating the GSR, a member of the PPM family of serine/threonine phosphatases dephosphorylates a phosphorylated substrate protein to activate an alternative σ -factor through a ‘partner-switching’ mechanism (Hecker et al., 2007), but how the phosphatase receives and is activated by an upstream signal has not been determined.

In this study, we address how the GSR phosphatase RsbU from *Bacillus subtilis* is regulated (Yang et al., 1996) and present evidence that this mechanism is widely conserved. RsbU dephosphorylates a single phospho-serine on its substrate protein (RsbV) to release the GSR transcription factor (σ^B) from inhibition by an anti-sigma factor (RsbW; Figure 1A). RsbU has an N-terminal four-helix bundle domain that dimerizes RsbU and is also the binding site for RsbT, which activates RsbU as a phosphatase (Figure 1C and D; Delumeau et al., 2004). RsbT is sequestered in a megadalton stress sensing

complex called the stressosome, and is released to bind RsbU in response to specific stress signals including ethanol, heat, acid, salt, and blue light (Hecker et al., 2007; Marles-Wright et al., 2008). We and others have reconstituted RsbU activation by RsbT (Delumeau et al., 2004; Ho and Bradshaw, 2021), but how RsbT activates RsbU was not known.

This ‘partner-switching’ mechanism is broadly conserved, and the activating phosphatases are observed to have diverse N-terminal sensory domains including PAS (Vijay et al., 2000; Greenstein et al., 2009; Martínez et al., 2009), response-receiver (de Been et al., 2010; Rodríguez-Martínez et al., 2023), GAF (Kint et al., 2019), and HAMP (Mittenhuber, 2002). These signaling domains are frequently found as N-terminal regulators of histidine kinases and GGDEF diguanylate cyclases in bacteria, but whether or not there are shared mechanistic features between the regulatory principles of PPM phosphatases and these other signaling effector families is not known (Möglich et al., 2009; Galperin, 2006).

We previously described a mechanism for allosteric control of the related *B. subtilis* phosphatase SpoII_E, which regulates endospore formation through a partner-switching mechanism analogous to RsbU control of the GSR (Ho and Bradshaw, 2021; Bradshaw et al., 2017). Two helices of the PPM domain ($\alpha 1$ and $\alpha 2$), which we refer to as the switch element, change conformation to recruit metal cofactor and activate SpoII_E (Bradshaw et al., 2017). Based on analysis of structural and genetic data, we conjectured that this conformational change exemplifies a common mechanism among diverse phosphatases. However, we could not propose a specific model for how unrelated regulatory domains modularly control the switch conformation. For RsbU, we found that RsbT enhances phosphatase activity by increasing the efficacy of metal cofactor binding and that substitution of an amino acid in $\alpha 1$ (RsbU^{M166V/L}) activates RsbU in the absence of RsbT (Ho and Bradshaw, 2021). These results supported our proposal that the switch element controls RsbU activation, but they did not reveal how RsbT binding to the N-terminus of RsbU is transduced to the switch.

One broadly conserved feature of GSR phosphatases (absent in SpoII_E) is an α -helical linker of variable length, which is N-terminal to the PPM phosphatase domain. Genetic data suggest that this linker regulates a paralogous *B. subtilis* GSR phosphatase (RsbP; Brody et al., 2009). Here, we report that binding of RsbT to the N-terminus of RsbU rigidifies an otherwise flexible linker to dimerize the C-terminal phosphatase domains through an interface mediated by the switch element. Importantly, and as we now report, this mechanism is generalizable to other PPM phosphatases and reveals a widespread feature of bacterial signaling.

Results

The linker controls RsbU activation through a predicted phosphatase dimer

We previously conducted a genetic screen (Ho and Bradshaw, 2021) to identify features of RsbU that are important for phosphatase regulation by isolating gain-of-function variants that are active in the absence of RsbT. Focusing on the phosphatase domain in our initial study, we reported identification of an amino acid substitution in $\alpha 1$ (RsbU^{M166V/L}) that links the switch element to RsbU activation (Ho and Bradshaw, 2021). From this screen, we isolated a second variant, RsbU^{Q94L}, located in the linker of RsbU between the N-terminal dimerization domain and the C-terminal phosphatase domain (Figure 1B–D). *rsbU*^{Q94L} strains activated σ^B in the absence of *rsbT*. This activation is comparable to when *rsbT* is overexpressed to exceed the capacity of the stressosome and is stronger than what we observe in strains expressing *rsbU*^{M166V/L} (Figure 1B).

To visualize how the Q94L and M166V/L substitutions could impact RsbU structure and activity, we generated an AlphaFold2 model of an RsbU dimer (Jumper et al., 2021; Mirdita et al., 2022; Figure 1D). The prediction of the complex was of high confidence (Figure 1—figure supplements 1–2) and had a dimer of the N-terminal region consistent with a previous experimental structure (Delumeau et al., 2004). In our model, the linkers form extended alpha-helices (82–112) that cross to connect dimers of the RsbU N-terminal domains and C-terminal phosphatase domains, with Q94 buried in the linker interface and M166 buried in the core of the phosphatase domain (Figure 1D).

The crossing linker helices have similar conformation to homologous linkers observed in crystal structures of *Pseudomonas aeruginosa* RssB, a phosphatase/adaptor protein that also controls the GSR (PDB 3F7A and 3EQ2, Figure 1—figure supplement 3). RssB has an N-terminal response-receiver

domain in place of the RsbU four helix bundle dimerization domain, suggesting that linker conformation is a conserved feature of PPM phosphatase dimers with divergent N-terminal regulatory domains (**Figure 1—figure supplement 3**). The linker helices in the RsbU prediction and the RssB structure splay apart as they enter the PPM domain with similar angle (pitch angles (α) based on Crick parameters for coiled-coils -17.6° and -18.4° , respectively) and pack against $\alpha-1$ of the PPM fold (**Grigoryan and Degrado, 2011**). Interestingly, we observed that other bacterial signaling proteins that are regulated by coiled-coil linkers had similar pitch angle for the helices connecting to the catalytic domains (for example the dimeric histidine kinase CheA, 1B3Q, $\alpha -16.8^\circ$, and the dimeric GGDEF diguanylate cyclase PadC, 5LLW, $\alpha -14.3^\circ$; **Bilwes et al., 1999; Gourinchas et al., 2017; Grigoryan and Degrado, 2011; Figure 1—figure supplement 3**). Thus, the linker of RsbU could be a conserved regulatory feature, analogous to linkers of histidine kinases and GGDEF diguanylate cyclases.

Analysis of the RsbU dimer model using Socket2 identifies amino acids 83–97 as a two-turn coiled coil, with Q94 unfavorably packing against leucine 90 (**Kumar and Woolfson, 2021; Figure 1—figure supplement 3**). To test whether hydrophobicity at position 94 promotes an active state, we tested the activity of RsbU alleles with different amino acid substitutions at position 94 for σ^B in wildtype and *rsbT* deleted strains. We found that substitutions of Q94 with amino acids that could pack at the intersection of the crossing linker helices (hydrophobic residues isoleucine, valine, and methionine, as well as tyrosine) resulted in mild, RsbT-independent activation of σ^B that was further heightened by RsbT (**Figure 1—figure supplement 3**). Aromatic hydrophobic residues (phenylalanine and tryptophan) drove constitutive activation of RsbU but did not bypass the requirement for *rsbT* on the chromosome (**Figure 1—figure supplement 1**). Substitutions of small or charged residues (alanine, glycine, glutamate, and asparagine) did not lead to activity in either strain (**Figure 1—figure supplement 3**). These results are consistent with a model in which burial of the amino acid at position 94, such as in the crossed helices of our prediction for RsbU dimer structure, drives RsbU activation.

RsbT binding to RsbU is influenced by the linker

One prediction of our hypothesis that RsbT stabilizes the crossed alpha helices of the RsbU dimer, is that RsbT should bind more tightly to RsbU^{Q94L} than to RsbU because the coiled-coil conformation that RsbT binds would be more energetically favorable. To test this, we measured the binding affinity of RsbT for RsbU using fluorescence anisotropy. From these data we obtained a K_D of $4.5 \pm 1 \mu\text{M}$, which decreased approximately 10-fold for RsbU^{Q94L} ($0.56 \pm 0.2 \mu\text{M}$; **Figure 2A**). Thus, RsbU^{Q94L} stimulates RsbU phosphatase activity by an on-pathway mechanism, supporting the idea that RsbT binding stabilizes the linker in a dimerized conformation to activate RsbU.

RsbU phosphatase activation is controlled by the linker

We previously found that RsbT activates RsbU by simultaneously decreasing the K_M for manganese cofactor and increasing the k_{cat} for dephosphorylation of RsbV-P (**Ho and Bradshaw, 2021**). To test whether RsbU Q94L substitution works by the same kinetic mechanism, we measured dephosphorylation of ^{32}P -phosphorylated RsbV by RsbU^{Q94L} (**Figure 2B**). The k_{cat} of RsbU^{Q94L} was nearly the same as for the basal activity of RsbU ($2.4 \pm 0.1 \text{ min}^{-1}$ compared to $1.4 \pm 0.1 \text{ min}^{-1}$, and stimulated more than 10-fold by RsbT, $15 \pm 0.4 \text{ min}^{-1}$; **Figure 2B; Ho and Bradshaw, 2021**). However, RsbU^{Q94L} had a K_M for MnCl_2 ($1.4 \pm 0.2 \text{ mM}$) that is more than 50-fold reduced compared to RsbU alone ($77 \pm 9 \text{ mM}$; **Ho and Bradshaw, 2021**), and similar to the $K_M^{\text{MnCl}_2}$ of RsbU in the presence of RsbT ($0.98 \pm 0.07 \text{ mM}$ **Ho and Bradshaw, 2021; Figure 2B**). Addition of RsbT did not change the K_M of RsbU^{Q94L} for MnCl_2 ($1.9 \pm 0.6 \text{ mM}$), but the maximum catalytic rate of dephosphorylation increased nearly four-fold to $9.0 \pm 0.64 \text{ min}^{-1}$, approaching the rate of RsbU stimulated by RsbT (**Figure 2B**). Thus, RsbU^{Q94L} substantially recapitulates RsbT activation by decreasing the K_M for Mn^{2+} , demonstrating that the linker transduces RsbT binding to control the active site of RsbU (**Figure 2C**). This is consistent with previous findings that the allosteric switch controlling phosphatase activity of SpoIIIE couples dimerization to cofactor interaction.

