Cargo engagement protects protease adaptors from degradation in a substrate-specific manner

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Protein degradation in bacteria is a highly controlled process involving proteolytic adaptors that regulate protein degradation during cell cycle progression or during stress responses. Many adaptors work as scaffolds that selectively bind cargo and tether substrates to their cognate proteases to promote substrate destruction, whereas others primarily activate the target protease. Because adaptors must bind their cognate protease, all adaptors run the risk of being recognized by the protease as substrates themselves, a process that could limit their effectiveness. Here we use purified proteins in a reconstituted system and in vivo studies to show that adaptors of the ClpXP protease are readily degraded but that cargo binding inhibits this degradation. We found that this principle extends across several adaptor systems, including the hierarchical adaptors that drive the Caulobacter bacterial cell cycle and the quality control adaptor SspB. We also found that the ability of a cargo to protect its adaptor is not simply subject-specific, as adaptors with artificial degradation tags were not protected even though cargo binding is unaffected. Our work points to an optimization of inherent adaptor degradation and cargo binding that ensures that robust adaptor activity is maintained when high amounts of substrate must be delivered and that adaptors can be eliminated when their tasks have been completed.

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2 The abbreviations used are: AAA, ATPases associated with diverse cellular activities; SW, swarmer; ST, stalked; cdGMP, cyclic di-GMP; Ni-NTA, nickel-nitrilotriacetic acid; SUMO, small ubiquitin-like modifier; PYE, peptone yeast extract.

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activity while allowing for a reset of protease complexes after the need has passed.

Results

The RcdA adaptor is degraded by a CpdR-activated ClpXP protease

We previously identified RcdA as an adaptor that delivers multiple cell cycle regulators exclusively to a CpdR-primed ClpXP (15). During additional characterization of RcdA-dependent degradation, we found that RcdA itself was degraded by the ClpXP protease in the presence of CpdR (Fig. 1, A and B). Consistent with this observation, cells lacking CpdR show higher steady-state levels of RcdA than wild-type cells (Fig. 1C). Translation shutoff experiments suggested that these increased levels are due to loss of RcdA degradation in ΔcpdR strains (Fig. 1, D and E). Together, these data show that the RcdA adaptor is degraded by ClpXP in a CpdR-dependent manner both in vivo and in vitro. We next sought to determine which sequence elements were important for RcdA degradation.

Previous work has shown that the C terminus of RcdA binds to a CpdR-primed ClpXP to deliver substrates for degradation (15). As expected, RcdAΔC was not degraded, even in the presence of CpdR, suggesting that the C terminus of RcdA is necessary for degradation (Fig. 2, B and C). We next used the fusion protein RcdAΔC−XB, where the substrate-binding domain of RcdA is appended with the ClpX-tethering motif of SspB (Fig. 2A) (15). This construct can deliver substrates to ClpXP even in the absence of CpdR (supplemental Fig. S1A) (15) but was not robustly degraded (Fig. 2, D and E). Therefore, simply tethering the cargo-binding domain of RcdA to ClpXP is insufficient for RcdA adaptor degradation.

Our results suggest that the RcdA degron is encoded by the same C-terminal residues needed for binding the CpdR-primed ClpX. RcdA ends in GG, a dipeptide sequence shown previously to be able to be recognized by ClpX in appropriately presented substrates (20). We mutated these residues to make an RcdADD variant that was fully capable of delivering cargo substrates in a CpdR-dependent manner (Fig. 2H and supplemental Fig. S1B) but was now substantially resistant to degradation compared with wild-type RcdA (Fig. 2, F and G). This allele supported CtrA degradation in vivo similar to wild-type RcdA (supplemental Fig. S1D) but was markedly stabilized (supplemental Fig. S1C), consistent with the in vitro results.

RcdA adaptor degradation is suppressed upon cargo binding

How is the RcdA adaptor able to effectively deliver its substrates to ClpXP given its own rather rapid degradation by the same protease? To address this question, we tested whether RcdA degradation was affected during delivery of its substrates. We found that addition of RcdA-dependent ClpXP substrates (TacA and CC2323) partially suppressed RcdA degradation (Fig. 3, A and B), suggesting that RcdA degradation is inhibited while it is actively operating as an adaptor. We considered two models for how this inhibition could come about: this suppression could be due to protection of the adaptor from the protease upon cargo binding, or suppression could arise from competition between the delivered substrates and RcdA degradation for the protease. To distinguish between these possibilities, we used substrate variants (TacADD and CC2323DD) that bind RcdA but are not recognized by the protease (supplemental Fig. S2, A and B) (15). Interestingly, addition of these variants resulted in even stronger suppression of RcdA degradation (Fig.
These results suggest that binding of cargo itself protects RcdA from protease recognition.

