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The UzcRS two-component system in *Caulobacter crescentus* integrates regulatory input from diverse auxiliary regulators

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Summary

The UzcRS two-component system in *Caulobacter crescentus* mediates widespread transcriptional activation in response to the metals U, Zn and Cu. Unexpectedly, a screen for mutations that affected the activity of the UzcR-regulated *urcA* promoter (P_{urcA}) revealed four previously uncharacterized proteins whose inactivation led to metal-independent induction of P_{urcA}. Using molecular genetics and functional genomics, we find that these auxiliary regulators control P_{urcA} expression by modulating the activity of UzcRS through distinct mechanisms. An ABC transporter with a periplasmic metallo-aminopeptidase domain forms a sensory complex with UzcRS, antagonizing metal dependent stimulation by virtue of its ATPase and peptidase domains. Two MarR-like transcription factors synergistically regulate UzcRS activity by repressing the expression of the membrane proteins UzcY and UzcZ, which stimulate UzcRS activity and enhance its sensitivity to a more environmentally-relevant U/Zn/Cu concentration range. Additionally, the membrane protein UzcX, whose expression is positively regulated by UzcR, provides a mechanism of feedback inhibition within the UzcRS circuit. Collectively, these data suggest that UzcRS functions as the core-signaling unit within a multicomponent signal transduction pathway that includes a diverse set of auxiliary regulators, providing further insight into the complexity of signaling networks.

Introduction

The ability to modulate intracellular gene expression in response to the extracellular environment is a critical task for microorganisms. This sense-and-respond function is commonly mediated by two-component systems (TCS) that are comprised of a membrane-bound sensor histidine kinase (HK) that perceives the environmental cue and a cytosolic response regulator (RR) that is regulated through reversible phosphorylation by the HK and carries out the gene regulatory function of the TCS (Hoch & Silhavy, 1995, Krämer & Jung, 2010). While this two-component paradigm is sufficient to mediate a response to certain environmental cues, a growing body of evidence supports the involvement of auxiliary regulators in the signal perception and

transduction process of TCS (Tetsch & Jung, 2009, Gebhard, 2012, Piepenbreier *et al.*, 2017, Mitrophanov & Groisman, 2008, Buelow & Raivio, 2010, Krämer & Jung, 2010).

In these multicomponent signal transduction pathways, the TCS functions as the base signaling module and integrates regulatory input from one or more accessory regulators. Transporters are well-suited as accessory regulators given their membrane-localization and ability to selectivity bind and translocate substrates (*e.g.*, nutrients, toxins, etc) across the membrane (Tetsch & Jung, 2009, Piepenbreier *et al.*, 2017). As such, transporters can affect HK activity indirectly by altering the concentration of the signaling substrate and/or directly through a protein-protein interaction with the HK that affects the phosphotransfer process (Unden *et al.*, 2016, Witan *et al.*, 2012, Dintner *et al.*, 2014). Proteins lacking enzymatic activity can also affect the phosphotransfer process through interactions with either the HK or RR (Buelow & Raivio, 2010, Mitrophanov & Groisman, 2008). For example, ternary complexes between TCS and inner membrane proteins serve as integration points for diverse signals/signaling pathways (Szurmant *et al.*, 2008, Schrecke *et al.*, 2013). While there is much to learn regarding the physiological function and regulatory mechanisms of accessory regulators, well-characterized examples point to regulatory complexity not easily attainable with a HK alone. This includes mechanisms for 1) the combinatorial integration of distinct signaling inputs (Cangelosi *et al.*, 1990, Unden *et al.*, 2016), 2) establishing regulatory connections between seemingly independent regulatory pathways (Eguchi *et al.*, 2007, Gerken *et al.*, 2009, Kato & Groisman, 2004), 3) altering the sensitivity of the HK for its cognate inducers (Cangelosi *et al.*, 1990), 4) directly sensing nutrient scavenging or detoxification functions (*e.g.*, flux sensing (Fritz *et al.*, 2015, Piepenbreier *et al.*, 2017)), and 5) fine-tuning response dynamics of the TCS (*e.g.*, positive and negative feedback loops (Kato *et al.*, 2007, Salazar *et al.*, 2016)). Further characterization of the regulatory networks involving TCS and their accessory regulators is paramount to understanding the microbial response to environmental signals.

In this study, we find that the TCS UzcRS in *Caulobacter crescentus* (*C. crescentus*) functions as the core element of a multicomponent signal transduction pathway that incorporates regulatory input from distinct auxiliary regulators. *Caulobacter sp.* are found in nearly all aquatic environments (Poindexter, 1981), including those that are nutrient poor (Poindexter, 1981) or characterized by high heavy metal content (*e.g.*, uranium (Utturkar *et al.*, 2013)). While the molecular networks that control cell cycle progression in *C. crescentus* have been well-studied (Curtis & Brun, 2010) less is known about the signal transduction pathways that enable a sense and respond functionality in the diverse environments that *C. crescentus* inhabits. Well-characterized sensory regulatory pathways in *C. crescentus* include the general stress response, which is governed by a extracytoplasmic family sigma factor σ^T, an antisigma factor, and the PhyK-PhyR and LovK-LovR TCS that coordinately control σ^T activity (Foreman *et al.*, 2012, Lourenço & Gomes, 2016), the PhoBR TCS, which mediates the response to phosphate limitation (Lubin *et al.*, 2016), and the oxygen sensory pathway FixLJ–FixK–FixT, which enables cell growth under hypoxic conditions (Crosson *et al.*, 2005). Recently, the UzcRS TCS, comprised of the HK UzcS and the RR UzcR, was identified as an activator of ~40 operons (Park *et al.*, 2016). UzcRS activity is strongly stimulated by the metals uranium, zinc, and copper, but not by several other tested divalent metals (Park *et al.*, 2016). Upon activation, UzcR binds extensively across the genome, activating transcription of genes which predominately encode uncharacterized proteins with predicted extracytoplasmic localization, including metallopeptidases (9), multidrug-resistant efflux pumps (3), TonB-dependent receptors (2), an outer membrane S-layer transporter (RsaFb), and numerous hypothetical proteins (Park *et al.*, 2016). Despite the central role of UzcRS in mediating U, Zn and Cu-dependent gene expression in *Caulobacter*, Δ*uzcR* and Δ*uzcS* mutants lack an obvious U/Zn/Cu tolerance phenotype (Park *et al.*, 2016), raising questions about the physiological role of UzcRS

During the course of screening for transposons that abolished metal-dependent activation of the UzcR-regulated *urcA* promoter (P_{urcA}) - a process that lead to the identification of *uzcR* and

97 *uzcS* (Park *et al.*, 2016) - 14 transposons were isolated that caused high P_{urcA} expression in the
98 absence of exogenously supplied metal (Table 1), raising the possibility that additional cellular
99 processes are involved in the regulation of the UzcRS activity. Eight mutants had transposons
100 located within CCNA_03681 or CCNA_03680 that encode an ABC transporter ATPase and an
101 ABC-2 family transporter fused to a C-terminal aminopeptidase domain, respectively. Based on
102 the data presented herein, these genes have been named *urtAP* (UzcRS Regulating Transporter
103 Atpase aminoPeptidase). Three transposons were mapped within CCNA_02866 (hereafter *uzcX*),
104 encoding a putative membrane protein of unknown function (DUF805 domain) that is located
105 within a prophage region of the genome (Scott & Ely, 2016) and is part of the UzcR regulon
106 (Park *et al.*, 2016). An additional three transposons either mapped to CCNA_03498 (1) or
107 CCNA_02289 (2), both encoding uncharacterized transcription factors of the MarR family
108 (hereafter given the names $marR_1$ and $marR_2$). Using a molecular genetics and functional
109 genomics approach, we show that all four proteins function as auxiliary regulators of UzcRS.
110 **Results**
111 **Identification of four negative regulators of UzcRS activity.**
112 To validate the phenotype of transposons that led to metal independent P_{urcA} induction, β-
113 galactosidase activity was tested in strains deleted for *urtAP*, *uzcX* or the MarR-like regulators.
114 All mutants exhibited elevated P_{urcA}-*lacZ* expression relative to WT in both complex and minimal
115 media in the absence of added metal inducer (Figure 1A; Figure S1A); loss of *urtAP* or $marR_1$
116 yielded a pronounced increase in P_{urcA}-*lacZ* activity (34-fold and 22-fold, respectively) while
117 deletion of *uzcX* or $marR_2$ yielded a more modest increase (10-fold and 6-fold, respectively).
118 Tandem deletion of $marR_1$ and $marR_2$ yielded synergistic induction of P_{urcA}-*lacZ* expression (47-
119 fold). Deletion of the third gene (CCNA_03679; putative thioesterase) within the *urtAP* operon
120 did not stimulate P_{urcA} activity (Figure 1A), consistent with the lack of transposon insertions
121 identified within this gene (see Figure S1B for operon diagram). P_{urcA}-*lacZ* expression was
122 further enhanced in all four single mutants by addition of the UzcRS inducers Zn and U (Figure

1A; Figure S1A), suggesting that metal stimulation of P_{urcA} expression is not dependent on the function of the negative regulators. To confirm that the elevated P_{urcA}-*lacZ* expression phenotypes were caused by regulator deletion, complementation tests were performed by placing the respective regulator under the control of the xylose-inducible promoter (P_{xyl} (Meisenzahl *et al.*, 1997)) at the *urcA* chromosomal locus. As expected, xylose-dependent induction of *urtAP*, *uzcX*, $marR_1$, and $marR_2$ complemented the respective deletion strain with regard to P_{urcA}-*lacZ* expression (Figure 1B), confirming a functional role for each in P_{urcA} regulation.

Given the strong UzcRS-dependent induction of P_{urcA} (Park *et al.*, 2016), we tested whether the effect of each mutant on P_{urcA} is mediated through UzcRS. P_{urcA}-*lacZ* expression was quantified in strains where *uzcX, urtAP,* $marR_1$, or $marR_2$ were deleted in tandem with *uzcR* or *uzcS*. Virtually no P_{urcA}-*lacZ* activity was detected in any double mutant, a phenotype identical to that of the Δ*uzcR* and Δ*uzcS* single mutants (Figure 1C), confirming the dependence on UzcRS. To directly test whether the negative regulators affect UzcRS activity, we attempted to quantify the *in vivo* levels of phosphorylated UzcR (UzcR-P) using phos-tag acrylamide gel electrophoresis followed by Western blotting (Barbieri & Stock, 2008). Although sufficient sensitivity was not achieved to quantitatively assess differences among single deletion mutants, the data indicated that UzcR-P levels were qualitatively increased in each negative regulator mutant relative to WT and in WT following Zn exposure (Figure 1D). Deletion of both *urtAP* and *uzcX* resulted in the highest UzcR-P levels of any condition tested, revealing that UzcR-P levels can be induced beyond the levels observed during Zn exposure or by deletion of any single negative regulator.