RsbT binds to the linker and N-terminal domain

To visualize how RsbT could control the linker to activate RsbU, we generated an AlphaFold2 prediction of a heterotetrameric complex between RsbT and RsbU (**Figure 3A**). In this model, RsbU adopts a conformation very similar to our prediction of the RsbU dimer (RMSD $1.36 \text{ \AA}$), suggesting that

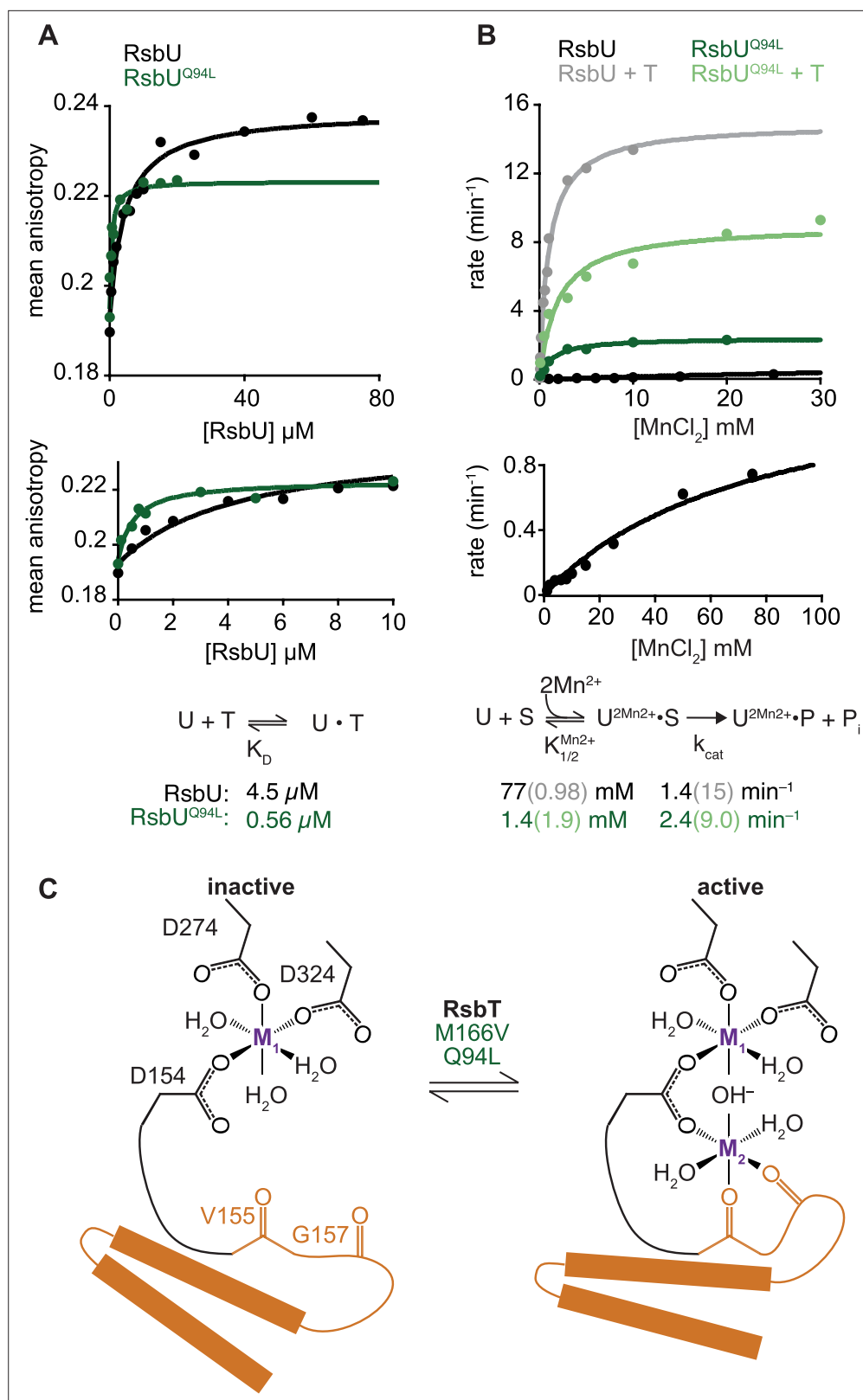


Figure 2. RsbU linker influences RsbT binding and phosphatase activity. **(A)** Mean anisotropy of RsbT-TMR (200 nM) is plotted as a function of RsbU concentration. The curves are fits to a quadratic binding equation using non-linear curve fitting (RsbU (black) $K_D = 4.5 \pm 0.96 \mu\text{M}$, RsbU^{Q94L} (green) $K_D = 0.56 \pm 0.15 \mu\text{M}$). The lower plot shows the same data plotted to display RsbU concentrations to 10 μM . Below is the reaction scheme of RsbT binding to RsbU

Figure 2 continued on next page

Figure 2 continued

with the values for the calculated K_D below. **(B)** The rate of dephosphorylation of RsbV-P is plotted as a function of concentration of $MnCl_2$ for RsbU (black) and RsbU^{Q94L} (green) in the presence (dark) and absence (light) of RsbT. Curves are fits to the Michaelis-Menten equation using non-linear curve fitting. The k_{cat} of wild-type RsbU is $15 \pm 0.35 \text{ min}^{-1}$ with RsbT and $1.4 \pm 0.077 \text{ min}^{-1}$ without RsbT and the k_{cat} of RsbU^{Q94L} is $9.0 \pm 0.64 \text{ min}^{-1}$ with RsbT and $2.4 \pm 0.084 \text{ min}^{-1}$ without RsbT. The $K_M^{MnCl_2}$ of wild-type RsbU is $0.98 \pm 0.069 \text{ mM}$ with RsbT and $77 \pm 9.0 \text{ mM}$ without RsbT and the $K_M^{MnCl_2}$ of RsbU^{Q94L} is $1.4 \pm 0.19 \text{ mM}$ with RsbT and $1.9 \pm 0.56 \text{ mM}$ without RsbT. The lower plot shows the data for wild-type RsbU in the absence of RsbT shown in the upper plot including higher $MnCl_2$ concentrations and with rescaled Y-axis. Below is a summary of a reaction scheme of RsbU dephosphorylating RsbV-P (denoted as S) with the $K_M^{MnCl_2}$ and k_{cat} values below. Data for RsbU are reproduced from **Ho and Bradshaw, 2021**. **(C)** A cartoon model of how the switch helices rotate to activate RsbU. Binding of RsbT or mutation moves the switch helices into place during activation to coordinate metal M2. The residues of RsbU that are hypothesized to coordinate metals are shown as sticks (based on homology to RsbX).

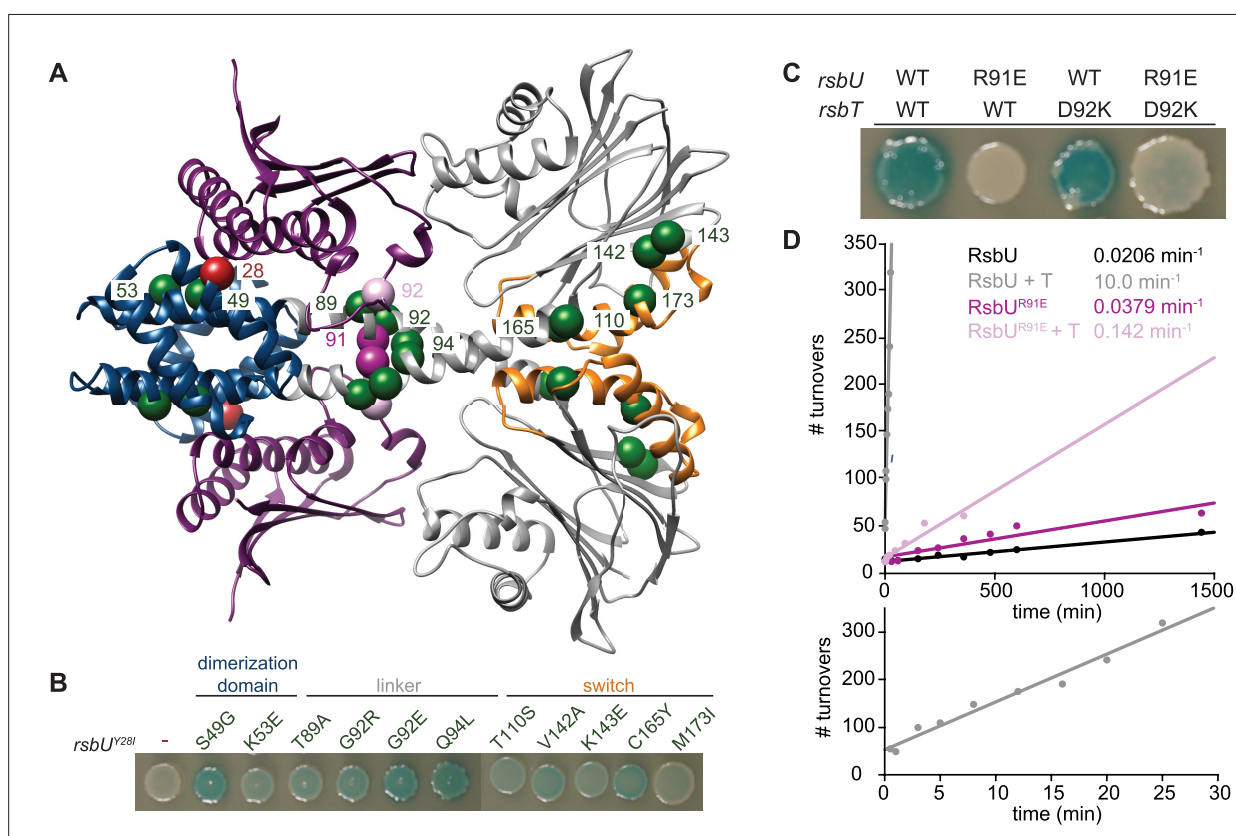


Figure 3. RsbT has a hybrid interface with RsbU N-terminal dimerization domain and linker. **(A)** An AlphaFold2 model of a heterotetrameric 2RsbT/2RsbU complex is shown in ribbon representation. RsbT (purple) binds with an interface that spans the N-terminal domains (blue) and α -helical linkers (grey) of both RsbU protomers. The PPM phosphatase domains are colored grey and the regulatory switch elements ($\alpha 1$ and $\alpha 2$) are colored orange. The $C\alpha$ positions of RsbU^{Y28} (red), RsbU^{Y28I} suppressor substitutions (green), RsbU^{R91} (dark pink), and RsbT^{D92} (pink) are shown as spheres with the residue numbers indicated. **(B)** Strains with a σ^B LacZ reporter were plated on IPTG/X-gal plates to induce expression of plasmid borne *rsbT* and *rsbU*^{Y28I} and visualize σ^B activity (indicated by blue pigmentation after 24 hr of growth at 37 °C). Additional amino acid substitutions in the *rsbU* gene are indicated above. **(C)** Strains with indicated changes to the RsbT and RsbU amino acid sequence were plated as in (B) and plates were imaged after 36 hr of growth at 37 °C. **(D)** R91E does not decrease the basal phosphatase activity of RsbU. Plots show dephosphorylation of RsbV-P (25 μ M) by RsbU (black) and RsbU^{R91E} (pink) (0.5 μ M) in the presence of 10 mM $MnCl_2$ and the presence or absence of 10 μ M RsbT (light colors). Linear fits of the data are shown, with the slopes indicating the observed rates (k_{obs}): RsbU $0.0206 \pm 0.00124 \text{ min}^{-1}$, RsbU + RsbT $10.0 \pm 0.628 \text{ min}^{-1}$, RsbU^{R91E} is $0.0379 \pm 0.00543 \text{ min}^{-1}$, RsbU^{R91E} + RsbT $0.142 \pm 0.0161 \text{ min}^{-1}$. The lower plot is RsbU in the presence of RsbT to 30 min.

The online version of this article includes the following figure supplement(s) for figure 3:

Figure supplement 1. RsbT binds to the linker.

our AlphaFold2 RsbU dimer model represents the active state of RsbU even when RsbT was not included in the prediction (a known feature of AlphaFold2 models [Chakravarty and Porter, 2022](#)). The predicted structure places RsbT in a hybrid interface that spans the N-terminal dimerization domain of both RsbU monomers and the linker of one monomer ([Figure 3A](#)). Supporting the validity of the AlphaFold2 2RsbT/2RsbU model, residues previously implicated in RsbT/U binding ([Hardwick et al., 2007](#); Y28 and E24 from one RsbU monomer, I74, and I78 from the other RsbU monomer, and RsbT R19, R23, and N24) are all buried in the predicted interface ([Figure 3—figure supplement 1](#)).