The cdGMP binding protein PopA is an upstream adaptor of RcdA during the hierarchical delivery of cell cycle substrates (15). Our above work suggests that cargo binding to RcdA alone is sufficient for inhibition of degradation. Because PopA directly interacts with RcdA (18, 19), we speculated that PopA would also protect RcdA from degradation. Consistent with this hypothesis, we found that PopA addition inhibited RcdA degradation (Fig. 3, E and F). Addition of cdGMP did not further affect this suppression (Fig. 3, E and F), validating prior observations that PopA binds RcdA regardless of cdGMP (18, 19). Similar to its stabilizing effect in vitro, overexpression of PopA or a cdGMP-insensitive variant (PopA-R357G) (18) reduces RcdA degradation in vivo (Fig. 3, G and H). Importantly, PopA was not degraded either in vivo or in vitro (Fig. 3E and supplemental Fig. S2, D–F), further supporting our working model that cargo binding alone is sufficient for RcdA stabilization even when that cargo is not delivered to the ClpXP protease.

**Cargo-mediated suppression of adaptor degradation is not a limited phenomenon**

To determine whether suppression of adaptor degradation upon binding of cargo is a general phenomenon, we monitored the stability of other known adaptors from *Caulobacter*. The adaptor SspB facilitates degradation of ssrA-tagged substrates by directly tethering them to the ClpXP protease (9, 10). The *Caulobacter* SspB was shown previously to be a ClpXP substrate in vitro (21), and we found that SspB degradation is suppressed in the presence of GFP-ssrA (Fig. 4, A and B). Similar to our work with RcdA, this inhibition does not depend on substrate degradation, as addition of the non-degraded GFP-ssrADD variant (which binds SspB as well as wild-type (23)) also stabilizes SspB degradation (Fig. 4, A and B).
CpdR is an adaptor that binds ClpXP to prime it for cell cycle-dependent activity (17). CpdR is degraded by the ClpXP protease in vivo (22), and we found it to be degraded on its own by ClpXP in vitro (Fig. 4, C and D) (17). When we monitored CpdR while it was delivering its substrate PdeA to ClpXP, we found that CpdR degradation was reduced (Fig. 4, C and D). Addition of the nondegradable PdeADD also suppresses CpdR degradation (Fig. 4, C and D). CpdR degradation was not strongly affected by RcdA alone (supplemental Fig. S3, A and B) or by RcdA-cargo complexes (supplemental Fig. S3, C and D), which we attribute to a relatively weak interaction between RcdA alone and CpdR-ClpX. In sum, we find that suppression

Figure 3. RcdA adaptor degradation is suppressed upon cargo binding. A, C, and E, RcdA degradation by ClpXP in the absence or presence of TacA/CC2323 (A) and TacADD/CC2323DD (C) in vitro. SDS-PAGE gel images are shown. B and D, bands corresponding to RcdA and ClpP were quantified, and normalized intensities were plotted. Data represent mean ± S.D. of three biological replicates (A) and two independent experiments (C). E, in vitro degradation of RcdA by ClpXP in the absence or presence of PopA and cdG. CpdR was used in all the reactions (A, C, and E). F, quantification of band intensities. Data represent mean ± S.D. of three biological replicates. G, RcdA degradation is inhibited in cells overexpressing PopA or a PopA variant that was deficient in cdG binding (PopA-R357G) (18). Protein expression was induced with 0.2% xylose throughout the course of the experiment. Translation was inhibited by addition of chloramphenicol. Aliquots were taken at the indicated time points for Western blotting. ClpP was used as a loading control. H, bands corresponding to RcdA and ClpP were quantified, and normalized intensities were plotted. Data represent mean ± S.D. of two independent experiments.
Figure 4. Cargo-mediated suppression of adaptor degradation is seen in other adaptor systems. A and C, degradation of the SspB adaptor was suppressed in the presence of a GFP-tagged ssrA or a nondegradable GFP-ssrADD. SDS-PAGE gel images are shown. B, bands corresponding to SspB and ClpP were quantified, and normalized intensities were plotted. Data represent mean ± S.D. of three biological replicates. C, CpdR degradation was inhibited in the presence of its cargo. Shown is in vitro degradation of CpdR by ClpXP in the absence or presence of the substrate PdeA or the nondegradable PdeADD variant. D, bands corresponding to CpdR and ClpP were quantified, and normalized intensities were plotted. Data represent mean ± S.D. of three biological replicates.