As an alternative to UzcR-P quantification, the effect of each negative regulator mutant on UzcRS activity was indirectly quantified by measuring the expression levels of 37 of the 42 UzcR direct regulon members (Park *et al.*, 2016) using custom nanostring codesets (Figure 1E, Table S1). As a control, the data were analyzed alongside previously generated nanostring data for WT and Δ*uzcR* strains following Zn exposure (Park *et al.*, 2016). The UzcR regulon was

induced in all negative regulator mutants, but to differing degrees (Figure 1E). Most regulon members (28/37 and 26/37, respectively) were induced by 2-fold or higher in the Δ*urtAP* and Δ$marR_1$ strains (Table S1), with the degree of induction slightly lower than that observed for WT cells following Zn exposure. Induction was more modest (4-fold or lower for each gene) for the Δ*uzcX* (22/37) and Δ$marR_2$ (8/37) strains (Table S1). Using the TFInfer algorithm (Asif *et al.*, 2010) to infer UzcR activity in each negative regulator mutant, revealed a hierarchy of UzcR activities that was consistent with the P_{urcA}-*lacZ* data (Figure S2). However, the higher predicted UzcR activity for the Zn-induced WT compared to any single negative regulator mutant contrasts with the P_{urcA}-*lacZ* reporter data. Examination of genome-scale UzcR DNA-binding in the Zn-induced WT compared to the Δ*urtAP* strain using ChIP-seq performed under the same growth conditions (PYE + 40 μM Zn) used for the P_{urcA}-*lacZ* assays, revealed higher overall UzcR occupancy in the Zn-induced WT strain (Figure S3), consistent with higher UzcR activity. This suggests that while the P_{urcA}-*lacZ* reporter is a good proxy for UzcR activity in the negative regulator mutants, it appears to underreport activity during Zn exposure, perhaps as a consequence of cellular Zn toxicity. Taken together, these data suggest that UzcX, UrtAP, $MarR_1$ and $MarR_2$ function as negative regulators of UzcRS activity, but inactivation of any single negative regulator does not induce maximal UzcRS activity.

RNA-seq highlights the specificity of the negative regulators for UzcRS

To determine the specificity of each negative regulator for UzcRS, we used RNA-sequencing to analyze the gene expression profiles of strains deleted for *uzcX, urtAP,* $marR_1$, or $marR_2$ in tandem with *uzcR* (Table S2). The *uzcR* null background, rather than WT, was employed to preclude pleiotropic effects of UzcR regulon induction (Figure 1E), facilitating identification of UzcRS-independent gene expression changes that may provide clues about the physiological function of each negative regulator. Surprisingly, only a few genes were differentially expressed in any double mutant relative to the *uzcR* control (Figure 2; Table 2). Two genes predicted to be cocistronic with each MarR regulator were induced by greater than 50-

fold in the respective *marR* mutant and represent candidates for MarR transcriptional control and UzcRS regulation (Figure 2; Table 2; described in detail below). Additionally, five operons that encode proteins with Zn import functions (Mazzon *et al.*, 2014) were induced to a similar level in all negative regulator mutants (Figure 2; Table 2). All five operons are regulated by the Zn-specific regulator Zur that functions to maintain Zn homeostasis (Mazzon *et al.*, 2014) and the differential expression is consistent with at least partial attenuation of Zur DNA-binding. However, subsequent experimentation revealed that a connection between the negative regulator mutants and Zur activity is subtle if at all, and that Zur regulon stimulation does not affect UzcRS activity in the presence or absence of added Zn (supplemental text; Figure 1E, Figure S4A-C). No other genes were induced in the Δ*uzcX*, Δ*urtAP,* or Δ$marR_1$ strains, while two uncharacterized sRNAs were weakly induced (~2-fold) in the Δ$marR_2$ strain. In sum, the RNA-seq data identify putative connector proteins between the MarR regulators and UzcRS, but do not yield genetic clues into the mechanism of action of UrtAP and UzcX. Nevertheless, the minimal UzcR-independent gene expression in the Δ*uzcX*, Δ*urtAP* null strains points toward a specific mechanism of UzcRS regulation by both regulators.

UrtAP forms a sensory complex with UzcS and both the ATPase and peptidase domains contribute to UzcRS regulation.

UrtAP possesses conserved domains with predicted transport and peptidase activities that could contribute to UzcRS regulation. Specifically, UrtAP is an ABC transporter with an accessory M1 family metallo-aminopeptidase domain containing a characteristic HEXXH Zn-binding motif (diagrammed in Figure 3A-C). Analysis of the transmembrane topology of UrtP using a combination of algorithmic prediction and alkaline phosphatase insertions revealed 13 transmembrane helices with the peptidase active site located in the periplasm (Figure 3A-B). From this data, UrtAP is predicted to couple transport (either import or export) with periplasmic aminopeptidase activity.

To determine whether the ATPase and peptidase domains of UrtAP are involved in UzcRS regulation, targeted mutations were made within the chromosomal copy of WT or HA-tagged *urtAP* (diagrammed in Figure 3C). First, the ATPase activity of UrtA was eliminated by substituting conserved residues within the Walker A or Walker B domains (K40N and E159Q, respectively) or by deleting *urtA* entirely. Substitutions analogous to K40N and E159Q have been shown to abolish ATP hydrolysis and transport activity of several ABC transporters (Davidson & Sharma, 1997, Orelle *et al.*, 2003, Tal *et al.*, 2013) without affecting complex formation between the ATPase and permease domains (Tal *et al.*, 2013, Hebbeln *et al.*, 2007). Both substitutions elevated P_{urcA}-*lacZ* expression to a similarly high level (16-fold higher than WT; Figure 3C; Figure S5A) without affecting UrtA protein abundance (Figure 3D), as evidenced by Western blots with HA-tagged UrtA. A similar result was observed for a Δ*urtA* strain. These results highlight the necessity of the ATPase domain for maintaining low basal UzcRS activity. However, the inability of any of these variants to fully recapitulate the level of P_{urcA}-*lacZ* activity observed in the Δ*urtAP* strain (34-fold increase; Figure 3C) suggests that loss of putative ATPase activity represents only a partial null phenotype.

Next, substitutions were made within the Zn-coordinating HEXXH motif (H934Y, H934Y); analogous substitutions abolished peptidase activity in other M1 family aminopeptidase enzymes (Wang & Cooper, 1993, Laustsen *et al.*, 2001). Both the H934Y and the H934Y/H938F variants increased P_{urcA}-*lacZ* expression in the absence of inducer (Figure 3C), although to a lower level than the K40N and E159Q variants. Importantly, western blots revealed that the levels of UrtP(H934Y)-HA were indistinguishable from UrtP-HA, suggesting that the substitutions in the HEXXH motif do not perturb protein stability (Figure 3D). Combining Zn coordination (H934Y) and ATPase (K40N) substitutions in tandem yielded a P_{urcA}-*lacZ* expression phenotype identical to the K40N variant (Figure 3C), providing genetic evidence of coupled ATPase and peptidase activities. In contrast to amino acid substitutions, deletion of the periplasmic aminopeptidase domain (amino acid residues 779-1195) yielded a P_{urcA}-*lacZ*

expression phenotype identical to the Δ*urtAP* strain (Figure 3C), supporting the essential role of this domain in the regulation of UzcRS.

The coupling of transporter activity with stimulus perception by a TCS is commonly mediated via complex formation between the transporter and the histidine kinase (Piepenbreier *et al.*, 2017). To test whether UrtAP interacts with UzcRS, a co-immunoprecipitation (Co-IP) assay was performed in a strain containing C-terminal HA-tagged UrtP (UrtP-HA) and N-terminal 3x FLAG-tagged UzcS expressed from their respective chromosomal loci. Importantly, the N-terminal FLAG tag and the C-terminal HA tag did not perturb the function of UzcS or UrtAP, respectively, given that P_{urcA}-*lacZ* expression levels in strains containing either fusion protein were indistinguishable from WT in the presence or absence of Zn (Figure S5A-B). UrtP-HA was immunoprecipitated in the strain expressing Flag-UzcS, but not in a control strain containing un-tagged UzcS (Figure 3E). This interaction was abolished in a strain deleted for *urtA* (Figure 3F). Since deletion of *urtA* did not affect UrtP-HA expression levels, we conclude that UrtA is required for the interaction of UrtAP with UzcS.

The interaction between UrtAP and UzcS was also tested in strains containing substitutions targeting the ATPase activity of UrtA (K40N) or the Zn-binding site in UrtP (H934Y). In contrast to the result with a *urtA* deletion, neither substitution impaired the interaction between UrtP and UzcS, as evidenced by the successful Co-IP of UrtP (Figure 3F). This result suggests that both mutations cause UzcRS activation without abolishing the interaction between UrtAP and UzcS. Collectively, these data support an interaction between UrtAP and UzcRS and suggest a functional role for the ATPase and peptidase domains in the negative regulation of UzcRS.

MarR-type transcription factors negatively regulate UzcRS activity by silencing the expression of the membrane proteins UzcY and UzcZ

The RNA-seq data revealed that deletion of each *marR* regulator resulted in upregulation of a three-gene transcription unit encoding the respective MarR regulator and two genes of

252 unknown function with predicted membrane localizations (*i.e.*, CCNA_03497-*marR*$_1$-
253 CCNA_03499 and CCNA_02291- CCNA_02291-*marR*$_2$; Figure 4A). Examination of the
254 sequence upstream of each operon revealed three adjacent copies of an inverted repeat that
255 overlap the putative -10 and -35 elements for CCNA_03497 and a single inverted repeat
256 overlapping the putative -35 element for CCNA_02291 (Figure S6A), consistent with the MarR
257 regulators functioning as direct repressors. The expression of the divergently oriented gene
258 CCNA_03496 was also increased in the Δ*marR*$_1$ strain, but to a much lesser degree than
259 CCNA_03497 or CCNA_03499 (Table 2). MarR-dependent regulation of the CCNA_03497
260 (hereafter *uzcY*) and CCNA_02291 (hereafter *uzcZ*) promoters was further confirmed by
261 quantifying the fluorescence of promoter-*gfp* fusions that encompass the inverted repeat elements
262 in WT, Δ*marR*$_1$, and Δ*marR*$_2$ backgrounds. P$_{uzcY}$ expression was strongly induced in the Δ*marR*$_1$
263 strain while P$_{uzcZ}$ expression was strongly induced in the Δ*marR*$_2$ strain (Figure S6B), confirming
264 MarR autoregulation and a lack of transcriptional cross-talk between the MarR modules.
265 Since the RNA-seq data revealed that the loss of MarR function failed to affect
266 expression of *uzcS*, *uzcX*, or *urtAP,* the most likely explanation for stimulation of UzcRS activity
267 in the MarR mutants is through upregulation of one or both of the putative membrane proteins
268 encoded within each *marR* operon. To test this hypothesis, each *marR* operon member was
269 deleted in tandem with the cognate *marR* regulator, and UzcRS activity was assessed by
270 quantifying P$_{urcA}$-*lacZ* expression (Figure 4B). Deletion of *uzcY*, but not CCNA_03499, was
271 sufficient to abrogate the high P$_{urcA}$-*lacZ* expression in the Δ*marR*$_1$ strain. Similarly, deletion of
272 *uzcZ*, but not CCNA_02290, abrogated the high P$_{urcA}$ expression phenotype of the Δ*marR*$_2$ strain.
273 These data suggest a role for UzcY and UzcZ in the activation of UzcRS.
274 To confirm the stimulatory role of UzcY and UzcZ on UzcRS activity, *uzcY* and *uzcZ*
275 were expressed from the xylose-inducible promoter (P$_{xyl}$ (Meisenzahl *et al.*, 1997)) on a low copy
276 plasmid. A xylose-dependent increase in P$_{urcA}$-*lacZ* expression was observed for both proteins
277 and required a functional copy of *uzcR* (Figure 4C). The high P$_{urcA}$-*lacZ* activity observed for