To gain further insight into how RsbT binding is transduced to RsbU linker conformation and phosphatase activity, we performed a genetic screen to isolate suppressors of RsbU^{Y28I}, which was previously observed to abolish binding to RsbT ([Hardwick et al., 2007](#)). After screening a library of PCR mutagenized *rsbU*^{Y28I} and *rsbT*, we identified 11 variants that introduced substitutions to RsbU that suppress *rsbU*^{Y28I}: S49G, K53E, T89A, G92E, G92R, Q94L, T110S, V142A, K143E, C165Y, and M173I ([Figure 3A–B](#)). We additionally isolated revertants that restored Y28 suggesting that the screen was near saturation, but did not isolate any suppressors in *rsbT*, which was also mutagenized.

Mapping the RsbU^{Y28I} suppressor mutations onto our 2RsbU/2RsbT model suggests that they fall into three classes ([Figure 3A and B](#)): (1) Substitutions in the RsbU N-terminal dimerization domain that map near the predicted RsbT binding site (S49G and K53E), supporting the predicted interface. (2) Substitutions that map near predicted contacts between RsbT and the RsbU linker (T89A, G92E/R, and Q94L), providing evidence that contacts between RsbT and the linker promote RsbU activation. (3) Substitutions that cluster around the regulatory switch element of the RsbU phosphatase domain (helices $\alpha 1$ and $\alpha 2$ of the PPM fold, T110S, V142A, K143E, C165Y, and M173I). We hypothesize that these substitutions rescue RsbU^{Y28I} inactivation by reducing the energetic cost (normally provided by RsbT binding) to transition the RsbU phosphatase domain to the active conformation. Together these genetic data provide independent support for the AlphaFold2 model of the 2RsbT/2RsbU complex, and suggest that RsbT activates RsbU by transmitting signals to the switch element in the RsbU phosphatase domain through a coiled-coil linker.

Contacts between RsbT and the RsbU linker drive phosphatase activation

To test the model that RsbT activates RsbU by directly interacting with the linker to dimerize the RsbU phosphatase domains, we introduced a charge swap at position R91 that would abolish a predicted salt-bridge with RsbT D92 ([Figure 3C](#)). RsbU^{R91E} abolished σ^B activity for cells overexpressing *rsbT* ([Figure 3C](#)), and in biochemical assays RsbT stimulation of RsbU^{R91E} phosphatase activity was abolished ([Figure 3D](#)). Finally, we introduced a potentially compensatory charge switch at position D92 of RsbT (RsbT^{D92K}; [Figure 3C](#)). Expression of *rsbT*^{D92K} partially restored σ^B activity to *rsbU*^{R91E} expressing cells, consistent with direct interaction between these residues ([Figure 3C](#)). Together, these results suggest that contacts between RsbT and the linker of RsbU are responsible for phosphatase activation.

The RsbU linker is flexible in the absence of RsbT

We considered two models for why RsbU is inactive in the absence of RsbT. One possibility is that inactive RsbU resembles the dimeric structure predicted by AlphaFold2 and that RsbT induces a subtle conformational change, for example changing the registry or pitch of the crossing linker helices. A second possibility is that inactive RsbU is dimerized by the N-terminal domains but that the linkers of inactive RsbU are flexible and that the phosphatase domains only interact with each other when RsbT orders the linkers into a crossing conformation. To distinguish between these two models, we performed small angle X-ray scattering coupled to size exclusion chromatography with multi-angle light scattering (SEC-MALS-SAXS) to assess the shape and flexibility of RsbU dimers in solution ([Table 1](#) and [Figure 4—figure supplements 1–2 S4A](#)). RsbU eluted as a single peak from the size exclusion column with an average molecular weight of 77 kDa (calculated dimeric molecular weight 77.3 kDa) determined by multi-angle light scattering (MALS; [Figure 4—figure supplement 1](#)). However, our sample had a minor contaminant of heterodimeric complexes of RsbU in which one monomer is C-terminally truncated that could influence the SAXS analysis ([Figure 4—figure supplement 1](#)). We therefore deconvoluted the SAXS profiles using evolving factor analysis (EFA) in RAW to isolate scattering profiles from the individual components ([Hopkins et al., 2017](#); [Meisburger et al., 2016](#); [Figure 4—figure supplement 2](#)). The EFA deconvolution identified a major component (MW 75.6 kDa calculated

Table 1. SAXS experimental details.

(a) Sample details		
	RsbU SASDU85	RsbU ^{Q94L} /RsbT SADSDU95
Organism	<i>B. subtilis</i>	<i>B. subtilis</i>
Description:	RsbU (1-335) Uniprot ID: P40399 GPG scar from 6His cleavage	RsbU ^{Q94L} (1-335) Uniprot ID: P40399; RsbT (1-133) Uniprot ID: P42411, 6His with 3 C cleavage site
Extinction coefficient ϵ (280 nm)	26,820 M ⁻¹ cm ⁻¹	RsbU ^{Q94L} : 26,820 M ⁻¹ cm ⁻¹ RsbT: 13,980 M ⁻¹ cm ⁻¹
Molecular mass <i>M</i> from chemical composition	38.6 kDa	RsbT: 14.3 kDa RsbU ^{Q94L} : 38.6 kDa
For SEC-SAX, loading volume/ concentration injection volume, flow rate	3.5 mg ml ⁻¹ 300 μ l, 0.6 ml min ⁻¹	2.5 mg ml ⁻¹ 300 μ l, 0.6 ml min ⁻¹
Solvent composition and source	20 mM HEPES pH 7.5, 100 mM NaCl, 5 mM DTT	20 mM HEPES pH 7.5, 100 mM NaCl, 5 mM DTT
(b) SAX data collection parameters		
Source, instrument and description	BioCAT facility at the Advanced Photon Source beamline 18ID with Pilatus3 X1 M (Dectris) detector	
Wavelength	1.033 Å	
Beam size (μ m ²)	150 (h) x 25 (v) focused at the detector	
Camera length	3.682 m	
<i>q</i> -measurement range	0.0027–0.33 Å ⁻¹	
Absolute scaling method	Glassy Carbon, NIST SRM 3600	
Basis for normalization to constant counts	To transmitted intensity by beam-stop counter	
Method for monitoring radiation damage	Automated frame-by-frame comparison of relevant regions using CORMAP algorithm (<i>Franke et al., 2015</i>) implemented in BioXTAS RAW	
Exposure time	0.5 s exposure time with a 1 s total exposure period (0.5 s on, 1.5 s off) of entire SEC elution	
Sample configuration	SEC-MALS-SAXS. Size separation used a Superdex 200 10/300 Increase GL column and a 1260 Infinity II HPLC (Agilent Technologies). UV data was measured in the Agilent, and MALS-DLS-RI data by DAWN HELEOS-II (17 MALS +1 DLS channels) and Optilab T-rEX (RI) instruments (Wyatt Technology). SAXS data was measured in a sheath- flow cell (<i>Kirby et al., 2016</i>), effective path length 0.542 mm.	
Sample temperature	22 °C	
(c) Software employed for SAXS data reduction, analysis and interpretation		
MALS-DLS-RI analysis	Astra 7 (Wyatt)	
SAX data reduction	Radial averaging; frame comparison, averaging, and subtraction done using BioXTAS RAW 2.1.4 (<i>Hopkins et al., 2017</i>). Deconvolution of overlapping peaks by EFA (<i>Meisburger et al., 2016</i>) as implemented in RAW.	
Basic analyses:	Guinier fit and molecular weight using BioXTAS RAW 2.1.4, P(r) function using GNOM (<i>Svergun, 1992</i>). RAW uses MoW and Vc molecular weight methods (<i>Rambo and Tainer, 2011; Piiadov et al., 2019</i>) ATSAS Version 3.2.1 (<i>Manalastas-Cantos et al., 2021</i>).	
FoXS	Calculated predicted scattering profiles from the model (FoXS) (<i>Schneidman-Duhovny et al., 2013; Schneidman-Duhovny et al., 2016</i>)	

Table 1 continued on next page

Table 1 continued

MultiFoxS	Calculated predicted scattering profiles from the multicomponent fits from a generated flexible structure ensemble (MultiFoXS) (<i>Schneidman-Duhovny et al., 2016</i>)	
(d) Structural parameters		
Figure 4 components	c0: RsbU dimer	c1: 2T/2U ^{Q94L} heterotetramer
Guinier Analysis		
I(0) (Arb.)	5.632±0.008	0.564±0.003
R _g	39.05±0.16 Å	35.06±0.43 Å
q-range	0.0036–0.0234 Å ⁻¹	0.0033–0.0336 Å ⁻¹
q _{max} R _g	0.916	1.178
Coefficient of correlation, r ²	0.995	0.88
Volume (adjusted V _p as SAXS MoW2)	116000 Å ³	124000 Å ³
MW, MoW2 method (ratio to expected)	96.2 kDa (1.25)	103.2 kDa (0.98)
MW, Vc method (kDa) (ratio to expected)	75.6 kDa (0.98)	83.9 kDa (0.80)
P(r) analysis		
I(0) (Arb.)	5.648±0.007	0.561±0.003
R _g	39.98±0.12 Å	34.87±0.36 Å
D _{max}	168 Å	176 Å
q-range	0.0035–0.3327 Å ⁻¹	0.005–0.3327 Å ⁻¹
χ ² (total estimate from GNOM)	1.257 (0.729)	1.374 (0.691)
FoXS		
R _g	34.05 Å	33.68 Å
χ ²	12.53	1.83
c ₁	1.02	1.04
c ₂	1.91	−1.77
MultiFoXS		
# of conformations	10,000	10,000
flexible residues	82–96	82–96
# of states	2	1
R _g	34.91 Å (71%) 45.69 Å (29%)	33.28 Å
χ ²	1.2	1.96
c ₁	1.0	0.99
c ₂	3.91	−0.5
Additional EFA components	c1: truncated dimer	c0: ambiguous
Guinier Analysis		
I(0) (Arb.)	1.042±0.004	0.478±0.003
R _g	28.14±0.17 Å	42.18±0.48 Å
q-range	0.0027–0.0461 Å ⁻¹	0.005–0.0305 Å ⁻¹
q _{max} R _g	1.298	1.295
Coefficient of correlation, r ²	0.931	0.948

Table 1 continued on next page

Table 1 continued

Volume (adjusted V_p as SAXS MoW2)	64,500 Å ³	197000 Å ³
MW, MoW2 method (ratio to expected)	53.6 kDa	163.5 kDa (1.55)
MW, V_c method (kDa) (ratio to expected)	48.8 kDa	145.3 kDa (1.37)
<i>P(r)</i> analysis		
$I(0)$ (Arb.)	1.055±0.004	0.486±0.004
R_g	29.29±0.13 Å	45.18±0.48 Å
D_{max}	98.0 Å	124 Å
q-range	0.0027–0.3327 Å ⁻¹	0.0027–0.3327 Å ⁻¹
χ^2 (total estimate from GNOM)	1.421 (0.924)	1.628 (0.84)
Additional MultiFoXS runs (Figure 4—figure supplement 3)		
	S6A, N-terminus constraint	S6B, N-terminus constraint
MultiFoXS		
# of conformations	10,000	10,000
flexible residues	82–86	82–92
# of states	2	2
R_g	34.09 Å (62%) 45.20 Å (38%)	33.54 Å (55%) 43.17 Å (45%)
χ^2	1.26	1.25
c_1	1.0	1.0
c_2	4.0	4.0
	S6C, N-terminus constraint	S6D, N-terminus constraint
MultiFoXS		
# of conformations	10,000	10,000
flexible residues	92–96	82–96
# of states	2	2
R_g	41.40 Å (61%) 31.92 Å (39%)	37.09 Å (47%) 34.08 Å (53%)
χ^2	1.26	5.4
c_1	1.01	1.01
c_2	3.91	0.94

from V_c method), which eluted earlier than a smaller component (MW 48.8 kDa calculated from V_c method; **Table 1** Guinier fits, and **Figure 4—figure supplement 2**). We conclude that the larger component is a RsbU dimer, while the smaller fragment is likely a heterodimeric complex of RsbU and the C-terminal truncation product of RsbU (**Figure 4—figure supplement 1**).