Inhibition of adaptor degradation by cargo binding is not due to global stabilization

We hypothesized that the cargo binding could affect adaptor degradation in one of two nonexclusive mechanisms. The first possibility is that cargo binding could cause adaptors to be generally more resistant to any forced unfolding. The second is that the adaptor degron itself could be masked upon cargo binding (Fig. 5A). We rationalized that if the first model was correct, then binding of cargo would stabilize the adaptor regardless of how it was recognized by ClpX. To test this, we generated a chimeric fusion protein, RcdAΔC−CtrA15, where we used the CtrA degron to drive degradation of an RcdA construct lacking its normal degron/tethering motif (RcdAΔC−CtrA15) (Fig. 5B). This chimera was slowly degraded by ClpXP alone (Fig. 5, C and D), but we did not observe any changes in degradation with the CC2323 cargo (Fig. 5, C and D). Importantly, RcdAΔC−CtrA15 still binds CC2323 strongly (supplemental Fig. S4A), suggesting that cargo binding alone is insufficient to generally protect an adaptor from degradation. To address whether this result was a consequence of the slower degradation of RcdAΔC−CtrA15, we also used the well-characterized ssrA degron, which is strongly recognized by ClpXP. RcdAΔC−ssrA is degraded rapidly by ClpXP alone, and, similar to our other results, degradation is not affected by the presence of the CC2323 cargo (Fig. 5, E and F). Taken together, these results disfavor a model where cargo binding alone is sufficient to generally protect adaptors from ClpX-mediated unfolding.

Our working hypothesis is that protection of RcdA degradation upon cargo binding arises from masking of its inherent degron rather than general stabilization (Fig. 5A). To explore this model, we fused the CtrA degron to the full-length RcdA protein (Fig. 5B). This RcdA−CtrA15 construct was degraded by ClpXP without CpdR, but cargo binding did not suppress degradation, although this construct was fully competent for cargo binding (Fig. 5, G and H, and supplemental Fig. S5A). Addition of CpdR increased degradation of this chimera (Fig. 5, G and H), presumably because of recognition of the combined degradation/tethering motif of RcdA by the CpdR-primed ClpX (15). Finally, addition of the non-degradable RcdA cargo CC2323DD substantially inhibited degradation of the fusion protein RcdA−CtrA15 (Fig. 5, G and H). Our conclusion is that the protective effect of cargo binding is specific for the recognition of the native degron of the adaptor that, in the case of RcdA, requires CpdR for its recognition. We next tested whether this paradigm could be applied to other cases of adaptor degradation.

The Caulobacter SspB protein contains a ClpX-binding motif at its C terminus (15, 23) and an N-terminal motif required for its degradation (supplemental Fig. S4B) (17, 24). Because we found that suppression of RcdA degradation upon cargo binding requires recognition of its native degron, we hypothesized that a similar mechanism might exist for SspB degradation. To test our hypothesis, we appended the CtrA degron to the C terminus of a minimized SspB variant that lacks its native degron but still delivers cargo (Fig. 6A and supplemental Fig. S4C). This fusion protein (ΔN9SspB−CtrA15) is degraded robustly by ClpXP, but addition of cargo does not protect it from degradation (Fig. 6, B and C). Similar to our above results, fusing the CtrA degron to the full-length SspB resulted in degradation by ClpXP and partial suppression of this degradation in the presence of cargo (Fig. 6, D and E). We note that, in cases where both the artificial and natural degrons are present, binding of cargo appears to only limit the protease recognition of the natural degron, leaving the artificial degron free to be engaged.
Finally, it is possible that binding of a protein cargo results in steric clashes that prevent recognition of the adaptor degron by the protease. To address this, we asked whether an adaptor binding peptide is sufficient to protect adaptors from degradation. Specifically, we used an ssrA-derived peptide (AANDN-NYA) that retains the SspB binding site but lacks the ClpXP recognition determinant (23). We found that addition of this peptide inhibits SspB degradation by ClpXP (Fig. 6, F). Thus, our data support a model where cargo binding leads to masking of the native degron of adaptors, likely through conformational changes.