278 P_{xyl}-*uzcZ* in the absence of xylose is puzzling, but could reflect leaky expression of UzcZ to a
279 level that is sufficient to stimulate UzcRS. Simultaneous expression of *uzcY* and *uzcZ* using P_{xyl}
280 for *uzcY* and a vanillate-inducible promoter (P_{van} (Thanbichler *et al.*, 2007)) for *uzcZ*, resulted in
281 synergistic activation of P_{urcA} (Figure 4D), explaining the synergistic effect of the Δ$marR_1$
282 Δ$marR_2$ double mutant (Figure 1A). In contrast, overexpression of CCNA_00113, encoding a
283 predicted membrane protein that is cocistronic with a paralogous MarR-type regulator in *C.*
284 *crescentus* (CCNA_00114; Figure S7A-C), using either P_{xyl} (Figure 4C) or by deleting the
285 cognate MarR-type regulator (Figure 5B) failed to induce P_{urcA}-*lacZ* expression (Figure 3B).
286 Given the transmembrane topology predictions for UzcY and UzcZ (Figure S6C) and the
287 localization of UzcY-mCherry and UzcZ-mCherry fusions to the periphery of the cell (Figure
288 S6D), we tested whether either protein interacts with UzcS using a Co-IP assay with HA-tagged
289 UzcY and UzcZ. HA-tagged UzcY and UzcZ both stimulated P_{urcA}-*lacZ* expression in a xylose-
290 dependent manner when the fusions were placed under the control of P_{xyl} (Figure S6E). However,
291 neither HA-UzcY nor HA-UzcZ co-immunoprecipitated with FLAG-UzcS (Figure 4E).
292 Collectively, these data confirm that the MarR regulators function as negative regulators of
293 UzcRS by maintaining low levels of UzcY and UzcZ; however, the regulation of UzcS by UzcY
294 and UzcZ does not appear to be mediated through a direct interaction.
295 **UzcX does not mediate UzcS regulation through a direct interaction.**
296 UzcX is annotated as a domain of unknown function (DUF805) containing protein and
297 predicted to contain four transmembrane helices and localize to the cytoplasmic membrane
298 (Figure 5A; Figure S8A). To confirm the membrane localization, mCherry was fused to the C-
299 terminus of UzcX at the chromosomal *uzcX* locus. Importantly, the C-terminal mCherrry fusion
300 did not perturb the ability of UzcX to negatively regulate UzcRS activity (Figure S8B). Using
301 superresolution, structured illumination microscopy (SIM), we found that the UzcX-mCherry
302 fluorescence was predominately localized to the periphery of cells (Figure 5B), supporting
303 membrane localization. Additionally, insertion of the *E. coli* alkaline phosphatase fragment

(PhoA$_{21\text{-}471}$) at four different locations within UzcX supported the topology prediction (Figure 5A).

Given the membrane localization of UzcX and the lack of an annotated enzymatic function, we hypothesized that UzcX regulates UzcRS activity through an interaction with membrane-bound UzcS. To test this, a Co-IP experiment was performed in a strain expressing FLAG-tagged UzcS and hemagglutinin-tagged UzcX (UzcX-HA) from their chromosomal loci. The C-terminal HA tag did not perturb the regulatory function of UzcX with respect to P$_{urcA}$-*lacZ* expression (Figure S8C). UzcX did not co-immunoprecipitate with UzcS under basal growth conditions or following Zn exposure (Figure 5C), where UzcX levels are significantly increased as a consequence of UzcR-mediated transcriptional activation of *uzcX* (Park et al., 2016). Chromosomally expressed UzcX-mCherry also failed to Co-IP with FLAG-UzcS under basal conditions (Figure S8D). Collectively, these data suggest that UzcX localizes to the inner membrane, but does not regulate UzcS through a direct interaction.

Loss of negative UzcRS regulation results in a hyperinducible phenotype.

Initial experiments indicated that UzcRS remains responsive to metal inducers despite the loss of negative regulation (Figure 1A). To further quantify how loss of negative regulation affects the sensitivity of UzcRS to metal inducers, we measured the expression of P$_{1968}$-*gfp*mut$_3$ (P$_{1968}$) in the negative regulator mutants over a range of Zn and Cu concentrations. The P$_{1968}$-*gfp*mut3 reporter was used in place of P$_{urcA}$-*lacZ* as it is responsive to a wider range of UzcR-P levels. Although loss of negative regulation elevated basal P$_{1968}$-*gfp*mut$_3$ expression relative to WT (Figure S9A-B), it also increased the metal sensitivity and signal amplitude of this promoter (Figure 6A-B; Figure S9A-B). This enhanced sensitivity was most striking in Δ*urtAP* and Δ*marR$_1$* strains where Zn concentrations as low as 2 μM (compared to 8 μM for WT) were sufficient to produce a detectable increase in fluorescence (Figure 6A). The sensitivity for U was similarly improved in the Δ*marR$_1$* strain, suggesting that this effect applies to all known UzcRS inducers (Figure S9C). Notably, the sensitivity enhancement in the negative regulator mutants

was significantly diminished at the higher range of Zn (16 μM and above) and Cu (10 μM) concentrations (Figure 6A-B), owing at least in part to greater Zn and Cu toxicity observed in these mutants (see below). From these data, we conclude that loss of negative regulator function hypersensitizes UzcRS to metal inducers.

Overexpression of ABC transporter but not *uzcX* abrogates metal induction. Since the loss of UzcX and UrtAP negative regulation sensitized UzcRS to metal inducers, we tested whether overexpression of UzcX or UrtAP would mitigate metal-dependent induction. Overexpression of the MarR repressors was not tested since wild type levels of these proteins appear sufficient to silence *uzcY* and *uzcZ* expression (Figure 5B). The chromosomal copy of *urtAP* and *uzcX* was placed under titratable control by replacing the previously mapped promoters (Zhou *et al.*, 2015) with P_{xyl} (Meisenzahl *et al.*, 1997), and P_{urcA}-*lacZ* expression was monitored over a range of Zn, Cu, and xylose concentrations. In the absence of xylose, P_{urcA}-*lacZ* expression in both strains was elevated relative to the isogenic WT strain at all Zn and Cu concentrations (Figure S9D-E), indicative of lower UzcX and UrtAP levels compared to WT. Overexpression of *urtAP* and *uzcX* reduced the metal sensitivity of P_{urcA} expression, but to differing degrees. Overexpression of *uzcX* modestly reduced metal-dependent induction of P_{urcA}-*lacZ* relative to WT (Figure 6C-D), demonstrating effective complementation but only minimal desensitization of the metal induction phenotype. In contrast, overexpression of UrtAP dramatically reduced metal-dependent induction of P_{urcA}-*lacZ*, effectively abolishing induction at Zn concentrations up to 30 μM (Figure 6C). A similar pattern was observed with Cu (Figure 6D). Overexpression of *urtAP* variants defective in ATPase activity (K40N) or Zn-coordination (H934Y, H938F) only modestly reduced the metal sensitivity of P_{urcA}-*lacZ* expression compared to WT, resembling the effect of UzcX overexpression (Figure 6C-D). This weakened ability of catalytically compromised UrtAP (K40N or H934Y, H938F) to dampen metal sensitivity implies that abrogation of metal induction by UrtAP is not simply a consequence of the aminopetidase HExxH motif reducing the active Zn

(or Cu) concentration via complexation. From these data, we conclude that UrtAP activity antagonizes metal-dependent induction of UzcRS.

Hyperactive UzcRS activity compromises cell fitness.

Given the pronounced effect of each negative regulator on the metal sensitivity of UzcRS, we wondered whether loss of negative regulator function affects cell fitness during metal exposure. To test this idea, the growth rate of strains deleted for each negative regulator individually and in tandem with *uzcR* was determined following exposure to Zn (Figure 7A). The Δ*uzcR* strain grew at the same rate as WT in the presence or absence of metal, consistent with the prior report that UzcR transcriptional activity was dispensable for U, Zn, and Cu tolerance (Park *et al.*, 2016). However, the growth rate of the Δ*urtAP* strain was significantly slower than WT during Zn exposure, a phenotype that was reverted by tandem deletion of *uzcR* (Figure 7A). In contrast, the Δ*uzcX* strain exhibited the same growth rate as WT in the presence of Zn, suggesting that loss of UzcX function during Zn exposure is not as detrimental to cell fitness as loss of UrtAP. The growth rates of Δ$marR_1$ and Δ$marR_2$ strains were also reduced relative to WT in the presence of Zn. However, in contrast to the Δ*urtAP* and Δ*uzcX* strains, tandem deletion of *uzcR* did not restore the growth rate of the *marR* mutants to WT levels. These data suggest that the reduced Zn tolerance of the Δ*urtAP* and Δ*uzcX* strains, but not the Δ$marR_1$ *or* Δ$marR_2$ strains, resulted from high UzcRS activity.

To test whether the decreased metal tolerance of the negative regulator mutants is specific to Zn, we also determined the growth rate following exposure to Ni, a metal that is toxic to *Caulobacter* at similar concentrations as Zn, but does not stimulate UzcRS activity. In contrast to the results for Zn, the growth rate of each mutant was indistinguishable from WT when exposed to Ni (Figure 7B), arguing against a general susceptibility to metals. We thus suspected that the reduced Zn tolerance is a product of hyperactive UzcRS activity owing to the synergistic effect of Zn and loss of UzcX/UrtAP regulation on UzcRS activity. In support of high UzcR-P levels causing reduced fitness, the Δ*urtAP* Δ*uzcX* double mutant, where UzcR-P levels

dramatically exceed the levels in either single mutant (Figure 1D), exhibited a *uzcR*-dependent growth defect even in the absence of metal (data not shown). As such, it is not surprising that *urtA, urtP* and *uzcX* were among the genes identified as conferring a high fitness cost when disrupted in a global transposon sequencing study (Christen *et al.*, 2011). It remains to be determined whether the toxicity associated with high UzcRS activity reflects induction of specific regulon member/s or the regulon as a whole.

Discussion

The UzcRS TCS was recently found to mediate transcriptional activation of a non-canonical extracytoplasmic stress response following exposure to the metals U, Zn, and Cu (Park *et al.*, 2016). In this work, we show that UzcRS is part of a multicomponent signaling pathway, integrating regulatory input from at least four previously uncharacterized proteins (Figure 8). The membrane protein UzcX and the ABC-transporter peptidase UrtAP act as negative regulators of UzcRS activity, whereas the membrane proteins UzcY and UzcZ, encoded within two independent, autoregulatory *marR* loci, function as condition-specific stimulators of UzcRS activity. This regulatory interplay has important implications for modulating the basal activity of UzcRS and its sensitivity to U/Zn/Cu. Collectively, our results suggest that additional signals can likely be linked to UzcRS activity, and that the physiological function of UzcRS extends beyond metal regulation.

Negative regulators are not required for metal perception by UzcRS.