Indicative of significant deviation between the RsbU structure in solution to the AlphaFold2 model, the scattering intensity profile ($I(q)$ vs. q) was a poor fit (χ^2 12.53) to a profile calculated from the AlphaFold2 model of an RsbU dimer using FoXS (**Schneidman-Duhovny et al., 2016; Schneidman-Duhovny et al., 2013; Figure 4A**). We therefore assessed the SAXS data for the RsbU dimer for features that report on flexibility (**Kikhney and Svergun, 2015**). First, the scattering intensity data lacked distinct features caused by the multi-domain structure of RsbU from the AlphaFold2 model (**Figure 4A**). This can be visualized more readily from a dimensionless Kratky plot, which is bell-shaped and peaks above 1.3, indicating that the RsbU dimer is non-globular (**Figure 4B**). Additionally, the data do not show the two peaks from the FoXS computed model of the AlphaFold2 dimer that reflect

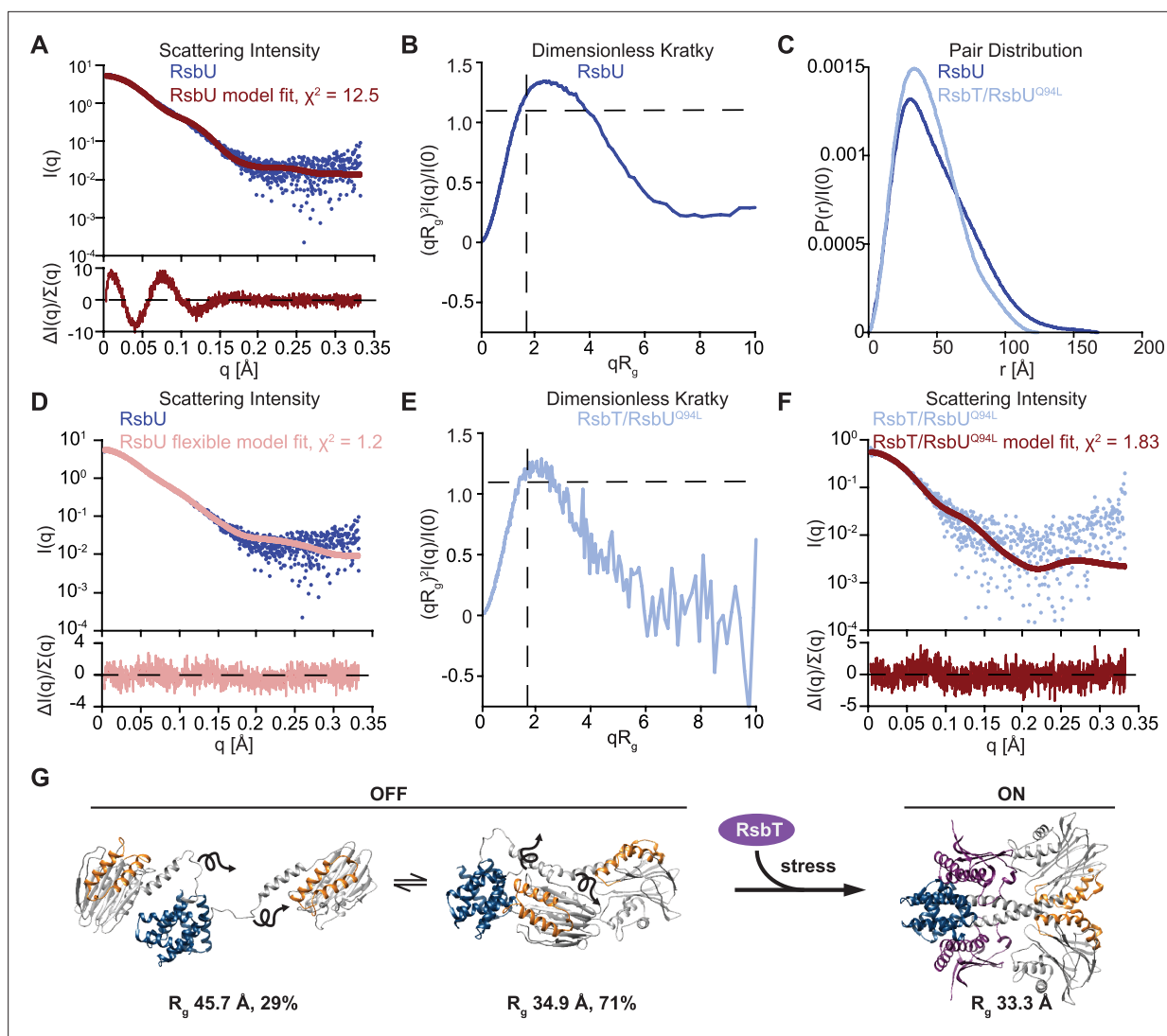


Figure 4. RsbU linker is flexible in the absence of RsbT, and is rigidified by RsbT binding. (A) $I(q)$ versus q plot of the dimer component of SAXS scattering data from RsbU (blue) overlaid with a FoXS generated profile fit of the AlphaFold2 RsbU dimer structure (red). The lower plot shows the uncertainty normalized residuals of the fit ((experimental-computed)/error), χ^2 of 12.53. (B) Dimensionless Kratky plot of SAXS scattering data from the dimer component of RsbU experimental data (blue). The experimental profile was logarithmically binned to reduce noise at high qR_g . (C) Normalized $P(r)$ plot from the RsbU dimer (blue) and heterotetrameric RsbT/RsbU^{Q94L} complex (light blue) is shown. (D) $I(q)$ versus q plot of the dimer component of SAXS scattering data from RsbU (blue) is shown overlaid with a MultiFoXS generated two-state profile fit with flexibility allowed for residues 82–96 (pink). The lower plot shows the uncertainty normalized residuals of the fit ((experimental-computed)/error), χ^2 of 1.2. (E) Dimensionless Kratky plot of the heterotetrameric component of RsbT/RsbU^{Q94L} complex is shown binned logarithmically to reduce noise at high qR_g values. (F) $I(q)$ versus q plot of the heterotetrameric RsbT/RsbU^{Q94L} complex (blue) overlaid with a FoXS generated profile fit of the AlphaFold2 RsbT/RsbU heterotetramer structure (red). The lower plot shows the uncertainty normalized residuals of the fits ((experimental-computed)/error), χ^2 of 1.83. (G) Schematic of the conformational change of RsbU upon RsbT binding. The two best fit flexible models of RsbU generated from MultiFoXS in panel (B) are shown with the calculated R_g and percentage of each model used in the fit. Squiggly arrows are shown to indicate that these models are approximate and likely represent an average of many flexible conformations.

The online version of this article includes the following source data and figure supplement(s) for figure 4:

Figure supplement 1. MALS estimate of molecular weight of RsbU dimer, RsbT/RsbU^{Q94L} complex, RsbT/RsbU complex, and RsbU^{Q94L}.

Figure supplement 1—source data 1. Original uncropped images of the gels shown in **Figure 4—figure supplement 1B and D**.

Figure supplement 1—source data 2. Original image files for the gels shown in **Figure 4—figure supplement 1B and D**.

Figure supplement 2. EFA deconvolution and Guinier analysis of RsbU dimer SAXS profiles.

Figure supplement 3. Varying constraints and flexibility of RsbU model.

Figure supplement 4. EFA deconvolution and Guinier analysis of RsbT/RsbU^{Q94L} SAXS profiles.

the extended two domain structure (Kikhney and Svergun, 2015; Receveur-Brechot and Durand, 2012; Figure 4—figure supplement 2). The scattering data had several additional characteristic features indicating flexibility: the $P(r)$ plot is smooth and has an extended tail (Cordeiro et al., 2017; Kikhney and Svergun, 2015; Receveur-Brechot and Durand, 2012; Figure 4C), R_g and $I(0)$ calculated from $P(r)$ are larger than when calculated from Guinier fits (Cordeiro et al., 2017; Kikhney and Svergun, 2015; Receveur-Brechot and Durand, 2012; Table 1), molecular weight calculated using the MoW2 modified Porod volume method is an overestimate (Rambo and Tainer, 2011; Table 1), and the R_g (39.05 ± 0.16 Å) was larger than the R_g of the AlphaFold2 dimer model (34.05 Å). Prompted by this evidence of flexibility, we performed multi-state modelling using MultiFoXS (Schneidman-Duhovny et al., 2016), which incorporates flexibility and heterogeneity. MultiFoXS produced a two-state solution with χ^2 of 1.2 (as compared to the one-state model with χ^2 of 12.5) and low residuals across the entire range of data (Figure 4D, Figure 4—figure supplement 2). We allowed flexibility for the linker residues 82–96 while keeping the N-terminal domain dimerized because a stable dimer of the N-terminal domain is observed in solution, was present in a previous X-ray crystal structure of C-terminally truncated RsbU{Delumeau:2004ep}, and C-terminally truncated RsbU coelutes as a heterodimer with the full-length protein (Figure 4—figure supplement 1). Importantly, the precise region of allowed linker flexibility does not have a substantial impact on the MultiFoXS fit (Figure 4—figure supplement 3), but constraining the C-terminal phosphatase domain dimer does not yield a good fit to the data (χ^2 of 5.4; Figure 4—figure supplement 3). The structural models predicted by MultiFoXS depict a dimerized N-terminal domain with flexible linkers and PPM domains that do not make contact. Notably, this approach also would include models in which both domains remain dimerized, suggesting that such models are not the best fit of the data. The two conformations that were selected as the best fit to the scattering data by MultiFoXS also have very different R_g values, (34.91 Å [71%] and 45.69 Å [29%]), indicating RsbU samples a range of conformational space that includes a more compact state(s) consistent with the AlphaFold2 model and a significantly extended state(s), emphasizing that the linker is highly flexible. Together, we conclude that the RsbU linker appears to be flexible in the absence of RsbT (Figure 4G).

RsbT rigidifies and compacts the RsbU dimer

To test the hypothesis that RsbT rigidifies the linker to produce an active RsbU dimer, we attempted SEC-MALS-SAXS analysis of the RsbT/U complex. RsbT/U eluted with molecular weight (determined by MALS) of 88 kDa compared to 105 kDa for a 2RsbT/2RsbU heterotetramer (Figure 4—figure supplement 1), suggesting that the RsbT/U complex had largely disassembled on the column. We therefore performed SEC-MALS-SAXS on the RsbT/RsbU^{Q94L} complex, which has a higher affinity (Figure 2A, Figure 4—figure supplement 4). The RsbT/U^{Q94L} complex eluted as a major peak with a higher molecular weight shoulder (Figure 4—figure supplements 1 and 4; also visible when we ran RsbU^{Q94L} alone. Figure 4—figure supplement 1), with the average molecular weight of the main peak (determined by MALS) of 125 kDa (Figure 4—figure supplement 1). This is larger than the predicted molecular weight of a heterotetrameric 2RsbT/2RsbU^{Q94L} complex (105 kDa), suggesting that some higher order complexes form. EFA deconvolution (Meisburger et al., 2016) identifies two components. The first deconvolved component had a SAXS determined molecular weight of 164 kDa, while the second had a SAXS molecular weight of 103 kDa, consistent with a 2RsbT/2RsbU complex (105 kDa). Together with the fact that RsbT co-elutes with RsbU^{Q94L} throughout the entire peak (Figure 4—figure supplement 1) and the MALS molecular weight, we assess that this second component is an 2RsbT/2RsbU hetero-tetramer. The identity of the higher molecular weight component of the scattering data is ambiguous, but could represent a larger assembly of RsbT/RsbU^{Q94L} or multimers of RsbU^{Q94L} that were observed when RsbU^{Q94L} was analyzed alone (Figure 4—figure supplement 1). Several lines of evidence demonstrate that the 2RsbT/2RsbU^{Q94L} complex lacks the flexibility of the 2RsbU complex. First, the R_g from the Guinier fit (35.06 ± 0.43 Å) matches well with the model computed from the AlphaFold2 prediction of the heterotetrameric complex of 2RsbT/2RsbU using FoXS (33.68 Å) and is smaller than the R_g we observed for the RsbU dimer (39.05 ± 0.16 Å). Second, the features of the scattering data that suggested flexibility for RsbU dimers all suggest that the RsbT/RsbU complex is more rigid (the quality of the FoXS fit, a lower peak of the dimensionless Kratky plot, and a shorter tail of the $P(r)$ plot; Cordeiro et al., 2017; Kikhney and Svergun, 2015; Receveur-Brechot and Durand, 2012; Figure 4C, E, F, Figure 4—figure supplement 4).