**Discussion**

Bacterial proteolytic adaptors facilitate degradation of regulatory proteins or poor-quality proteins. These auxiliary factors work in a variety of ways, such as directly tethering substrates to their cognate protease or priming either the protease or substrate (9, 17, 25). Because adaptors must be in close physical proximity to the protease, understanding how adaptors avoid being destroyed impacts our general understanding of adaptor-mediated proteolysis. Here we highlight cases from the model bacterium *C. crescentus* to show how ClpXP adaptors are selec-
Cargo binding protects SspB from degradation initiated by natural degron recognition. A, schematic depicting full-length SspB, a SspB variant lacking its native N terminus degron and appended with a C-terminal CtrA degron, and a full-length SspB appended with the CtrA degron. The N-terminal three residues (GSH) are part of the linker that remains after cleavage by the thrombin protease. B and D, degradation of the ΔN9SspB–CtrA15 variant by ClpXP was not inhibited in the presence of a cargo (GFP-ssrADD), whereas degradation of SspB–CtrA15 was partially inhibited (D). SDS-PAGE gel images are shown (B). Because of overlapping ClpP and SspB–CtrA15 bands, Western blots using anti-SspB antibody are shown to detect SspB–CtrA15. Untagged ClpP was used for SspB–CtrA15 degradation reactions to specifically detect SspB–CtrA15. C and E, quantification of band intensities. The degradation of SspB–CtrA15 was normalized to the zero time point. Data represent mean ± S.D. of three biological replicates (B) and two biological replicates (D). F, SspB adaptor degradation is protected in the presence of ssrA-derived peptide (AANDNNYA). An in vitro degradation experiment was performed in the absence or presence of ssrA peptide. G, bands corresponding to SspB and ClpP were quantified, and normalized intensities of SspB over ClpP were plotted. Data represent mean ± S.D. of two independent experiments.

Figure 6. Cargo binding protects SspB from degradation initiated by natural degron recognition. A, schematic depicting full-length SspB, a SspB variant lacking its native N terminus degron and appended with a C-terminal CtrA degron, and a full-length SspB appended with the CtrA degron. The N-terminal three residues (GSH) are part of the linker that remains after cleavage by the thrombin protease. B and D, degradation of the ΔN9SspB–CtrA15 variant by ClpXP was not inhibited in the presence of a cargo (GFP-ssrADD), whereas degradation of SspB–CtrA15 was partially inhibited (D). SDS-PAGE gel images are shown (B). Because of overlapping ClpP and SspB–CtrA15 bands, Western blots using anti-SspB antibody are shown to detect SspB–CtrA15. Untagged ClpP was used for SspB–CtrA15 degradation reactions to specifically detect SspB–CtrA15. C and E, quantification of band intensities. The degradation of SspB–CtrA15 was normalized to the zero time point. Data represent mean ± S.D. of three biological replicates (B) and two biological replicates (D). F, SspB adaptor degradation is protected in the presence of ssrA-derived peptide (AANDNNYA). An in vitro degradation experiment was performed in the absence or presence of ssrA peptide. G, bands corresponding to SspB and ClpP were quantified, and normalized intensities of SspB over ClpP were plotted. Data represent mean ± S.D. of two independent experiments.

Adaptor proteolysis has been observed in other protease systems. For example, the MecA adaptor activates the ClpC unfoldase to target the ComK protein for degradation by the ClpCP protease in Bacillus subtilis and is itself degraded (25, 26). The cyanobacterial adaptor NblA delivers phycobilisomes to the Clp protease for degradation, and purified NblA is degraded in vitro (27, 28). By contrast, some adaptors remain stable regardless of delivery of their substrates. For example, the RssB adaptor delivers RpoS to ClpXP in Escherichia coli, but RssB itself is not degraded (29). The ClpS adaptor delivers N-end rule substrates to ClpAP and is itself not degraded, although ClpS contains an N-terminal determinant sufficient for ClpAP recognition (30). In this case, the current model is that the core of ClpS contains a tightly folded domain that prevents its degradation during delivery even when ClpA engages this adaptor (30).

This work yields molecular insights into adaptor degradation and its control. A clear next step of study is to understand the physiological relevance of adaptor degradation. When CpdR was first identified, the degradation of CpdR was speculated to serve as a mechanism to release the ClpXP protease from its subcellular polar location (22). Similarly, MecA degradation was suggested to be important for the dynamic assembly and disassembly of the protease complex ClpCP (31). By contrast, the stability of the RssB adaptor is thought to enable its recycling for repeated rounds of RpoS delivery (28, 32). Collectively, our work shows that adaptors are more resistant to proteolysis when loaded with cargo. This suggests that, regardless of the cellular outcome of adaptor degradation (changes in protease location or protease assembly), adaptor degradation is highly
**Experimental procedures**