The finding that UzcRS remains sensitive (actually is hypersensitized) to the metal inducers (Zn, Cu and U) despite loss of UzcX, UrtAP, or MarR-mediated negative regulation suggests that an alternative mechanism is involved in mediating metal perception by UzcRS. While many HK that employ co-sensing strategies (*e.g.*, PhoBR and BceAB (Piepenbreier *et al.*, 2017)) lack prototypical periplasmic sensing domains (*i.e.*, are intramembrane sensing HKs (Mascher *et al.*, 2006)) relying instead on the accessory regulator for signal perception, UzcS contains a predicted ~124 AA periplasmic domain where signal perception likely occurs (Figure 8). Initial attempts to

structurally characterize the UzcS periplasmic domain and assay for metal binding were unsuccessful due to aggregation of the purified domain. Nevertheless, swapping this domain with a similar-sized periplasmic domain from another HK in *C. crescentus* or deleting segments of the domain unexpectedly led to high UzcRS activity (data not shown), supporting the involvement of this domain in signal transmission. As such, there are intriguing parallels between UzcRS and the global regulatory TCS CpxAR in *E. coli,* which incorporates regulatory input from two accessory proteins, neither of which are explicitly required for sensing the envelope stressors that stimulate CpxRA activity (Vogt & Raivio, 2012). Instead, mutational data point to a role for the CpxA periplasmic domain in envelope stress perception (DiGiuseppe & Silhavy, 2003), with the accessory regulators enabling additional sensory or regulatory capabilities (*e.g.*, NlpE stimulates CpxA in response to surface adhesion (Otto & Silhavy, 2002)).

UrtAP forms a sensory complex with UzcS and modifies its sensitivity to U/Zn/Cu

UrtAP belongs to an uncharacterized family of ABC transporters that contain a large C-terminal (~600 AAs), periplasmic, M1 family aminopeptidase domain with a conserved HEXXH catalytic Zn coordination motif. Despite the presence of homologs throughout alphaproteobacteria and within some members of beta and gamma proteobacteria, virtually nothing is known about this family of transporters. The closest characterized analogs are in the ABC transporter maturation and secretion protein (AMS) family that have established roles in the processing and export of certain bacteriocins, competence factors, and peptide pheromones in Firmicutes (Young & Holland, 1999, Gebhard, 2012). Specifically, AMS enzymes utilize a small (~160 amino acid), N-terminal, cytoplasmic cysteine peptidase domain to cleave a leader peptide concomitantly with export (Young & Holland, 1999). In some cases (*e.g.*, SunT group transporters (Gebhard, 2012, Eran *et al.*, 2007)), the expression of the AMS enzyme is regulated by a TCS that senses the mature peptide product, forming a positive feedback loop. We propose that UrtAP also couples peptidase and transport activities (Figure 8), but this process contributes

to the negative regulation of UzcRS activity and, consequentially, is required for optimal cell fitness.

An interaction between UrtAP and UzcRS provides a plausible mechanism by which UrtAP could exert regulatory control over UzcRS. Recent data have revealed several examples of transporters that regulate TCS activity through a direct interaction (Piepenbreier *et al.*, 2017). In some cases, complex formation alone is sufficient for regulation (i.e., an activity switch (Steinmetz *et al.*, 2014)). Alternatively, a growing number of TCS transporter pairs appear to employ a flux sensing mechanism, whereby the activity of the histidine kinase is conformationally coupled to the transport process (Piepenbreier *et al.*, 2017, Fritz *et al.*, 2015, Gardner *et al.*, 2014). We argue that the interaction between UrtAP and UzcS alone is insufficient to mediate UzcS regulation and, instead, propose a working model whereby UrtAP transport activity acts as a repressing signal for UzcS based on the following observations. Conserved residues within the ATPase and Zn coordination sites of UrtAP are critical for maintaining low basal UzcRS activity but are not required for complex formation. Second, overexpression of catalytically active UrtAP severely dampened the sensitivity of UzcRS to its metal inducers, whereas overexpression of UrtAP lacking these conserved residues merely restored UzcRS sensitivity to WT levels. It remains to be determined whether this regulatory control by UrtAP is exerted by inducing a conformational change in UzcS that affects signal perception/transduction and/or by altering the level of the inducing signal (*e.g.*, U/Zn/Cu or a byproduct of U/Zn/Cu stress) through its transport/peptidase activity.

The coupling of transporter activity with stimulus perception by a TCS enables the coordination of gene expression based on the cells ability to perform a critical function such as scavenging of nutrients or detoxifying an environmental toxin. In *C. crescentus*, the critical role of the ABC transporter aminopeptidase UrtAP in maintaining low UzcRS activity provides an intriguing link between the sensory and output responses of UzcRS. The UzcR regulon includes nine peptidase enzymes that are predicted to localize to the cell envelope, including two

458 aminopeptidase enzymes. UzcR also weakly stimulates *urtAP* expression (~3-fold *uzcR*-
459 dependent induction during Zn exposure), although this regulation appears indirect, based on the
460 lack of an upstream ChIP-seq peak (Park *et al.*, 2016). It is thus tempting to speculate that the
461 UzcR-activated extracytoplamic peptidases function within the same physiological pathway as
462 UrtAP and perhaps serve a compensatory function when the UrtAP substrate accumulates beyond
463 a certain threshold.

464 **UzcX provides a mechanism of feedback inhibition within the UzcRS system**

465 Our data indicate that low level basal expression of the previously uncharacterized
466 membrane protein UzcX, encoded within a prophage region of the *C. crescentus* genome (Scott &
467 Ely, 2016), is required for proper regulation of UzcS. Disruption of UzcX function through *uzcX*
468 deletion elicits both a minor increase in basal UzcRS activity and a greater sensitivity to the metal
469 inducers. Since *uzcX* is directly activated by UzcR, this regulation provides a negative feedback
470 mechanism within the UzcRS system (Figure 8). The lack of a direct interaction between UzcX
471 and UzcS, however, suggests that the function of UzcX is distinct from previously characterized
472 small membrane protein regulators such as LiaF, YycH/YycI, and MgrB, which exert feedback
473 control through complex formation with the target histidine kinase (Szurmant *et al.*, 2007, Jordan
474 *et al.*, 2006, Lippa & Goulian, 2009). UzcX is a DUF805 domain family protein, which despite
475 having a broad taxonomic distribution within bacteria lacks a characterized function. This family
476 consists of small proteins (~110 to 180 amino acids) with 3 or 4 transmembrane domains and a
477 speculated role as a transport system (Saier *et al.*, 2016). This speculative function is intriguing
478 in light of the synergistic effect between *uzcX* and *urtAP* deletions on UzcRS activity; synergistic
479 phenotypes are commonly the result of genes that act in parallel pathways that converge at a
480 common node (Perez-Perez *et al.*, 2009). Whether UzcX is a transporter and whether it shares a
481 common transport intermediate with UrtAP remains to be determined.

482 **UzcRS activity is linked to the function of two autoregulatory MarR modules**

MarR family transcription factors are ubiquitously distributed throughout bacteria and coordinate gene expression changes in response to a variety of small molecules (*e.g.*, phenolics, antibiotics, etc.) via ligand-mediated attenuation of DNA binding (Grove, 2013). Here, we present evidence that connects the transcriptional activity of two previously uncharacterized MarR-like transcription factors with UzcRS activity. We propose that upon signal perception, presumably through binding of a cognate ligand, MarR DNA binding is attenuated, leading to derepression of the 'connector (Mitrophanov & Groisman, 2008)' membrane protein (UzcY or UzcZ) that functions to stimulate UzcRS activity through an unknown mechanism (Figure 8). Since neither MarR regulator appears responsive to the known UzcRS inducers U (Hu *et al.*, 2005), Zn (Park *et al.*, 2016) or Cu (data not shown), as evidenced by the lack of *uzcY* or *uzcZ* induction following exposure to these metals, the MarR modules likely broaden the number of signals integrated within the UzcRS system. In this regard, the function of UzcY and UzcZ in connecting the activity of MarR regulators with stimulation of UzcRS is analogous to the membrane proteins B1500 (Eguchi *et al.*, 2007) and MzrA (Gerken *et al.*, 2009) that serve a signal integration function by connecting the activity of the EvgAS and CpxRS TCS with stimulation of PhoPQ and EnvZ/OmpR TCS, respectively.

In addition to modulating the basal activity of UzcRS, UzcY and UzcZ enhance the magnitude of the response to metal inducers. In the absence of metal inducer, expression of *uzcY* or *uzcZ* only partially stimulates UzcR regulon induction. However, when *uzcY* or *uzcZ* induction is coupled with addition of U, Zn, or Cu, the effect on UzcRS activity is synergistic. As such, UzcY and UzcZ function as signal amplifiers within the UzcRS system, enabling the metal sensitivity of UzcRS, in theory, to be fine-tuned by the concentration of MarR inducer. Indeed, we find that the activity of UzcRS at a given Zn concentration can be scaled in a UzcY concentration-dependent manner (Figure S10). What benefit this enhanced U/Zn/Cu sensitivity affords the cell remains to be determined. In one respect, the signal amplification mechanism has the effect of shifting the responsiveness of UzcRS to a more environmentally relevant range of

metal concentrations (e.g., Cu and Zn concentrations in surface and drinking waters commonly range from nM to low μM (National_Research_Council, 1977)). However, attenuation of MarR1/2 binding is toxic when combined with high metal concentrations (>10 μM Zn), and the actual benefit of metal-dependent stimulation of UzcRS remains tenuous (see below). Ultimately, understanding the physiological rationale for this synergistic effect will require examination of the cellular effects of UzcY and UzcZ beyond the transcript level, given the minimal perturbation in gene expression observed upon $MarR_1$, or $MarR_2$ derepression.

Is UzcRS induction by U/Zn/Cu physiologically relevant?

There remains a disconnect between the potent induction of UzcRS by metal inducers and the functions encoded by the ensuing transcriptional response. The UzcRS regulon does not resemble a metal resistance response or a canonical extracytoplasmic stress response (e.g., CpxAR or SigmaE (Yamamoto & Ishihama, 2005, Egler *et al.*, 2005, Yamamoto & Ishihama, 2006)), nor does it confer increased U, Zn, or Cu tolerance (Park *et al.*, 2016). In fact, we show that elevated UzcRS activity actually reduces tolerance to Zn. The lack of an apparent connection between the function of the upstream protein regulators of UzcRS and U/Zn/Cu further challenges the physiological relevance of these metals as the inducing signal. As such, our results are consistent with the previously raised possibility that a stress caused in common by all three metals is responsible for UzcRS induction (Park *et al.*, 2016). Based on the putative roles of the negative regulars UrtAP and UzcX in peptide cleavage/transport and the UzcRS-dependent induction of 1) three multidrug resistance pumps, including ~50-fold activation of CCNA_02172-2174 (uncharacterized multidrug resistance ABC transporter), 2) a TolC-like outer membrane transporter RsaFb, and 3) the aforementioned suite of extracytoplamic peptidases, it is tempting to speculate that the physiologically relevant inducing signal is peptide-based. U/Zn/Cu could stimulate UzcRS activity by either disrupting an intrinsic process (e.g., peptide recycling), leading to the accumulation of an endogenous peptide, or by mimicking the physiological effects of a hostile exogenous peptide (e.g., an antimicrobial peptide such as a bacteriocin (Schoenlein & Ely,

1983)), triggering futile UzcRS induction. A further understanding of the nature of the physiological inducer awaits characterization of the substrate specificities of UrtAP/UzcX and CCNA_02172-2174.