Third, fitting the scattering profile ($I(q)$ vs. q) to the AlphaFold2 model yielded a χ^2 of 1.83 without requirement for flexibility (**Figure 4F**). As expected, based on the lack of evidence of flexibility in the scattering profile and $P(r)$ function, the model is not improved by the use of multi-state modeling by MultiFoXS, which was run as a control (**Schneidman-Duhovny et al., 2016; Figure 4—figure supplement 4**). Together, these data support the validity of the AlphaFold2 model of the heterotetrameric 2RsbT/2RsbU complex, and additionally suggest that RsbT rigidifies the otherwise flexible linker of RsbU to dimerize the phosphatase domains (**Figure 4G**). Together with our biochemical data linking RsbU linker conformation to RsbT binding, metal cofactor interaction, and phosphatase activity, this provides a comprehensive model for how RsbU is activated (**Figure 4G**).

Regulated dimerization is an evolutionarily adaptable mechanism to control bacterial phosphatases

B. subtilis has a second GSR-initiating PPM phosphatase, RsbP, that has an N-terminal PAS domain and dephosphorylates RsbV to activate σ^B in response to energy stress. Our AlphaFold2 prediction of an RsbP dimer shows an extended alpha-helical linker connecting dimeric PAS domains and phosphatase domains. Like RsbU, the interface between the dimerized phosphatase domains is predicted to be mediated by $\alpha 1$ and $\alpha 2$ of the phosphatase domain. We found that previously identified constitutively activating mutations in RsbP (**Brody et al., 2009**) map to similar positions as the gain-of-function mutations we identified here for RsbU in their respective predicted structures, namely the interface between $\alpha 1$ and $\alpha 2$ of the phosphatase domains and the linker. RsbP is activated by a partner-protein, RsbQ, that has analogous function to RsbT for RsbU. An AlphaFold2 prediction of the heterotetrameric 2RsbP/2RsbQ complex places the two molecules of RsbQ in a hybrid interface between the PAS domains and linkers, similar to how RsbT contacts RsbU. These predictions suggest a testable hypothesis that RsbP is controlled through an activation mechanism similar to that of RsbU (**Figure 5A**).

To further probe the generality of the linker-mediated phosphatase dimer we observe for RsbU, we generated AlphaFold2 predictions for dimers of other documented GSR initiating phosphatases (*C. difficile* RsbZ [N-terminal tandem GAF domains; **Figure 5B**], *S. coelicolor* OsaC [N-terminal GAF and PAS domains], and *M. tuberculosis* Rv1364c [N-terminal PAS domain, C-terminally fused to an anti- σ factor domain and an anti-anti- σ factor domain]), as well as from examples of common domain architectures of PPM phosphatases selected from InterPro (response receiver [*Streptomyces coelicolor*, MY40396.1], GAF [*Rhodococcus aetherivorans*, WP_029544106.1], and HAMP [*Synechocystis* sp., WP_010874143.1] domains; **Figure 5—figure supplement 1**). In each case, AlphaFold2 produced high-confidence predictions of phosphatase domains dimerized through a shared $\alpha 0$, $\alpha 1$, $\alpha 2$ interface, connected to dimeric N-terminal domains by crossing α -helical linkers (**Figure 1—figure supplements 1–2, Figure 5—figure supplement 1**). Consistent with a model in which the stability of the linker plays a conserved regulatory role, the AlphaFold2 models for many of the predicted structures have unfavorable polar residues buried in the coiled-coil interface (positions *a* and *d*, for which non-polar residues are most favorable; **Figure 5—figure supplement 2**). From this analysis, we speculate that linker-mediated phosphatase domain dimerization is an evolutionarily conserved, adaptable mechanism to control PPM phosphatase activity. This mechanistic/structural congruence provides a framework for how distantly related phosphatases can be regulated to control diverse biological processes in bacteria (**Figure 5E**).

Discussion

Since the ground-breaking discovery of σ^B , the first conditionally activated cellular transcription factor to be identified, 45 years ago (**Haldenwang and Losick, 1979**), a persistent unanswered question has been how σ^B is turned on. Here, our findings reveal how RsbU is activated as a phosphatase in the decisive step of σ^B activation. Below, we describe how this mechanism is broadly applicable to other bacterial signaling pathways.

RsbT activates RsbU through a conserved phosphatase dimer

Using unbiased genetic screens, biochemical reconstitution, biophysical approaches, and structural prediction, we have discovered that RsbT activates RsbU by rigidifying an otherwise flexible linker region to allosterically activate RsbU as a phosphatase. Our structural prediction, supported by

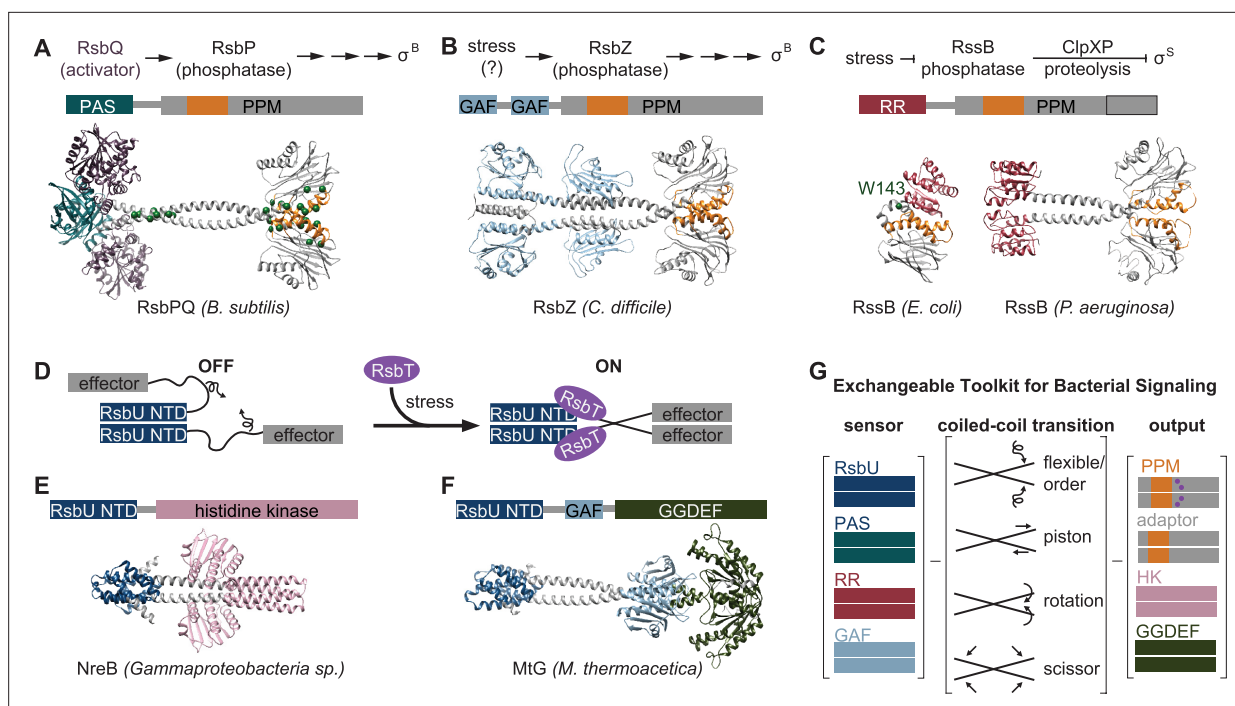


Figure 5. An exchangeable toolkit for bacterial signal transduction. **(A)** An AlphaFold2 model of the *B. subtilis* energy stress phosphatase RsbP is shown bound to its activating α/β -hydrolase protein RsbQ (purple) with a schematic of the σ^B activation pathway shown above. RsbP has an N-terminal PAS domain (turquoise) that is connected to the PPM phosphatase domain by a predicted coiled coil linker. The α positions of bypass suppressor mutations that render RsbP active in the absence of RsbQ (Brody et al., 2009) are shown as green spheres. The switch element of the PPM phosphatase domain ($\alpha 1$ and $\alpha 2$) is colored orange. **(B)** An AlphaFold2 model of the *C. difficile* GSR phosphatase RsbZ is shown with a schematic of the σ^B activation pathway shown above. RsbZ has tandem N-terminal GAF domains (blue) that connect to the PPM phosphatase domain by a predicted coiled coil linker. The switch element of the PPM phosphatase domain ($\alpha 1$ and $\alpha 2$) is colored orange. **(C)** Experimental structures of the GSR initiating adaptor protein RssB from *E. coli* (left, 8T85) and *P. aeruginosa* (right, 3F7A) are shown with a schematic of the σ^S activation pathway shown above. The α positions of a bypass suppressor mutation (W143R) that renders RssB insensitive to anti-adaptor proteins is shown as a green sphere. The switch element of the PPM phosphatase domain ($\alpha 1$ and $\alpha 2$) is colored orange. The black outline shows the portion of the PPM phosphatase domain that is missing in the *E. coli* RssB adaptor protein, which lacks phosphatase activity. **(D)** A cartoon schematic of activation by RsbT of proteins with RsbU NTD through ordering of a flexible linker. The protein is depicted with dimerized RsbU N-terminal dimerization domains (blue) with flexible linkers (black) that extend into two effector domains (grey). During stress conditions, RsbT (purple) activates the protein through binding to the N-terminal dimerization domains and linkers, rigidifying the linkers to dimerize the effector domains. **(E)** An AlphaFold2 model of a dimer of the histidine kinase NreB from a *Gammaproteobacteria* species (MBI3545564.1) is shown with the RsbU N-terminal domain colored blue and the histidine kinase domain colored pink. **(F)** An AlphaFold2 model of a dimer of the GGDEF diguanylate cyclase MtG from *M. thermoacetica* (WP_011392981.1) is shown with RsbU N-terminal domain colored blue, GAF domain colored light blue, and GGDEF domain colored green. **(G)** Schematic illustrating how various sensor domains (RsbU N-terminal dimerization domain, PAS, response receiver (RR), and GAF) can control various output activities (serine/threonine phosphatase (PPM), histidine kinase (HK), protease adaptor, and diguanylate cyclase (GGDEF)). We hypothesize that the shared regulatory mechanism through a dimeric coiled-coil linker makes these regulatory domains modularly exchangeable across effector domains. Known mechanisms for how allosteric regulation is transmitted through the linker are listed and shown with arrow diagrams.

The online version of this article includes the following figure supplement(s) for figure 5:

Figure supplement 1. AlphaFold2 predictions of varying signaling PPM phosphatases.