### Bacterial strains and culture conditions

The *Caulobacter* and *E. coli* strains used in the study are tabulated in supplemental Table S1. *E. coli* strains were grown in lysogeny broth liquid medium or lysogeny broth agar plates at 37 °C with the appropriate antibiotic (100 μg/ml ampicillin, 50 μg/ml kanamycin, or 50 μg/ml spectinomycin). *Caulobacter* strains were grown in peptone yeast extract (PYE) liquid medium or PYE agar plates at 30 °C with the appropriate antibiotic (25 μg/ml spectinomycin or 5 μg/ml kanamycin). *Caulobacter* strains grown in PYE liquid medium were supplemented with 0.2% xylose to induce gene expression wherever required.

### Molecular cloning and generation of chimeric constructs

PopA and PopA-R357G were PCR-amplified and cloned into pENTR plasmids. The constructs were then moved into *xylose-inducible expression plasmids*, which appends an M2 epitope tag on the N terminus of the protein using Gateway-based cloning (33). RcdA and RcdADD were PCR-amplified and cloned into pET23SUMO and pRXMCS2 vectors by the Gibson assembly method (34). RcdAΔC, RcdAΔC~XB, and CC2323DD were PCR-amplified with the appropriate primers and then cloned into the pET23SUMO expression plasmid by the Gibson assembly method. Chimeric variants of RcdA and SspB were generated by round-the-horn site-directed mutagenesis. RcdAΔC~CtrA15 and RcdAΔC~ssrA constructs were generated in the pET23SUMO-RcdAΔC vector by appending the 15 C-terminal residues of CtrA or the 14 residues that constitute the ssrA tag, respectively. The RcdA~CtA15 fusion construct was generated in the pET23SUMO-RcdA vector by designing appropriate primers to append the last 15 residues of CtrA onto the C terminus of full-length RcdA. SspB~CtrA15 and ΔN9SspB~CtrA15 were created by appending the 15 C-terminal residues of CtrA to a SspB variant that either contained or lacked the N-terminal nine residues, respectively. All chimeric constructs and mutant proteins were confirmed by sequencing. Oligonucleotide sequences are available upon request.

### Protein expression and purification

All proteins were expressed in BL21(DE3)pLysS *E. coli* strains. The cells were grown to an optical density (A600) between 0.4 and 0.8, induced with 0.4 mM isopropyl 1-thio-β-D-galactopyranoside for 3–5 h at 37 °C with shaking and then centrifuged at 5000 rpm for 10 min. The pellets were suspended in lysis buffer containing 50 mM Tris (pH 8.0), 300 mM NaCl, 10 mM imidazole, 10% glycerol, and 5 mM β-mercaptoethanol and frozen at ~80 °C. The cell suspension containing 1 mM PMSF was lysed using a Microfluidizer system (Microfluidics, Newton, MA). The clarified lysate was loaded onto a pre-equilibrated Ni-NTA column. The SUMO tag was cleaved by Ulp1-his protease, whereas the hexahistidine tag was cleaved by thrombin protease. The tags were then further removed by Ni-NTA affinity chromatography. Proteins were further purified balanced with cargo occupancy, an ideal mechanism for ensuring protease activity when activity is truly needed.

**Cargo binding protects adaptor degradation**

RcdA is constitutively degraded in a CpdR-dependent manner in the absence of cargo. Upon binding of cargo such as TacA, RcdA degradation is suppressed, with levels of RcdA remaining sufficiently high to ensure the delivery of substrate. This suppression is maintained if the cargo is not destroyed; for example, in the case of PopA. The enlarged image highlights the substrate binding domain, primed protease-binding region, and degron of RcdA during substrate delivery.
by size exclusion chromatography using a Sephacryl 200 16/60 column. The protease components ClpX and ClpP were purified as described previously (21).