In sum, the integration of multiple auxiliary regulators within the UzcRS signaling pathway is consistent with an emerging theme in bacterial signal transduction where components beyond the HK and RR play integral roles in the signal perception and transduction within TCS (Buelow & Raivio, 2010, Tetsch & Jung, 2009, Gebhard, 2012, Mitrophanov & Groisman, 2008, Piepenbreier *et al.*, 2017). Although the precise properties afforded to UzcRS by this complex regulation remain to be characterized, our data point toward intriguing possibilities, including modulation of signal sensitivity, broadening of signal specificity, and facilitating connections between distinct regulatory pathways. With the exception of *uzcX*, the negative regulators are not encoded within the UzcR regulon and are located in disparate parts of the genome, precluding their facile identification through bioinformatic or targeted approaches. As such, the integration of multiple layers of auxiliary regulation within TCS pathways and the establishment of regulatory links between otherwise independent signal transduction pathways may be underestimated.

Experimental Procedures

Bacterial strains, media and materials. All strains were derived from wild type *C. crescentus* strain NA1000 (ATCC 19089). Growth experiments were performed in (i) PYE medium, containing 0.2% (wt/vol) Bacto peptone (Difco), 0.1% yeast extract (Difco), 1 mM $MgSO_4$, and 0.5 mM $CaCl_2$, (ii), modified M5G medium (10 mM PIPES, pH 7, 1 mM NaCl, 1 mM KCl, 0.05% NH_4Cl, 0.01 mM Fe/EDTA, 0.2% glucose, 0.5 mM $MgSO_4$, 0.5 mM $CaCl_2$) supplemented with 1.3 mM inorganic phosphate or (iii) M5G medium supplemented with 5 mM glycerol-2-phosphate as the phosphate source (M5G-G2P) to facilitate uranium solubility at the start of growth assays. U, Zn and Cu stocks were prepared as previously described (Park *et al.*, 2016). All strains were grown at 30°C with shaking at 220 RPM in Erlenmeyer flasks or at 1000

562 RPM in 96-well plates in a PHMP-4 Thermoshaker (Grant Instruments). *Escherichia coli* HST08
563 (Clontech) was used for cloning following standard procedures.
564 **Transposon screen with a P_{urcA}-*lacZ* reporter**. Previously, ~60,000 transposon mutants in a
565 DMP89 strain background (chromosomal P_{urcA}-*lacZ* fusion) were screened on X-gal plates for
566 altered P_{urcA} activity (Park *et al.*, 2016). Mutants (4) exhibiting a white colony phenotype led to
567 the identification of *uzcR* and *uzcS*. An additional 29 mutants exhibited a dark blue colony
568 phenotype independent of Zn, which was clearly distinguishable from the fainter blue color
569 produced by mutants with WT P_{urcA}-*lacZ* expression. Nested semi-arbitrary PCR was used to
570 map the location of each transposon as described previously (Christen *et al.*, 2010). β-
571 galactosidase assays were performed to eliminate mutants that yielded a dark blue phenotype
572 through a mechanism independent of P_{urcA} activity (*e.g.*, by affecting colony morphology or X-gal
573 transport). As a result, 14 mutants with transposons located within the *acrAB-nodT* operon were
574 eliminated as false positives given a P_{urcA}-*lacZ* expression phenotype that was indistinguishable
575 from WT (data not shown). A clean deletion of this operon transformed with pNJH123 also
576 failed to exhibit a P_{urcA} expression phenotype that differed from WT (data not shown).
577 Transposons within *E. coli acrA and acrB* were also reported to cause a false positive dark blue
578 colony phenotype on X-gal plates (O'Reilly & Kreuzer, 2004). Lastly, a transposon that caused a
579 dark blue phenotype within *parD*$_3$ (antitoxin protein) was described previously (Park *et al.*,
580 2016). The remaining 14 transposon mutants form the basis of this study.
581 **Clean deletions and site-directed mutagenesis.** In frame deletions were obtained by a two-step
582 *sacB* counterselection procedure (Stephens *et al.*, 1996). Approximately 600 bp regions flanking
583 the 5′ and 3′ regions of each target gene were amplified using the primers described in Table S3
584 (UR for upstream region and DR downstream region) and cloned into the HindIII and BamHI-
585 digested suicide plasmid pNPTS138 using In-Fusion cloning (Clontech). The pNPTS138
586 deletion plasmids were transformed into *C. crescentus* by electroporation and primary integrants
587 were selected on PYE plates containing 25 μg ml^{-1} kanamycin. Counterselection for the second

588 chromosomal crossover event, resulting in gene deletion, was selected for by overnight growth in
589 liquid PYE media followed by plating on PYE agar containing 3% sucrose. Deletions were
590 confirmed by colony PCR and strains are listed in Table S4.
591 Site-directed mutagenesis of the chromosomal copy of *urtAP* was performed using the
592 double-crossover allele replacement strategy described above. For *urtAP*, approximately 600-
593 1000 bp fragments surrounding each mutation were PCR amplified using the primers described in
594 Table S3 (UR for upstream region and DR downstream region) and cloned into the HindIII and
595 BamHI-digested pNPTS138 using In-Fusion cloning (Clontech). Chromosomal integration and
596 counter selection were performed as described above and successful substitutions were confirmed
597 by sequencing.
598 N-terminal HA-tagged UrtA variants and C-terminal HA tagged UrtP variants were
599 constructed as follows. A 1310-bp fragment containing the *urtA* gene was amplified using the
600 CCNA_03681-3680_UR_F and K40N_DR_R primers and cloned into the HindIII and BamHI-
601 digested pNPTS138 using In-Fusion cloning. An HA tag was added to the 5' end of *urtA* by
602 amplifying the resulting vector with primers HA-tag_for_3681_F and HA-tag_for_3681_R and
603 ligating using In-Fusion cloning, forming pDMP1031. The same primers were used to amplify
604 pDMP422 (*urtA*:K40N) and pDMP461 (*urtA*:E159Q), forming pDMP1029 and pDMP1030. A
605 2492-bp fragment containing a portion of the *urtP* gene was amplified using primers
606 H934Y_UR_F and CCNA_03681-3680_DR_R and cloned into the HindIII and BamHI-digested
607 pNPTS138 using In-Fusion cloning. An HA tag was added to the 3' end of *urtP* by amplifying
608 the resulting vector with primers pepN_HA_tag_F and pepN_HA_tag_R and ligating using In-
609 Fusion cloning, forming pDMP1031. The same primers were used to amplify pDMP940
610 (*urtP*:H934Y), forming pDMP1032. Chromosomal integration and counter selection were
611 performed as described above and the HA tag sequence and *urtAP* mutations were confirmed by
612 sequencing.

Chromosomal P_{xyl}-*urtAP*, P_{xyl}-*uzcX*, *uzcX-mCherry,* and *uzcX-HA* constructs. Guided by global transcription start site mapping data for *Caulobacter* (Zhou *et al.*, 2015), the native *urtA* and *urcX* promoters were replaced with the xylose-inducible promoter using the double-crossover allele replacement strategy described above. For CCNA_03681, the 645-bp region 218 bp upstream of the start codon was amplified with Pxyl_3681_UR_F and Pxyl_3681_UR_R, the 590-bp region of CCNA_03681 starting at the start codon was amplified with Pxyl_3681_DR_F and Pxyl_3681_DR_R, and a region containing the P_{xyl} promoter and ribosome binding site was amplified from pRXMCS-6 using Pxyl_F and Pxyl_R. All three fragments were cloned into the HindIII and BamHI-digested pNPTS138 using In-Fusion cloning, forming pDMP356. The K40N substitution was introduced into pDMP356 using primers K40N_UR_R and K40N_DR_F to form pDMP942. For *uzcX*, the 580-bp region 91 bp upstream of the start codon was amplified with 2866_up_F and Pxyl_2866_UR_R, the 579-bp region of CCNA_02866 starting with the start codon was amplified with Pxyl_2866_DR_F and Pxyl_2866_DR_R, and both fragments were cloned into pNPTS138 along with the P_{xyl} fragment, forming pDMP357. The resulting plasmids were electroporated into DMP90 to form DMP370, DMP369 and DMP644. pDMP357 was electroporated into DMP248 (H934Y, H938F) to form DMP392. To make the chromosomal *uzcX-mcherry* fusion, a gblock (uzcX-mcherry_trimmed (Table S3)) containing *mCherry* fused to the 5' end of *uzcX* was synthesized (IDT) and a DNA fragment containing the 520 bp 3' of the *uzcX* stop codon was PCR amplified from chromosomal DNA using urcX_mCherry_DR_F and urcX_mCherry_DR_R primers. Both fragments were cloned into the HindIII and BamHI-digested pNPTS138 using In-Fusion cloning, forming pDMP1081, which was used to integrate the *uzcX*-mCherry fusion at the *uzcX* chromosomal locus using the double-crossover allele replacement strategy described above. The suicide plasmid for chromosomal *uzcX*-HA integration was constructed by amplifying pDMP1081 with the primer pair uzcX_HA_F and uzcX_HA_R and ligating using In-Fusion cloning to form pDMP1127.

For assays with P_{xyl}-*urtAP* and P_{xyl}-*uzcX* constructs**,** cells were grown overnight to mid-exponential phase in PYE supplemented with 200 μM d-xylose in order to produce sufficient levels of UrtAP and UzcX to maintain low P_{urcA}-*lacZ* expression. Overnight cultures were washed 1x with fresh PYE then subcultured into PYE containing various concentrations of d-xylose. At mid-exponential phase, cells were treated with 40 μM Zn for 1 hour then harvested for β-galactosidase assays.

Construction of promoter-*gfp* transcriptional fusions. The DNA sequence regions from -9 to -165, +1 to -208, -2 to -227, -15 to -139, -9 to -132 with respect to the translation initiation site of CCNA_03497, CCNA_02290, CCNA_02291, CCNA_01585, and CCNA_01586, respectively, were amplified with the primers listed in Table S3. Amplified DNA was digested with EcoRI and BamHI and cloned into the similarly digested pDMP460 to construct promoter -*gfp* fusions (Table S4).