Figure supplement 2. Coiled-coil analysis of structures of bacterial signaling enzymes.

our genetic and structural data suggest that RsbU linkers assume a crossed α -helical conformation that dimerizes the phosphatase domains (Figure 4G). This dimerization activates RsbU through a conserved switch element at the dimer interface, decreasing the K_M for metal cofactor and increasing the catalytic rate. Our structural predictions suggest that this is a widespread mechanism to regulate the activity of bacterial protein serine/threonine phosphatases. While RsbU activation is controlled by a flexible-to-rigid transformation of the linker, it is possible that other conformational changes of the linker could be alter the PPM dimer to control phosphatase activity through the conserved switch element.

A general mechanism for general stress response activation

Escherichia coli and *B. subtilis* are the most extensively studied models for GSR activation (Battesti et al., 2011; Hecker et al., 2007). However, key mechanistic features of the activation mechanisms differ between the two systems. The *E. coli* GSR is initiated by stabilizing the alternative sigma factor σ^S (RpoS) against degradation by ClpXP (Figure 5C). Under non-stressed conditions, degradation of σ^S is mediated by the adapter protein, RssB, which is inhibited by anti-adaptors (IraD, IraM, and IraP) and stimulated by phosphorylation on its N-terminal response-receiver domain during stress. Historically, the differences between these systems were interpreted to mean that they operate by distinct mechanistic principles. However, RssB contains a C-terminally truncated (and therefore inactive) PPM phosphatase domain, suggesting some aspects of its regulatory mechanism could be shared with RsbU (Battesti et al., 2013).

Our findings reveal that *E. coli* RssB and *B. subtilis* RsbU use three shared regulatory features to control GSR activation through their distinct downstream mechanisms. First, the positioning of analogous flexible linkers controls GSR activation by RssB and RsbU (Brugger et al., 2023a; Brugger et al., 2023b; Dorich et al., 2019). The C-terminal pseudo-phosphatase domain of RssB is joined to its N-terminal σ^S binding response-receiver domain by a predominantly α -helical linker (termed the segmented helical linker, or SHL) of similar length to the linker of RsbU (Dorich et al., 2019). The SHL folds to occlude the σ^S interacting face of the response-receiver domain when bound to anti-adaptors, and is remodeled to promote σ^S interaction when the response-receiver domain is phosphorylated (Brugger et al., 2023a; Brugger et al., 2023b; Dorich et al., 2019). Supporting the hypothesis that the SHL is mechanistically related to the RsbU linker, substitution of W143, the homologous residue to Q94 of *B. subtilis* RsbU, blocks regulation by IraD and IraP (Battesti et al., 2013). Second, the interface between the linker and $\alpha 1$ of the phosphatase domain is critical for regulation of both RssB and RsbU (Brugger et al., 2023a; Dorich et al., 2019). W143 packs in a hydrophobic pocket formed with the $\alpha 1$ helix of the pseudo-phosphatase domain of phosphorylated RssB and IraD-bound RssB (Brugger et al., 2023a; Dorich et al., 2019). Substitution of residues in $\alpha 1$, including A216 (corresponding to M166 of RsbU), block regulation of RssB by anti-adaptors, underscoring the mechanistic similarity (Battesti et al., 2013). Third, conformational change of the switch helices ($\alpha 1$ and $\alpha 2$) of the phosphatase domain is of shared regulatory importance (Battesti et al., 2013). HDX experiments demonstrate that there is decreased exchange of the $\alpha 1$ helix of the pseudo-phosphatase domain of RssB when σ^S is bound, suggesting that the conformational dynamics of this region are reduced by substrate recognition (Brugger et al., 2023a).

These striking mechanistic similarities between RssB from *E. coli* and RsbU from *B. subtilis* suggest that key regulatory features were conserved when an ancestral phosphatase lost enzymatic activity and became a dedicated adapter protein in *E. coli* and related γ -proteobacteria. Interestingly, *P. aeruginosa* has a homologue of RssB/RsbU that functions as an adapter for σ^S degradation, yet has a catalytically active, full-length phosphatase domain and resides in an operon with an *rsbV* homologue (*rssC*; Rodríguez-Martínez et al., 2023). Crystal structures of *P. aeruginosa* RssB revealed that it forms a dimer with crossing linkers and dimerized phosphatase domains with striking similarity to the dimer predicted for RsbU (Figure 5, Figure 5—figure supplement 2C). Intriguingly, adapter protein function of *P. aeruginosa* RssB requires RssC (Rodríguez-Martínez et al., 2023), and *E. coli* RssB has been observed to weakly dimerize (Dorich et al., 2019), further supporting a direct mechanistic link between phosphatase regulation and adapter protein function. While not all GSR regulatory mechanisms involve a phosphatase or adapter protein related to RsbU/RssB, these findings suggest that linker-mediated control of a PPM phosphatase is a broadly conserved mechanism for GSR initiation across bacterial phyla.

An exchangeable toolkit for signal transduction

Histidine kinases and GGDEF diguanylate cyclases are the two most prevalent families of bacterial signal transduction proteins. A striking feature of their regulatory mechanisms is that they are often active in dimeric complexes that are allosterically controlled by N-terminal regulatory domains that propagate signals through crossing α -helical linkers (Zschiedrich et al., 2016; Randall et al., 2022; Gourinchas et al., 2017). Our findings that the crossing α -helical linkers are structurally similar (Fig. S2C) and that shared regulatory domains (response receiver, HAMP, and PAS domains) connect phosphatase dimers by a coiled-coil linker (Figure 5A-C, Figure 5—figure supplement 1) suggests that

this shared allosteric regulatory architecture allows modular exchange of N-terminal domains between PPM phosphatases, histidine kinases, and GGDEF diguanylate cyclases (**Figure 5D**). In support of this, the RsbU N-terminal domain and RsbT were previously shown to control GGDEF diguanylate cyclase activity in *Moorella thermoacetica* (Quin et al., 2012), histidine kinases with RsbU N-terminal domains are found in operons with *rsbT* and stressosome genes in some *Proteobacteria* species (Heinz et al., 2022), and AlphaFold2 prediction of these dimeric complexes reveals coiled-coil linkers to the effector domains (**Figure 5E–F**). Similarly, the PAS/ α/β hydrolase module that controls RsbP phosphatase activity in *B. subtilis* is found coupled to histidine kinases and GGDEF diguanylate cyclases across bacterial phyla (Nadezhdin et al., 2011).

While the specific mechanism discovered here for linker control of RsbU activity (flexibility controlled by a binding partner) is distinct from the models for linker control of histidine kinases and GGDEF diguanylate cyclases (piston shift, scissoring, and helical rotation; Zschiedrich et al., 2016; Randall et al., 2022), the shared dimeric architecture suggests that N-terminal regulatory domains could be modularly exchanged across effector domain families (**Figure 5G**). Allosteric control of PPM switch element conformation could theoretically be achieved by piston shift, scissoring, or helical rotation. Future characterization of PPM phosphatases will be required to determine how widespread linker flexibility is as a regulatory mechanism and how modularly sensory domains can be exchanged across signaling effector families.

Materials and methods

Protein expression and purification

All proteins were expressed in *E. coli* BL21 (DE3) cells grown at 37 °C to an OD₆₀₀ of 0.4 and induced at 16 °C for 14–18 hr with 1 mM isopropyl β -D-1-thiogalactopyranoside (IPTG) unless otherwise specified. Cells were harvested and purified as follows:

RsbU and RsbU variants

Cell pellets were resuspended in lysis buffer with 1 mM phenylmethylsulfonyl fluoride (PMSF; 50 mM K•HEPES, pH 7.5, 100 mM NaCl, 20 mM imidazole, 10% glycerol, 0.5 mM dithiothreitol (DTT)), and were lysed using two passes in a microfluidizer at 10,000 PSI. Cell lysates were cleared by spinning at 16,000 RPM for 45 min in an Avanti JA-20 rotor. Cleared lysates were then run over a HisTrap HP column on an AKTA FPLC, washed with lysis buffer, and eluted with a gradient to 200 mM imidazole. RsbU was run on a Superdex200 16/600 column equilibrated with lysis buffer on an AKTA FPLC. Fractions were pooled and the 6-His tags were cleaved with 3 C protease in dialysis to lysis buffer overnight at 4 °C. Cleaved tags and 3 C protease were subtracted by passing over a column containing equilibrated Ni-NTA resin. 2 mM EDTA was added to cleaved protein prior to gel filtration run. RsbU was then further purified on a Superdex200 16/600 column equilibrated with 20 mM K•HEPES pH 7.5, 100 mM NaCl, 2 mM DTT on an AKTA FPLC. Fractions were pooled, concentrated to 200 μ M, and flash-frozen and stored at –80 °C.

RsbT/U and RsbT/RsbU^{Q94L} co-expression

Cell pellets were resuspended in lysis buffer with 1 mM PMSF (50 mM K•HEPES pH 8.0, 200 mM NaCl, 20 mM imidazole, 10% glycerol, 0.5 mM DTT), and were lysed using two passes in a microfluidizer at 10,000 PSI. Cell lysates were cleared by spinning at 16,000 RPM for 45 min in an Avanti JA-20 rotor. Cleared lysates were then run over a HisTrap HP column on an AKTA FPLC, washed with lysis buffer, and eluted with a gradient to 200 mM imidazole. 2 mM EDTA was added to protein prior to gel filtration run. RsbT/RsbU was then further purified on a Superdex200 16/600 column equilibrated with 20 mM K•HEPES pH 8.0, 100 mM NaCl, 2 mM DTT on an AKTA FPLC. Fractions were pooled, concentrated to 200 μ M, and flash-frozen and stored at –80 °C.

RsbT

Cells were resuspended in lysis buffer with 1 mM PMSF (50 mM K•HEPES pH 8.0, 100 mM NaCl, 20 mM imidazole, 10% glycerol, 2 mM MgCl₂, 0.1 mM ATP, 0.5 mM DTT), and were lysed using two passes in a microfluidizer at 10,000 PSI. Cell lysates were cleared by spinning at 16,000 RPM for 45 min in an Avanti JA-20 rotor. Cleared lysates were then run over 2 mL of cells of Ni-NTA resin

slurry equilibrated with lysis buffer. The resin was then washed with 10 CV of lysis buffer. Ni-NTA resin was resuspended with 10 mL of lysis buffer with 400 mM imidazole, and was incubated for 10 min. RsbT was eluted off column, elution was tracked by Bradford Reagent. Additional elution buffer was added until all protein was eluted off column. The 6-His tags were cleaved with 3 C protease in dialysis to lysis buffer overnight at 4 °C. Cleaved tags and 3 C protease were subtracted by passing over a column containing equilibrated Ni-NTA resin. Protein was concentrated to approximately 50 μ M, was flash-frozen and stored at –80 °C.

RsbV

RsbV was expressed in *E. coli* BL21 (DE3) cells grown at 37 °C to an OD₆₀₀ of 0.4 and induced at 37 °C for 3–4 hrs with 1 mM IPTG. Cells were harvested and resuspended in lysis buffer with 1 mM PMSF (50 mM K•HEPES pH 8.0, 200 mM NaCl, 20 mM imidazole, 10% glycerol, 0.5 mM DTT), and were lysed using two passes on a microfluidizer at 10,000 PSI. Cell lysates were cleared by spinning at 16,000 RPM for 45 min in an Avanti JA-20 rotor. Cleared lysates were then run over a HisTrap HP column on an AKTA FPLC, washed with lysis buffer, and eluted with a gradient to 200 mM imidazole. 2 mM EDTA was added to protein prior to gel filtration run. The 6-His tags were cleaved with 3 C protease in dialysis to lysis buffer overnight at 4 °C. Cleaved tags and 3 C protease were subtracted by passing over a column containing equilibrated Ni-NTA resin. Cleaved protein was then further purified on a Superdex75 16/60 equilibrated in 20 mM K•HEPES pH 7.5, 150 mM NaCl, 10% glycerol, 2 mM DTT. Fractions were pooled, concentrated to approximately 100 μ M, and flash-frozen and stored at –80 °C.