**In vivo and in vitro protein degradation assays**

The synchronization experiment was performed as described previously (15). Protein stability was measured by blocking the synthesis of proteins. Different strains of *Caulobacter* were grown to OD$_600$ ~ 0.3 in PYE medium with the appropriate antibiotics. Protein expression was induced with xylose for the times indicated in the figure legends, and then translation was blocked by addition of 30 µg/ml chloramphenicol. Equal volumes of samples were collected at different time points, as indicated in figures for Western blot analysis. Steady-state protein levels were measured by growing *Caulobacter* wild-type and Δ*pdR* strains to exponential phase. Equal numbers of cells were then used for Western blot analysis. *In vitro* degradation assays were carried out at 30 °C and monitored by the loss of the protein of interest over time using SDS-PAGE gel analysis. Gels were scanned by an Odyssey CLx imaging system (LI-COR Biosciences) and quantified using ImageJ software (National Institutes of Health) to measure the change in band intensity over time. The concentrations used in the final reaction volume otherwise mentioned separately below were as follows: 2 µM each TacA, TacADD, CC2323, CC2323DD, RcdA, RcdAAΔC, RcdAAΔC~Xb, RcdAAΔC~CtrA$_{15}$, RcdA~CtrA$_{15}$, RcdADD, CpdR, PopA, SspB, GFP-ssrA, GFP-ssrADD, ΔN9SspBΔC10~RcdA$_{19}$, ΔN9SspB, ΔN9SspB~CtrA$_{15}$, SspB~CtrA$_{15}$, 40 µM cdG, 0.2 µM ClpX$_{60}$, and 0.4 µM ClpP$_{14}$; 1 µM each $h_6$TacA and $h_6$CtrA for Fig. 2H; 8 µM CpdR, 8 µM PdeA, and 8 µM PdeADD for Fig. 4C; 0.05 µM ClpX$_{60}$ and 0.1 µM ClpP$_{14}$ for Fig. 5E; 0.1 µM ClpX$_{60}$ and 0.2 µM ClpP$_{14}$ for Fig. 5G; 8 µM each CpdR, RcdA, RcdADD, CC2323DD, and PopA for *supplemental Fig.* S4E. The reaction was initiated by addition of ATP regeneration mixture, which consisted of 4 mM ATP, 5 mM creatine phosphate, and 75 µM/ml creatine kinase. The buffer used for the reaction was H buffer, which consisted of 20 mM HEPES-KOH (pH 7.5), 100 mM KCl, 10 mM MgCl$_2$, 10% glycerol, and 5 mM β-mercaptoethanol.

**Immunoblot analysis**

Cultures samples withdrawn at the different time points indicated in the figures were centrifuged at 15,000 rpm for 2 min. After removal of the supernatant, the pellets were resuspended in 2× SDS-PAGE sample buffer containing 40 mM DTT. The samples were boiled at 95 °C for 10 min and then centrifuged to remove cellular debris. After centrifugation, the extracts were resolved on 10–15% SDS-PAGE gels. Proteins from the gel were then transferred to a PVDF membrane. After blocking the membrane with 3% milk-TBST (Tris-buffered saline (50 mM Tris, 150 mM NaCl, pH 7.5) + 0.1% Tween-20) buffer, the membranes were probed with polyclonal rabbit anti-CtrA (1:5000 dilution), anti-McpA (1:10,000 dilution), anti-RcdA (1:5000 dilution), anti-ClpP (1:5000 dilution), anti-SspB (1:20,000 dilution), or monoclonal mouse anti-FLAG M2 (1:5000 dilution, Sigma) antibodies overnight at 4 °C. After washing off the excess primary antibody, the membrane was probed with goat anti-rabbit or goat anti-mouse (Millipore) secondary antibodies conjugated to HRP enzyme. Proteins on the membrane were then visualized by the luminescence from the HRP substrate using a chemiluminescence detection system (Syngene).

**In vitro pulldown assays**

Ni-NTA affinity resin was used to pull down the protein that binds to hexahistidine-tagged bait protein. The resin was pre-equilibrated with H buffer supplemented with 20 mM imidazole. His$_6$-CC2323 (5 µM) was incubated with RcdA (10 µM), RcdAAΔC~CtrA$_{15}$ (10 µM), RcdAAΔC~ssrA (10 µM), or RcdA~CtrA$_{15}$ (10 µM), and his$_6$-RcdA (5 µM) was incubated with CC2323DD (10 µM) either alone or together in a 250-µl final volume of H buffer containing 20 mM imidazole and 50 µl of pre-equilibrated Ni-NTA resin at 4 °C for 1 h. After 1 h of incubation, the resin was spun down at 700 × g for 2 min to collect the flow-through. The resin was washed twice with H buffer supplemented with 20 mM imidazole at 350 × g for 1 min. The bound complex was eluted with H buffer containing 200 mM imidazole by spinning at 700 × g for 5 min. The fractions collected were then analyzed by SDS-PAGE gels.

**Author contributions**—K. K. J. and P. C. were involved in conceptualization, design, data analysis, and interpretations of the results. K. K. J. and M. S. performed the experiments. K. K. J. and P. C. wrote the manuscript.

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