Construction of plasmid-borne and chromosomal xylose-inducible expression constructs. DMP946 (pBBR-oriV, catR), containing the *C. crescentus* xylose-inducible promoter (-299 to 61 bp relative to the TSS) was used as a low-copy vector backbone for xylose-inducible gene expression. *uzcX*, $marR_1$, $marR_2$, *uzcY, uzcZ,* and CCNA_00113 were amplified from the *C. crescentus* NA1000 chromosome with the primers described in Table S3 and cloned into DMP946 that was linearized using Pxyl_vector_F and Pxyl_R, replacing *gfp* to generate pDMP1082, pDMP1020, pDMP1025, pDMP588, pDMP589, and pDMP590. A plasmid-borne P_{xyl}-*urtAP* fusion was constructed by amplifying *urtAP* (codon optimized for *E. coli*) from pDMP605 using the primers urtAP_comp_F and urtAP_comp_R and cloning into linearized DMP946. To construct P_{xyl}-*uzcY*-mCherry (mCherry fused to the C-terminus of UzcY), the mCherry gene was amplified from the synthetic gblock uzcX-mcherry_trimmed (Table S3) with mcherry_F and mcherry_R and cloned into DMP593 that was linearized with the 3497-mcherry_F and 3497-mcherry_R primers, forming DMP795. P_{xyl}-mCherry-*uzcZ* (mCherry fused to the N-terminus of UzcZ) was constructed by amplifying mcherry with mcherry_F and

mcherry_R_not_stop and cloning into DMP594 that was linearized with the 2291_for_N-mcherry_F and 2291_for_N-mcherry_F primers, forming DMP819. The gene encoding mCherry in pDMP819 was swapped with an HA tag by PCR amplifying with the primers uzcZ_HA_F / uzcZ_HA_R and ligating using In-Fusion cloning to form pDMP1129. An HA tag was added to the N-terminal end of UzcY by amplifying pDMP588 with the primers N-term_HA_for_uzcY_F / N-term_HA_for_uzcY_F and ligating using In-Fusion cloning, forming pDMP1144.

P_{xyl} fusions with *urtAP, uzcX, marR$_1$,* and *marR$_2$* were integrated at the chromosomal *urcA* locus as follows for complementation experiments. The plasmid-borne P_{xyl} fusions were amplified using the HRP_chrome_int_F and HRP_chrome_int_R primers and cloned into the double recombination vector pDMP82, which was linearized with the urcA_loc_DR_F and urcA_loc_DR_F primers, using In-Fusion cloning. The native GTG start codon of $MarR_2$ was replaced with ATG by linearizing the entire P_{xyl}-CCNA_02289 expression plasmid with primers 2289_ATG_F and 2289_ATG_R and re-ligating using In-Fusion cloning to form pDMP1075. The double-crossover allele replacement strategy was then used to integrate the P_{xyl} fusions into the *urcA* locus of strains deleted for *urtAP, uzcX, marR$_1$,* and *marR$_2$*.

Co-Immunoprecipitation Assay. A *C. crescentus* strain expressing N-terminal 3x FLAG-tagged UzcS from the chromosomal locus was constructed by cloning both the 3xFLAG_uzcS_gblock_trimmed and a portion of the *uzcS* gene, which was amplified with the FLAG_urcS_F and FLAG_urcS_R primers, into the BamHI and HindIII digested pNPTS138 suicide vector using In-Fusion cloning. The resulting pDMP1115 vector was used to incorporate FLAG-tagged UzcS into strains expressing either HA-tagged variants of UrtAP and UzcX expressed from their native chromosomal loci, or HA-tagged variants of UzcY, UzcZ, expressed from P_{xyl} on a low-copy plasmid. For assays, each culture was grown in 300 ml PYE at 30°C to mid-exponential phase. Xylose was added to 0.5 mM one hr prior to harvesting for strains harboring P_{xyl} plasmids. The cells were harvested by centrifugation at 10,000 xg (4°C) for 15 min. Cells were then washed with 1x PBS (pH 6.8), centrifuged at 10,000 xg (4°C) for 15 min

690 then resuspended in 30 ml PBS (pH 6.8) containing 1% formaldehyde and allowed to cross-link
691 at room temperature for 30 minutes. Cells were centrifuged at 10,000 xg (4°C) for 15 min,
692 washed once with 1x PBS (pH 6.8), centrifuged, frozen in dry ice, and stored at −80°C. The cell
693 pellets were thawed and resuspended in 1.4 ml B-PER™ Complete Bacterial Protein Extraction
694 Reagent (ThermoFisher), followed by addition of EDTA to 1mM and rocking for 15 min at room
695 temperature. Triton X-100 and ETDA were added to 1% and 2 mM, respectively, and the
696 samples were incubated on ice for 60 min. Insoluble cell debris was pelleted via centrifugation
697 (20,000xg, 10 min at 4°C) and the lysate was incubated with 40 µl of FLAG-M2 agarose
698 (FLAGIPT-1 kit; Sigma) overnight on a nutator at 4°C. Beads were washed three times with 1x
699 wash buffer (50 mM Tris-HCl, 150 mM NaCl) and proteins were eluted by incubating with 100
700 µl of 5 µg/µl 3× FLAG peptide solution for 30 min at 4°C. Western blots were performed with the
701 following antibody dilutions;anti-HA.11 epitope tag antibody (Covance; 1:1000), anti-FLAG M2
702 antibody (Sigma; 1:1000), and mCherry polyclonal antibody (Thermofisher; 1:1000).
703 **UrtAP and UzcX topology analysis.** To validate the topology prediction for UrtP, *urtAP* was
704 codon optimized for expression in *E. coli* and placed under the control of the rhamnose-inducible
705 promoter. To accomplish this, *urtAP* was synthesized as three, ~1500 bp gblock fragments
706 (Table S3) that were ligated into pDMP58 that was linearized using the primers Prham_vector_F
707 and Prham_vector_R using In-Fusion cloning, producing pDMP605. The *E. coli phoA* gene
708 (amino acids 21-471) was inserted at eight different locations within *urtAP* using the primers
709 listed in Table S3. *Escherichia coli* HST08 (Clontech) cells containing each plasmid were plated
710 on LB containing 0.2% rhamnose, 30 µg ml^{-1} kanamycin and 80 µg ml^{-1} 5-Bromo-4-chloro-3-
711 indolyl phosphate (X-Phos). To validate the UzcX topology analysis, the *E. coli phoA* gene
712 (amino acids 21-471) was inserted at four different locations within *uzcX* as follows. *uzcX-phoA*
713 fusions were synthesized as gblocks (Table S3) and ligated into pDMP271 that was linearized
714 using the primers pBAD_ABCpepN_F and pBAD_ABCpepN_R using In-Fusion cloning,
715 placing each *uzcX-phoA* under the control of the arabinose-inducible promoter (pBAD).

716 *Escherichia coli* HST08 (Clontech) cells containing each plasmid were plated on LB containing
717 0.005% arabinose, 30 μg ml^{-1} Ampicillin and 80 μg ml^{-1} X-Phos.
718 **β-Galactosidase assays.** Strains possessing pNJH123, a plasmid-borne translational P_{urcA}-
719 *lacZ* fusion (Hillson *et al.*, 2007), or the chromosomal P_{urcA}-lacZ fusion (DMP90) were used for
720 all assays. For experiments with Zn, cells were grown in PYE at 30°C to an OD600 of 0.1,
721 before addition of Zn to 40 μM. After 1 hour of Zn exposure, cell growth and any further protein
722 synthesis were terminated by the addition of tetracycline to a final concentration of 2 μg ml^{-1}, and
723 cells were placed on ice. For experiments with U, cells were grown overnight in M5G-G2P,
724 washed once to remove inorganic phosphate and inoculated into M5G-G2P with or without 50
725 μM uranyl nitrate. Aliquots were removed at various time points for assays. β-Galactosidase
726 activity was assayed as previously described (Miller, 1972).
727 **Phos-tag acrylamide SDS-PAGE.** UzcR phosphorylation was analyzed by Phos-tag acrylamide
728 SDS-PAGE following the method of Barbieri et al (Barbieri & Stock, 2008). Cells were grown to
729 an OD_{600} of 0.3, centrifuged at 20,000 *g* for 1 min, decanted, and then lysed by adding 55 μl of
730 BugBuster master mix (Novagen) and pipetting up and down for ~10 seconds. 18 μL of 4× SDS
731 loading buffer (Barbieri & Stock, 2008) was added to each sample and 7 μl of the cell lysate was
732 loaded onto a 10% (wt/vol) acrylamide gel containing 25 μM Phos-tag acrylamide and 50 μM
733 $MnCl_2$ and run at 150 V at 4 °C for two hours. Controls with phosphorylated and
734 unphosphorylated UzcR were run with 7 μl of lysates prepared from a Δ*uzcR* strain. UzcR was
735 phosphorylated as described previously (Park *et al.*, 2016). The gel was washed for 20 min with
736 50 ml Transfer buffer (Tris-HCl, glycine, 20% methanol) containing 1 mM EDTA and 20 min
737 with 50 ml Transfer buffer lacking EDTA, and then transferred to a nitrocellulose membrane at
738 100 V for 2 hour using a Mini Trans-Blot Cell (Biorad). Primary antibody incubation was
739 performed overnight at 4°C with a 1:1,000 dilution of the affinity purified UzcR antibody (Park *et*
740 *al.*, 2016) in TBS-T (Tris-buffered saline supplemented with 0.1% Tween-20) with 3% (w/v)

741 powdered milk. A goat anti-rabbit antibody (Bio-Rad, 1:5000 dilution) conjugated to
742 horseradish peroxidase (HRP) was used as the secondary antibody. The Pierce ECL Western
743 Blotting Substrate detection kit and a ChemiDoc XRS+ imager (Bio-Rad) were used to detect
744 HRP chemiluminescence.
745 **RNA-seq.** RNA was isolated from triplicate Δ*uzcR,* Δ*urtAP*Δ*uzcR*, Δ*uzcX*Δ*uzcR,* Δ*marR1*Δ*uzcR,*
746 Δ*marR2*Δ*uzcR* strains grown to mid-exponential phase (OD_{600} of ~0.28) in M5G media
747 supplemented with 1.32 mM inorganic phosphate using the hot phenol method (Jahn *et al.*, 2008).
748 Total RNA was quantified with a NanoDrop 2000 (ThermoFisher) and its integrity verified using
749 an E-gel system (ThermoFisher). Library preparation was performed at the UC Berkeley Vincent
750 J. Coates Genomics Sequencing Laboratory as previously described (Park *et al.*, 2016). Samples
751 were normalized and pooled, then run on an Illumina HiSeq 4000 (50 bp read length, single end)
752 at the UC Berkeley Vincent J. Coates Genomics Sequencing Laboratory.
753 Sequencing data was mapped to the *C. crescentus* NA1000 reference genome sequence
754 (GenBank accession number NC_011916.1) using Bowtie v1.1.1 (-t -S -m 1); all samples
755 contained at least 16 million reads that mapped to unique locations within the genome. HTSeq-
756 0.6.1p1 (Anders *et al.*, 2015) was used to quantify the number of counts per genomic feature; the
757 output was converted to a .bam file, sorted, and indexed using samtools-0.1.19. RSeQC-2.3.9
758 (Wang *et al.*, 2012) was then used to normalize the counts data and generate wig files for
759 visualization in MochiView (Homann & Johnson, 2010). The DESeq package in R (Anders &
760 Huber, 2010) was used to generate differential expression data for each sample as compared to
761 wild type cells. Genes that exhibited a greater than 2-fold change in expression with a p-value <
762 0.01 were considered DE.
763 **ChIP-seq.** Assays were performed with a Δ*urtAP* strain grown to mid-exponential phase in PYE
764 as described previously (Park *et al.*, 2016).
765 **mRNA profiling using Nanostring custom gene expression codesets.** Cells were grown in
766 duplicate in M5G supplemented with 1.3 mM phosphate to exponential phase (OD_{600} of 0.2) at

30°C. 1.5 ml of each culture was quenched with 188 µl of stop solution (95% ethanol and 5% phenol), centrifuged at 20,000 x g for 5 min at 4°C, and then frozen on dry ice. Total RNA was isolated using an RNeasy column (QIAGEN) as previously described (Park *et al.*, 2016). RNA (100 ng) was hybridized for 17 hours at 65°C to custom nCounter gene expression codesets (Nanostring) containing a panel of 50 selected *Caulobacter crescentus* target genes (Park *et al.*, 2016), including 37 genes under direct UzcR transcriptional control. Following hybridization, samples were prepped in the nCounter Prep Station and data was collected in the nCounter Digital Analyzer according to the manufacturer's instructions. The NanoStringDiff package (Wang *et al.*, 2016) was used for data normalization and differential expression analysis. Normalization parameters are estimated from the positive controls, negative controls and housekeeping genes (CCNA_01297, CCNA_01448, CCNA_03876, and CCNA_01320) incorporated within the nCounter codeset. Previously generated nanostring data for WT and Δ*uzcR* strains exposed to 10 µM Zn (Park *et al.*, 2016) were analyzed as positive and negative controls, respectively. Genes that exhibited a greater than 2-fold change in expression with a p-value < 0.01 were considered DE. The activity of UzcR was approximated using the TFInfer algorithm (Asif *et al.*, 2010) with the $\log_2$ fold change (mutant/WT) values for each UzcR regulon member.