RsbW

Cell pellets were resuspended in lysis buffer with 1 mM PMSF (20 mM K•HEPES pH 7.5, 200 mM NaCl, 20 mM imidazole, 10 mM MgCl₂, 10% glycerol, and 0.5 mM DTT), and were lysed using two passes on a microfluidizer at 10,000 PSI. Cell lysates were cleared by spinning at 16,000 RPM for 45 min in an Avanti JA-20 rotor. Cleared lysates were then run over a HisTrap HP column on an AKTA FPLC, washed with lysis buffer, and eluted with a gradient to 200 mM imidazole. The 6 H tag was left uncleaved to aid removal after phosphorylation reactions. RsbW was then further purified on a Superdex75 16/60 equilibrated in 50 mM K•HEPES pH 7.5, 150 mM NaCl, 10% glycerol, and 2 mM DTT. Fractions were pooled, concentrated to approximately 100 μ M, and flash-frozen and stored at –80 °C.

RsbV-P

RsbVW coexpression plasmid was expressed in *E. coli* BL21 (DE3) cells grown at 37 °C to an OD₆₀₀ of 0.4 and induced at 37 °C for 3–4 hr with 1 mM IPTG. Cells were harvested and resuspended in lysis buffer with 1 mM PMSF (50 mM K•HEPES pH 7.5, 50 mM KCl, 20 mM imidazole, 0.5 mM DTT), and were lysed using two passes on a microfluidizer at 10,000 PSI. Cell lysates were cleared by spinning at 16,000 RPM for 45 min in an Avanti JA-20 rotor. Cleared lysates were then run over a HisTrap HP column on an AKTA FPLC, washed with lysis buffer, and eluted with a gradient to 200 mM imidazole. The 6-His tags were cleaved with 3 C protease in dialysis to lysis buffer overnight at 4 °C. Cleaved tags and 3 C protease were subtracted by passing over a column containing equilibrated Ni-NTA resin. RsbVW was run on a Superdex75 16/60 column equilibrated with lysis buffer on an AKTA FPLC. Fractions of RsbVW coelution were pooled and RsbV was phosphorylated in dialysis at room temperature in lysis buffer with 5 mM ATP and 10 mM MgCl₂. RsbV-P Fractions were pooled, concentrated to 200 μ M, and flash-frozen and stored at –80 °C. Dialyzed RsbVW were then concentrated and run on Superdex75 16/60 column equilibrated with 20 mM K•HEPES pH 7.5, 150 mM NaCl, 10% glycerol, 2 mM DTT. RsbV-P Fractions were pooled, concentrated to 200 μ M, and flash-frozen and stored at –80 °C.

SEC-MALS-SAXS

Small Angle X-ray Scattering (SAXS) was performed at BioCAT (beamline 18ID at the Advanced Photon Source, Chicago) with in-line size exclusion chromatography (SEC) to separate sample from aggregates and other contaminants and ensure optimal sample quality. In-line multiangle light scattering (MALS), dynamic light scattering (DLS) and differential refractive index (dRI) measurements were recorded for additional biophysical characterization (SEC-MALS-SAXS). The samples were loaded on a Superdex

200 10/300 Increase GL Column (Cytiva) run by a 1260 Infinity II HPLC (Agilent Technologies) at 0.6 mL/min. The flow passed in order through an Agilent UV detector, a MALS detector and a DLS detector (DAWN Helios II, Wyatt Technologies), and an RI detector (Optilab T-rEX, Wyatt) before the SAXS flow cell. The flow cell consists of a 1.0 mm ID quartz capillary with ~20 μm walls. A coflowing buffer sheath is used to separate sample from the capillary walls, helping prevent radiation damage (Kirby *et al.*, 2016). Scattering intensity was recorded using a Pilatus3 X1 M (Dectris) detector that was placed 3.682 m from the sample giving us access to a q -range of 0.0027 $\text{\AA}^{-1}$ to 0.33 $\text{\AA}^{-1}$. 0.5 s exposures were acquired every 1 s during elution and data was reduced using BioXTAS RAW 2.1.4 (Hopkins *et al.*, 2017; Hopkins 2024). Buffer blanks were created by averaging regions flanking the elution peak and subtracted from all measured profiles. Because of the heterogeneity in the samples, EFA was used to deconvolve the scattering profiles of the overlapping components (Meisburger *et al.*, 2016). These scattering profiles were used for subsequent analysis in RAW. The quality of the deconvolution was assessed based on the mean weighted χ^2 for each deconvoluted component and the χ^2 of the combined fit to each frame of the dataset (Figure 4—figure supplement 2B–E, Figure 4—figure supplement 4B–E). RAW was used to run GNOM from the ATSAS package (version 3.2.1) (Svergun, 1992; Manalastas-Cantos *et al.*, 2021). Description of each SAXS sample and results of the analysis are presented in (Table 1; Trewthella *et al.*, 2017). The composition of each scattering component was determined based on the SAXS derived molecular weights, the MALS profile, and gels from the purification of the protein or protein complex (Figure 4—figure supplement 1; Table 1). Molecular weights and hydrodynamic radii were calculated from the MALS and DLS data respectively using the ASTRA 7 software (Wyatt). All samples were run in 20 mM HEPES pH 7.5, 100 mM NaCl, 5 mM DTT. Wild-type RsbU was run at a concentration of 3.5 mg/mL, RsbT/RsbU^{Q94L} was run at a concentration of 2.5 mg/mL, RsbU^{Q94L} was run at a concentration of 3.5 mg/mL, RsbT/RsbU was run at a concentration of 2.5 mg/mL. FoXS was used to calculate predicted scattering profiles from the model, and was compared to the experimental scattering profiles (Schneidman-Duhovny *et al.*, 2016; Schneidman-Duhovny *et al.*, 2013). Output parameters calculated using FoXS are provided in Table 1. MultiFoXS was used to generate a flexible structure ensemble with multicomponent fits and was compared to the experimental scattering profiles (Schneidman-Duhovny *et al.*, 2016). MultiFoXS input and output parameters are provided in Table 1.

Fluorescence anisotropy

We site-specifically labeled RsbT with tetramethylrhodamine 5-maleimide (TMR) dye at its unique cysteine (position 6). We added increasing concentrations of RsbU and RsbU^{Q94L} to 200 nM RsbT^{TMR} and measured the change in anisotropy (Figure 2A). All measurements were done at 25 °C in 50 mM K•HEPES pH 7.5, 100 mM NaCl, and 10% glycerol. All measurements of spectra were conducted with a slit width of 7 nm, and anisotropy measurements were conducted with a slit width of 7 nm. The excitation wavelength was 551 nm and the emission wavelength was 577 nm. Anisotropy measurements were taken 30 s apart for six measurements, and then were averaged and plot against its corresponding concentration of RsbU. Anisotropy data was then fit to a quadratic binding curve using KaleidaGraph, reported errors are the error of the fit.

Phosphatase assays

Phosphatase assays were performed with RsbV-P that was labeled with ³²P by incubating RsbV (40 μM), 6His-RsbW (45 μM), and 100 μCi of γ -³²P ATP overnight at room temperature in 50 mM K•HEPES pH 7.5, 50 mM KCl, 10 mM MgCl₂, and 2 mM DTT. Unincorporated nucleotide was removed by buffer exchange using a Zeba spin column equilibrated in 50 mM K•HEPES pH 8.0, 100 mM NaCl. 6H-RsbW was then removed by Ni-NTA resin equilibrated in 50 mM K•HEPES pH 8.0, 100 mM NaCl, 20 mM imidazole. The flow-through fraction from the Ni-NTA resin containing RsbV-³²P was then exchanged into 50 mM K•HEPES pH 8.0, 100 mM NaCl buffer using 3 subsequent Zeba spin columns to remove all unincorporated nucleotide and free phosphate. Labeled RsbV-³²P was aliquoted and frozen at –80 °C for future use.

All phosphatase assays were performed at room temperature in 50 mM K•HEPES pH 7.5, 100 mM NaCl. The concentrations of enzyme, substrate, MnCl₂, and MgCl₂ were varied as indicated. 10 μM RsbT was additionally added to reactions as indicated. Reactions were stopped with 0.5 M EDTA, pH 8.0 and run on PEI-Cellulose TLC plates developed in 1 M LiCl₂ and 0.8 M acetic acid and imaged

Table 2. *B. subtilis* strains.
All *B. subtilis* strains are in the background of PY79.

Strain #	Genotype	Reference
RB01	Δ rsbPQ Δ rsbU rsbV-FLAG amyE::ctc-lacZ Pspank-rsbU	Ho and Bradshaw, 2021
RB02	Δ rsbPQ Δ rsbTU rsbV-FLAG amyE::ctc-lacZ Pspank-rsbU	Ho and Bradshaw, 2021
RB03	Δ rsbPQ Δ rsbTU rsbV-FLAG amyE::ctc-lacZ Pspank-rsbTU	Ho and Bradshaw, 2021
RB04	Δ rsbPQ Δ rsbU rsbV-FLAG amyE::ctc-lacZ Pspank-rsbU ^{Q94L}	This study
RB05	Δ rsbPQ Δ rsbTU rsbV-FLAG amyE::ctc-lacZ Pspank-rsbU ^{Q94L}	This study
RB06	Δ rsbPQ Δ rsbU rsbV-FLAG amyE::ctc-lacZ Pspank-rsbU ^{M166V}	Ho and Bradshaw, 2021
RB07	Δ rsbPQ Δ rsbTU rsbV-FLAG amyE::ctc-lacZ Pspank-rsbU ^{M166V}	Ho and Bradshaw, 2021
RB08	Δ rsbPQ Δ rsbTU rsbV-FLAG amyE::ctc-lacZ Pspank-rsbTU ^{Y28I}	This study
RB09	Δ rsbPQ Δ rsbTU rsbV-FLAG amyE::ctc-lacZ Pspank-rsbTU ^{Y28I,S49G}	This study
RB10	Δ rsbPQ Δ rsbTU rsbV-FLAG amyE::ctc-lacZ Pspank-rsbTU ^{Y28I,K53E}	This study
RB11	Δ rsbPQ Δ rsbTU rsbV-FLAG amyE::ctc-lacZ Pspank-rsbTU ^{Y28I,T89A}	This study
RB12	Δ rsbPQ Δ rsbTU rsbV-FLAG amyE::ctc-lacZ Pspank-rsbTU ^{Y28I,G92R}	This study
RB13	Δ rsbPQ Δ rsbTU rsbV-FLAG amyE::ctc-lacZ Pspank-rsbTU ^{Y28I,G92E}	This study
RB14	Δ rsbPQ Δ rsbTU rsbV-FLAG amyE::ctc-lacZ Pspank-rsbTU ^{Y28I,Q94L}	This study
RB15	Δ rsbPQ Δ rsbTU rsbV-FLAG amyE::ctc-lacZ Pspank-rsbTU ^{Y28I,T110S}	This study
RB16	Δ rsbPQ Δ rsbTU rsbV-FLAG amyE::ctc-lacZ Pspank-rsbTU ^{Y28I,V142A}	This study
RB17	Δ rsbPQ Δ rsbTU rsbV-FLAG amyE::ctc-lacZ Pspank-rsbTU ^{Y28I,K143E}	This study
RB18	Δ rsbPQ Δ rsbTU rsbV-FLAG amyE::ctc-lacZ Pspank-rsbTU ^{Y28I,C165Y}	This study
RB19	Δ rsbPQ Δ rsbTU rsbV-FLAG amyE::ctc-lacZ Pspank-rsbTU ^{Y28I,M173I}	This study
RB20	Δ rsbPQ Δ rsbTU rsbV-FLAG amyE::ctc-lacZ Pspank-rsbTU ^{R91E}	This study
RB21	Δ rsbPQ Δ rsbTU rsbV-FLAG amyE::ctc-lacZ Pspank-rsbT ^{D92K} U	This study
RB22	Δ rsbPQ Δ rsbTU rsbV-FLAG amyE::ctc-lacZ Pspank-rsbT ^{D92K} U ^{R91E}	This study
RB23	Δ rsbPQ Δ rsbU rsbV-FLAG amyE::ctc-lacZ Pspank-rsbU ^{Q94I}	This study
RB24	Δ rsbPQ Δ rsbTU rsbV-FLAG amyE::ctc-lacZ Pspank-rsbU ^{Q94I}	This study
RB25	Δ rsbPQ Δ rsbU rsbV-FLAG amyE::ctc-lacZ Pspank-rsbU ^{Q94V}	This study
RB26	Δ rsbPQ Δ rsbTU rsbV-FLAG amyE::ctc-lacZ Pspank-rsbU ^{Q94V}	This study
RB27	Δ rsbPQ Δ rsbU rsbV-FLAG amyE::ctc-lacZ Pspank-rsbU ^{Q94Y}	This study
RB28	Δ rsbPQ Δ rsbTU rsbV-FLAG amyE::ctc-lacZ Pspank-rsbU ^{Q94Y}	This study
RB29	Δ rsbPQ Δ rsbU rsbV-FLAG amyE::ctc-lacZ Pspank-rsbU ^{Q94F}	This study
RB30	Δ rsbPQ Δ rsbTU rsbV-FLAG amyE::ctc-lacZ Pspank-rsbU ^{Q94F}	This study
RB31	Δ rsbPQ Δ rsbU rsbV-FLAG amyE::ctc-lacZ Pspank-rsbU ^{Q94W}	This study
RB32	Δ rsbPQ Δ rsbTU rsbV-FLAG amyE::ctc-lacZ Pspank-rsbU ^{Q94W}	This study
RB33	Δ rsbPQ Δ rsbU rsbV-FLAG amyE::ctc-lacZ Pspank-rsbU ^{Q94M}	This study
RB34	Δ rsbPQ Δ rsbTU rsbV-FLAG amyE::ctc-lacZ Pspank-rsbU ^{Q94M}	This study
RB35	Δ rsbPQ Δ rsbU rsbV-FLAG amyE::ctc-lacZ Pspank-rsbU ^{Q94N}	This study
RB36	Δ rsbPQ Δ rsbTU rsbV-FLAG amyE::ctc-lacZ Pspank-rsbU ^{Q94N}	This study
RB37	Δ rsbPQ Δ rsbU rsbV-FLAG amyE::ctc-lacZ Pspank-rsbU ^{Q94E}	This study
RB38	Δ rsbPQ Δ rsbTU rsbV-FLAG amyE::ctc-lacZ Pspank-rsbU ^{Q94E}	This study