Structured illumination microscopy (SIM). Strains DMP500, DMP793, and DMP865 were grown to mid-exponential phase in PYE supplemented with 1 mM xylose (for DMP793, and DMP865) and 1 µg ml^{-1} chloramphenicol. DMP500, containing a chromosomal *uzcX-mCherry* fusion at the *uzcX* locus, was treated with 40 µM $ZnCl_2$ for 1.5 hr prior to harvesting to induce expression of the *uzcX-mCherry* fusion. Cells were harvested, fixed with 4% paraformaldehyde in 1x PBS pH 7 for 30 min at room temperature, and then washed once with 1x PBS pH 7. Fixed cells were concentrated (5-fold) in 1x PBS, spotted on a M5G-1.25% agarose pad, topped with a #1.5 coverslip, sealed with nail polish, and imaged. Z-series images of cells harboring protein-

mCherry fusions were acquired on a DeltaVision OMX three- dimensional (3D) SIM superresolution system (GE Healthcare) equipped with a 60x 1.42 NA oil immersion objective. Deconvolution and alignment were performed using Softworx imaging software and images were processed using ImageJ (Schneider *et al.*, 2012).

Accession number. All RNA-seq data are available for download from the NCBI Gene Expression Omnibus (GEO) under accession number GSE108075

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Author Contributions:
(DMP and YJ) Conception and design of the work
(DMP, KWO, YJ) Data collection, analysis and interpretation
(DMP) Drafting the article
(DMP, KWO, YJ) Critical revision of the article

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Figure Legends

Figure 1. Identification of negative regulators of UzcRS. (A) Genes containing transposon insertions that led to high P_{urcA}-*lacZ* activity were deleted and the resulting strains were transformed with plasmid-borne P_{urcA}-*lacZ* (pNJH123)(Hillson *et al.*, 2007). Cells were harvested at mid-exponential phase with or without 1 h exposure to Zn (40 μM), and P_{urcA}-*lacZ* activity was determined using β-galactosidase assays. Error bars represent the standard deviation of triplicate measurements. The lack of Zn induction in the $marR_1$ $marR_2$ double mutant in PYE, in contrast to the observation in minimal media (Figure S1), likely reflects near maximum levels of P_{urcA} expression in this condition. (B) Complementation of negative regulator mutants. Each mutant was placed under the control of P_{xyl} and chromosomally integrated into the respective deletion strain. 100 μM xylose was used to induce all regulators, except for $MarR_2$, which required 1 mM xylose for effective complementation. (C) Effects of deleting negative regulators of P_{urcA} in tandem with *uzcR* or *uzcS* on P_{urcA}-*lacZ* expression. (D) Effect of P_{urcA} regulatory mutations and

1027 Zn on *in vivo* UzcR-P levels assessed by a representative Phos-tag SDS-PAGE followed by
1028 Western blot. Phos-tag retards the migration of UzcR□P relative to unphosphorylated UzcR. As
1029 a control, purified UzcR was either phosphorylated with the small molecule phosphate donor
1030 carbamoyl phosphate as previously described ((Park *et al.*, 2016); labeled as UzcR-P) or
1031 subjected to the same treatment lacking carbamoyl phosphate (labeled as UzcR). Arrows depict
1032 shifted complexes. (E) Global UzcR-dependent transcriptional regulation in negative regulator
1033 mutants. Gene expression profiles of 37 of 42 members of the UzcR regulon were assayed in
1034 WT, Δ*uzcX,* Δ*urtAP,* Δ$marR_1$, Δ$marR_2$, and Δ*zur* cells without exposure to metal using nCounter
1035 gene expression codesets. Data depict the $\log_2$ fold change expression value for each strain
1036 relative to WT cells with no inducer and are visualized using the heatmap.2 function in R. Data
1037 for WT and Δ*uzcR* cells exposed to 10 μM Zn were analyzed and plotted as positive and negative
1038 controls. Note that CCNA_02287 is the only UzcR-repressed gene assayed with the codesets.
1039
1040 **Figure 2. Negative regulator mutants cause minimal UzcR-independent gene expression**
1041 **changes.** Volcano plots depicting DE genes for pairwise comparisons of RNA-seq data for
1042 individual negative regulator mutants deleted in tandem with *uzcR* compared to the Δ*uzcR* control
1043 strain. Genes that exhibited a greater than twofold change in expression with a *p*-value of <0.05
1044 were considered DE. Genes under Zur transcriptional control (Mazzon *et al.*, 2014) are depicted
1045 in red while those likely under the direct control of the respective MarR regulator are depicted in
1046 black. Genes marked with an asterisk were plotted as $-\log_{10}$ padj values of 84 for visualization
1047 purposes. Both had *padJ* values of 0, indicating that the biological difference is strongly
1048 significant compared to the variability of replicates.
1049 **Figure 3. UrtAP forms a sensory complex with UzcS and both the ATPase and peptidase**
1050 **domains contribute to UzcRS regulation**. (A) Diagram of UrtP transmembrane topology using
1051 the Protter program (Omasits *et al.*, 2014). (B) PhoA (*E. coli* alkaline phosphatase) insertions
1052 suggest that UrtP contains 13 transmembrane domains with the aminopeptidase domain localized

to the periplasm. *E. coli* HST08 cells expressing different *urtAP-phoA* fusions were plated on LB containing X-phos indicator (see methods for details). Locations of PhoA insertions are depicted with roman numerals in panel A. (C) Effects of substitutions of conserved amino acid residues within the Walker A, Walker B and Zn coordination site of UrtAP or deletion of the entire aminopeptidase domain on P_{urcA}-*lacZ* expression. Amino acid substitutions or deletions were introduced into the chromosomal copy of *urtAP* and P_{urcA}-*lacZ* activity was determined using β-galactosidase assays in cells harboring pNJH123 (Hillson *et al.*, 2007). The location of conserved regions within the coding regions of *urtA* and *urtP* are diagrammed below the plot with substituted residues bolded. Black rectangles depict the transmembrane helices of the ABC-2 domains. (D) Western blot of chromosomally encoded WT and mutant HA-tagged UrtA and UrtP variants using lysates from cells grown to mid-exponential phase. HA-tagged UrtA and UrtP lysates were run on 12% and 7.5% polyacrylamide gels, respectively. Western blots were performed with a monoclonal anti-HA.11 epitope tag antibody (Covance; MMS-101P) and a goat anti-mouse HRP-conjugated secondary antibody (Biorad). (E) Whole cell extracts and samples from Co-IP experiments performed with anti-FLAG M2 agarose beads were subjected to western blot with an anti-HA antibody. The genotype of strains used in Co-IP experiments is depicted above the corresponding gel lane. A plus denotes the presence of chromosomally encoded FLAG-tagged *uzcS* and HA-tagged *urtP* while a blank indicates a WT copy of *uzcS*. Samples were run on Any kD polyacrylamide gels (Biorad). (F) The effect of UrtAP variants on the UrtP-HA interaction with FLAG-UzcS. Samples from Co-IP experiments performed with anti-FLAG M2 agarose beads and whole-cell extracts were subjected to Western blot with anti-HA and anti-FLAG antibodies. The *urtAP* genotype for each lane is depicted above the gel.

Figure 4. MarR regulators repress UzcRS function by silencing expression of the membrane proteins UzcY and UzcZ. (A) Operon diagram and RNA-seq expression profile of the autoregulatory $marR_1$ (top) and $marR_2$ (bottom) operons. The RNA-seq data for Δ$marR_1$ Δ*uzcR* (top; blue), Δ$marR_2$ Δ*uzcR* (middle; red), and Δ*uzcR* (bottom; black) is depicted for each

operon. Blue lines depict locations of transposons mapped within *marR*$_1$ and *marR*$_2$. The red arrow depicts the approximate location of the P$_{uzcZ}$ transcription start site. (B) Effect of deleting each gene within the autoregulatory MarR operons individually and in tandem with the respective *marR* regulator on P$_{urcA}$-*lacZ* expression. The data for the deletion of genes encoding an additional paralogous MarR-like regulator (CCNA_00114) is also included, with an operon diagram for this regulator displayed in Figure S7. Fold induction was calculated by dividing the β-galactosidase activity of each strain by that of WT. Error bars represent the standard deviation, determined using the propagation of errors (Ku, 1966). (C) Effect of *uzcY*, *uzcZ*, and CCNA_00113 (negative control) overexpression on P$_{urcA}$-*lacZ* activity. DMP90 (chromosomal P$_{urcA}$-*lacZ* fusion), harboring a low-copy plasmid containing each gene under the control of P$_{xyl}$ was grown to early exponential phase in PYE medium supplemented with various concentrations of xylose. Fold activation was determined by dividing the β-galactosidase activity at each xylose concentration by that of DMP90, and error bars represent the standard deviation, determined using the propagation of errors (Ku, 1966). (D) Effect of tandem expression of *uzcY* and *uzcZ* on P$_{urcA}$-*lacZ* activity. DMP90 harboring a low-copy plasmid containing *uzcY* under the control of P$_{xyl}$ (Meisenzahl *et al.*, 1997) and *uzcZ* under the control of P$_{van}$ (Thanbichler *et al.*, 2007) was grown to early exponential phase in PYE medium supplemented with various concentrations of xylose and vanillate, and P$_{urcA}$-*lacZ* activity was determined by β-galactosidase assays. (E) WT and FLAG-tagged *uzcS* strains harboring a low-copy plasmid containing N-terminal HA-tagged *uzcY* or *uzcZ* under the control of P$_{xyl}$ were subjected to Immunoprecipation using anti-FLAG M2 agarose beads. HA-UzcY and HA-UzcZ expression was induced with 0.5 mM xylose for 1 hr prior to harvesting cells. Co-IP samples and cell lysates were analyzed by Western blot with anti-HA and anti-FLAG antibodies. A plus sign denotes the presence of chromosomally encoded FLAG-tagged *uzcS*, or xylose-induced HA-tagged *uzcY / uzcZ* expression. Samples were run on Any kD polyacrylamide gels (Biorad).

Figure 5. UzcX localizes to the membrane but does not interact with UzcS.

(A) The transmembrane topology of UzcX predicted by the PolyPhobius algorithm is depicted using the Protter program (Omasits *et al.*, 2014). PhoA (*E. coli* alkaline phosphatase) insertions support the predicted topology of UzcX. *E. coli* HST08 cells expressing different *uzcX-phoA* fusions were plated on LB containing X-phos indicator. Locations of PhoA insertions are depicted with roman numerals. (B) Subcellular localization of UzcX-mCherry. DMP500, an otherwise WT strain containing a chromosomal uzcX-mCherry fusion was grown to early exponential phase, subjected to a one hour exposure to 40 μM Zn, and imaged using 3D structured illumination microscopy. A Representative Z-stack image is presented. (C) Whole cell extracts and samples from Co-IP experiments performed with anti-FLAG M2 agarose beads were subjected to Western blot with an anti-HA antibody. The genotype of strains used in Co-IP experiments is depicted above the corresponding gel lane. A plus denotes the presence of chromosomally encoded FLAG-tagged *uzcS* and HA tagged *uzcX* while a blank indicates a WT copy of *uzcS*. The Co-IP data for ccells grown to mid-exponential phase in PYE with or without a one-hour exposure to $ZnCl_2$ are depicted on the bottom and top panels, respectively. Samples were run on Any kD polyacrylamide gels (Biorad).

Figure 6. Negative regulators affect the sensitivity of UzcRS to metal inducers. Zn (A) and Cu (B) sensitivity profile of P_{1968}-*gfp* in strains deleted for UzcRS negative regulators. Fluorescence was quantified following a two-hour exposure of mid-exponential phase cells to each metal concentration and normalized to the OD_{600}. The change in normalized fluorescence was calculated by subtracting the fluorescence of the no metal condition from the fluorescence at each metal concentration. See Figure S9A-B for the fluorescence data prior to subtraction of the no metal signal. Error bars represent the standard deviation, determined using the propagation of errors (Ku, 1966). Effect of overexpression of *uzcX, urtAP*, and mutant *urtAP* variants on the Zn (C) and Cu (D) induction profile of P_{urcA}-*lacZ* (top plots). The chromosomal promoters of *uzcX* and *urtAP* (Zhou et al., 2015) were swapped with P_{xyl} in a strain containing a chromosomal P_{urcA}-*lacZ* fusion (DMP90). The expression of each chromosomal *urtAP* variant was induced with 10

1131 mM xylose as P_{urcA}-*lacZ* expression decreased with increasing xylose concentrations up to 10
1132 mM. Fold repression (bottom plots) was determined by dividing the β-galactosidase activity of
1133 DMP90 at each metal concentration by the expression with each xylose-induced urtAP variant.
1134 **Figure 7. Loss of negative regulation weakened Zn tolerance.** Effect of negative regulator
1135 mutants on the doubling time following exposure to the UzcRS inducer Zn (A) and the non-
1136 inducer Ni (B). The top and bottom plots reflect data from two independent experiments. Error
1137 bars depict the standard deviation of biological triplicates.
1138 **Figure 8. Multicomponent signaling pathway governing an extracytoplasmic stress response**
1139 **in *Caulobacter*.** The UzcRS TCS is the core signaling unit of this multicomponent signaling
1140 pathway and functions as a U/Zn/Cu-dependent global activator of genes encoding
1141 extracytoplasmic functions (Park *et al.*, 2016). The activity of UzcRS is influenced by at least
1142 four auxiliary regulatory proteins. In the absence of inducer, a conserved membrane protein
1143 UzcX and an ABC transporter with a C-terminal periplasmic metallo-aminopeptidase domain
1144 UrtAP function synergistically to maintain UzcRS in an off state. *uzcX* is encoded in the UzcRS
1145 regulon, providing a mechanism of feedback inhibition. UrtAP forms a sensory complex with
1146 UzcS and both the ATPase and peptidase domains are critical for regulating basal UzcRS and
1147 antagonizing metal-dependent stimulation of UzcS. Note that UrtAP is arbitrarily drawn as an
1148 exporter since we have not experimentally determined its polarity. Lastly, the DNA binding
1149 activity of two independent MarR-like transcription factors is linked to UzcRS activity. We
1150 propose that upon signal perception, presumably through binding of cognate substrate, MarR
1151 DNA binding is antagonized, leading to derepression of the membrane proteins UzcY and UzcZ
1152 that stimulate UzcRS activity in a synergistic manner. Arrows are colored based on interactions
1153 that occur under normal conditions of growth conditions (*i.e.* no added inducer; black) or
1154 following exposure to U/Zn/Cu (blue) or an unidentified stressor/s (magenta). Dashed lines
1155 represent either indirect interactions or unconfirmed direct interactions. Direct and indirect UzcR
1156 transcriptional control is based on previously published ChIP-seq and RNA-seq data (Park *et al.*,

2016); dashed arrows reflect a *uzcR*-dependent change in transcription, but no upstream ChIP-seq peak.

Table 1. Transposon mutants with deregulated P_{urcA}-*lacZ* activity

Gene	# of Tn5 hits	Colony color	P_{urcA}-*lacZ* activity[b] -Zn	P_{urcA}-*lacZ* activity[b] +Zn	Clean deletion[c]	Function
CCNA_02289 (*marR*$_2$)	2	dark blue	265	3788	yes	MarR-like regulator
CCNA_03498 (*marR*$_1$)	1	dark blue	140	3753	yes	MarR-like regulator
CCNA_03680, CCNA_03681 (*urtAP*)	8	dark blue	2225	6440	yes	aminopeptidase N - ABC transporter
CCNA_02866 (*uzcX*)	3	dark blue	403	4544	yes	conserved membrane protein
[d]parD$_3$	1	dark blue	138	2502	ND	Antitoxin of ParDE$_3$ system
[a]WT PurcA-lacZ	NA	blue	20	2900	NA	

[a]Parent strain for reference uninduced and Zn-induced P_{urcA} activity
[b]activity in Miller Units of one representative transposon mutant
[c]In frame deletion. Yes indicates confirmation of phenotype
[d]UzcRS phenotype characterized previously (Park *et al.*, 2016).

Table 2. UzcRS-independent gene expression in negative regulator mutants

			Δ*uzcX* Δ*uzcR* / Δ*uzcR*[b]		Δ*urtAP* Δ*uzcR* / Δ*uzcR*		Δ*marR*$_1$ Δ*uzcR* / Δ*uzcR*		Δ*marR*$_2$ Δ*uzcR* / Δ*uzcR*	
Gene or operon	**gene name**	**Function**	**log_2 Fold Change**	**padj**	**log_2 Fold Change**	**padj**	**log_2 Fold Change**	**padj**	**log_2 Fold Change**	**padj**
CCNA_03497	*uzcY*	hypothetical protein	-0.01	1.00E+00	-0.04	1.00E+00	8.92	0.00E+00	0.00	9.04E-01
CCNA_03499		hypothetical protein	-0.20	8.07E-01	-0.19	1.00E+00	7.29	0.00E+00	0.13	5.12E-01
CCNA_03496		hypothetical protein	0.10	8.20E-01	0.04	1.00E+00	1.63	2.71E-84	0.00	8.00E-01
CCNA_02291	*uzcZ*	hypothetical protein	-0.14	9.40E-01	0.11	1.00E+00	0.32	1.82E-01	8.54	0.00E+00
CCNA_02290		hypothetical protein	0.01	1.00E+00	0.03	1.00E+00	-0.13	6.31E-01	5.74	1.47E-175
CCNA_00214-CCNA_R0101	*znuL**	TonB-dependent receptor, sRNA	3.22	3.34E-37	2.75	1.77E-29	3.05	1.03E-37	3.04	1.60E-36
CCNA_01586-CCNA_01588	*znuGHI**	ABC transporter	4.72	1.88E-29	3.76	2.98E-20	4.73	7.39E-32	4.46	2.34E-28
CCNA_01585	*znuK**	TonB-dependent receptor	5.08	6.11E-19	4.11	2.65E-13	5.19	4.48E-21	5.31	4.64E-21
CCNA_00700	*znuM**	TonB-dependent receptor	2.72	2.35E-16	2.13	1.15E-10	2.58	4.25E-17	2.53	1.05E-15
CCNA_00322	*zrpW**	hypothetical protein	2.91	1.55E-07	2.02	1.32E-03	2.73	5.84E-08	2.74	1.94E-07
CCNA_02805-CCNA_02807[a]	*-, czrC, czcrB,-*	Hypothetical protein, RND system	-1.65	4.86E-04	-1.43	1.86E-03	-0.87	3.45E-02	-1.10	7.89E-03
CCNA_01316	*rplN*	LSU ribosomal protein L14P	-0.79	5.55E-01	-1.55	9.19E-04	-1.60	8.72E-05	-1.18	2.89E-03
CCNA_01997		ribosome recycling factor (RRF)	-1.00	4.60E-01	-1.36	4.93E-02	-1.49	4.56E-03	-0.81	1.06E-01
CCNA_02823		hypothetical protein	-0.95	2.64E-03	-0.91	1.24E-03	-1.07	1.61E-06	-1.03	7.10E-06
CCNA_02625	*ftsQ*	cell division protein FtsQ	-0.81	1.17E-01	-0.92	3.68E-03	-1.08	4.08E-05	-0.79	3.16E-03
CCNA_00531		LSU ribosomal protein L12P (L7/L12)	-0.69	3.03E-01	-0.96	3.68E-03	-1.13	4.32E-05	-0.79	3.77E-03
CCNA_03987		hypothetical protein	-0.46	7.58E-01	-0.17	1.00E+00	0.33	4.61E-01	1.65	3.35E-08
CCNA_R0092		sRNA	0.37	8.01E-01	0.45	1.00E+00	0.32	6.59E-01	1.42	1.77E-06
CCNA_R0053		sRNA	-0.24	9.45E-01	0.38	1.00E+00	0.97	6.34E-03	1.25	2.15E-04
CCNA_01178	-	hypothetical protein	0.28	8.32E-01	0.12	1.00E+00	0.13	8.51E-01	1.08	1.13E-03

CCNA_02823	-	hypothetical protein	-0.95	2.64E-03	-0.91	1.24E-03	-1.07	1.61E-06	-1.03	7.10E-06
CCNA_03901	-	hypothetical protein	-0.10	9.70E-01	-0.12	1.00E+00	-0.40	2.87E-01	-1.26	7.82E-05
CCNA_00572	-	PTS system, N-acetylglucosamine-specific IIBC component	-1.61	1.15E-01	-1.24	3.12E-01	-0.97	8.03E-02	-1.48	6.52E-03

Displays all genes that were differentially expressed in at least one negative regulator mutant

[a]data depicted for CCNA_02807. Likely part of CCNA_02805-CCNA_02811 operon, but only CCNA_02805-CCNA_02807 exhibit DE in at least one strain
*Part of Zur regulon. All Zur-regulated genes were grouped into operons.
[b]Depicts data for only biological duplicate data (replicates 1 and 2) since Zur-dependent gene induction was not observed for replicate 3 (see Figure S4C). All other pairwise comparisons were performed in triplicate.

1168