Table 2 continued on next page

Table 2 continued

Strain #	Genotype	Reference
RB39	Δ rsbPQ Δ rsbU rsbV-FLAG amyE::ctc-lacZ Pspank-rsbU ^{Q94A}	This study
RB40	Δ rsbPQ Δ rsbTU rsbV-FLAG amyE::ctc-lacZ Pspank-rsbU ^{Q94A}	This study
RB41	Δ rsbPQ Δ rsbU rsbV-FLAG amyE::ctc-lacZ Pspank-rsbU ^{Q94G}	This study
RB42	Δ rsbPQ Δ rsbTU rsbV-FLAG amyE::ctc-lacZ Pspank-rsbU ^{Q94G}	This study
RB43	Δ rsbPQ Δ rsbU rsbV-FLAG amyE::ctc-lacZ Pspank-rsbU ^{T89A}	This study
RB44	Δ rsbPQ Δ rsbU rsbV-FLAG amyE::ctc-lacZ Pspank-rsbU ^{C165Y}	This study

on a Typhoon scanner. Phosphatase assays were performed more than three independent times as separate experiments. Multiple turnover reactions were performed with trace RsbV-³²P and an excess of cold RsbV-P. Number of turnovers were calculated for each timepoint from (1 – fraction substrate)*[-substrate]/[enzyme]. Data shown in figures is from a single representative experiment, and reported errors are the error from the fit.

B. subtilis strain construction

B. subtilis cells were grown in Lennox lysogeny broth (LB, Sigma-Aldrich) as liquid medium or as plates supplemented with 15% Bacto Agar (Difco). Antibiotics were used as appropriate for maintenance of plasmids or for selection of transformants: MLS (50 µg/mL erythromycin, 250 µg/mL lincomycin), kana-mycin (10 µg/mL). For plates used to visualize σ^B reporter activity, 80 µg/mL of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) and 1 mM IPTG were added. All *B. subtilis* strains were derived from the PY79 strain background and are listed in the table of strains. Plasmids based on pHB201 (Bron et al., 1998) were introduced to *B. subtilis* strains using natural competence (Harwood and Cutting, 1990). All *B. subtilis* strains used in this study are listed in Table 2, *E. coli* strains are listed in Table 3.

Genetic screens

Genetic screens were performed as described previously (Ho and Bradshaw, 2021). Plasmids containing *rsbU* or *rsbT/rsbU*^{Y28I} were subjected to PCR mutagenesis using GoTaq DNA polymerase mix (Promega) supplemented with 2 mM additional MgCl₂. Pools of mutagenized inserts were assembled into digested plasmid using isothermal (Gibson) assembly and transformed into *E. coli* DH5α cells. Mutation rate of approximately one single-nucleotide polymorphism per kilobase of DNA was confirmed by sequencing individual clones. Plasmids were then introduced to *B. subtilis* using natural competence and cells were plated on selective medium containing IPTG and X-gal for two days at 37 °C. Blue colonies were selected, restruct on plates with and without IPTG, and plasmids recovered from single colonies that retested as σ^B positive were sequenced using Sanger sequencing. For the RsbU^{Y28I} suppressor screen, two independent pools of PCR mutagenized plasmid were screened. In addition to the reported variants that were selected, we identified simple revertants that restored Y28 in both pools, and mutations in the promoter and RBS that we predict enhanced protein production. These variants were not studied further. Individual amino acid changing mutations were rebuilt in the parental plasmid using PCR (QuickChange) mutagenesis and the phenotypes were validated by replating on indicator plates. All strains shown in the figures are from these rebuilt plasmids.

AlphaFold2 structure predictions

AlphaFold2 predictions were performed using ColabFold (Mirdita et al., 2022). Alphafold2_multimer_v2 was used in unpaired_paired mode with no templates with 3 recycles, 200 iterations, and greedy pairing strategy. The predicted aligned error plots for all AlphaFold2 structures are shown in Figure 1—figure supplement 1. The predicted local distance difference test scores (pLDDT) mapped onto the structures are shown in Figure 1—figure supplement 2.

Table 3. *E. coli* strains.

Strain #	Genotype	Reference
RB45	BL21 (DE3) pET47b 6H-3C-rsbT	Ho and Bradshaw, 2021
RB46	BL21 (DE3) pET47b 6H-3C-rsbU	Ho and Bradshaw, 2021
RB47	BL21 (DE3) pET47b 6H-3C-rsbV	Ho and Bradshaw, 2021
RB48	BL21 (DE3) pET47b 6H-3C-rsbW	Ho and Bradshaw, 2021
RB49	BL21 (DE3) pET47b 6H-3C-rsbVW	This study
RB50	BL21 (DE3) pET47b 6H-3C-rsbU ^{Q94L}	This study
RB51	BL21 (DE3) pET47b 6H-3C-rsbU ^{M166V}	Ho and Bradshaw, 2021
RB52	BL21 (DE3) pET47b 6H-3C-rsbTU	This study
RB53	BL21 (DE3) pET47b 6H-3C-rsbU ^{R91E}	This study
RB54	BL21 (DE3) pET47b 6H-3C-rsbTU ^{Q94L}	This study
RB55	DH5 α Pspank-rsbU	Ho and Bradshaw, 2021
RB56	DH5 α Pspank-rsbTU	Ho and Bradshaw, 2021
RB57	DH5 α rsbU ^{Q94L}	
RB58	DH5 α Pspank-rsbU ^{M166V}	Ho and Bradshaw, 2021
RB59	DH5 α Pspank-rsbTU ^{Y28I}	This study
RB60	DH5 α Pspank-rsbTU ^{Y28I,S49G}	This study
RB61	DH5 α Pspank-rsbTU ^{Y28I,K53E}	This study
RB62	DH5 α Pspank-rsbTU ^{Y28I,T89A}	This study
RB63	DH5 α Pspank-rsbTU ^{Y28I,G92R}	This study
RB64	DH5 α Pspank-rsbTU ^{Y28I,G92E}	This study
RB65	DH5 α Pspank-rsbTU ^{Y28I,Q94L}	This study
RB66	DH5 α Pspank-rsbTU ^{Y28I,T110S}	This study
RB67	DH5 α Pspank-rsbTU ^{Y28I,V142A}	This study
RB68	DH5 α Pspank-rsbTU ^{Y28I,K143E}	This study
RB69	DH5 α Pspank-rsbTU ^{Y28I,C165Y}	This study
RB70	DH5 α Pspank-rsbTU ^{Y28I,M173I}	This study
RB71	DH5 α Pspank-rsbTU ^{R91E}	This study
RB72	DH5 α Pspank-rsbT ^{D92K} U	This study
RB73	DH5 α Pspank-rsbT ^{D92K} U ^{R91E}	This study
RB74	DH5 α Pspank-rsbU ^{Q94I}	This study
RB75	DH5 α Pspank-rsbU ^{Q94V}	This study
RB76	DH5 α Pspank-rsbU ^{Q94Y}	This study
RB77	DH5 α Pspank-rsbU ^{Q94Y}	This study
RB78	DH5 α Pspank-rsbU ^{Q94F}	This study
RB79	DH5 α Pspank-rsbU ^{Q94W}	This study
RB80	DH5 α Pspank-rsbU ^{Q94M}	This study
RB81	DH5 α Pspank-rsbU ^{Q94N}	This study
RB82	DH5 α Pspank-rsbU ^{Q94E}	This study
RB83	DH5 α Pspank-rsbU ^{Q94A}	This study

Table 3 continued on next page

Table 3 continued

Strain #	Genotype	Reference
RB84	DH5 α Pspank-rsbU ^{Q94G}	This study
RB85	DH5 α Pspank-rsbU ^{T89A}	This study
RB86	DH5 α Pspank-rsbU ^{C165Y}	This study

Socket2 analysis of AlphaFold2 structure predictions

Socket2 coiled-coil predictions were performed using Socket2 Colab (Kumar and Woolfson, 2021). Socket2 was used with a packing cutoff of 7.0 Å. Analysis done using Socket2 is shown in **Figure 5—figure supplement 2**.

Materials availability statement

All strains, plasmids, and coordinate files reported in this manuscript are available by request to the corresponding author.

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Additional files

Supplementary files

MDAR checklist

Data availability

SAXS data were deposited in SASDB under accession codes SASDU85 and SASDU95. All other data generated or analyzed during this study are included in the manuscript and supporting files.

The following datasets were generated:

Author(s)	Year	Dataset title	Dataset URL	Database and Identifier
Baral R, Kumar RP, Hopkins JB, Watkins MB, Bradshaw N	2025	Wild-type phosphoserine phosphatase RsbU dimer	https://www.sasbdb.org/data/SASDU85/	SASDB, SASDU85
Baral R, Kumar RP, Hopkins JB, Watkins MB, Bradshaw N	2025	Hyperactive variant of phosphoserine phosphatase RsbU (Q94L) bound to the activator serine/threonine-protein kinase RsbT (heterotetrameric complex)	https://www.sasbdb.org/data/SASDU95/	SASDB, SASDU95

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