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Transmembrane redox control and proteolysis of PdeC, a novel type of c-di-GMP phosphodiesterase

Susanne Herbst, Martin Lorkowski, Olga Sarenko, T Kim L Nguyen, Tina Jaenicke, Regine Hengge

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Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 5 September 2017

Thank you for submitting your manuscript for consideration by the EMBO Journal. We have now received a full set of referee reports on your manuscript, which are included below for your information.

As you can see from the comments, all three referees express interest in the presented mechanism of c-di-GMP phosphodiesterase regulation. However, they also raise several substantive concerns, which need to be addressed before they can support publication here. Based on the overall interest expressed in the reports I would like to invite you to submit a revised version of your manuscript in which you address the comments of all three referees, particularly focusing on the physiological relevance of PdeC regulation, as requested by all referees, and further analysis of disulfide bond formation (reviewers #2 and #3) and PdeC proteolysis (reviewer #1). Please note that it is The EMBO Journal policy to allow only a single major round of revision and that it is therefore important to resolve the main concerns at this stage.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website: http://emboj.embopress.org/about#Transparent_Process

We generally allow three months as standard revision time. Please contact us in advance if you would need an additional extension. As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of the conceptual advance presented by your study. However, upon publication of any related work please contact me as soon as possible to discuss how to proceed.

Please feel free to contact me if have any further questions regarding the revision. Thank you for the opportunity to consider your work for publication. I look forward to your revision.

REFEREE REPORTS

Referee #1:

General comment
The "CSS-motif" domain entry in the Pfam database has been created several years ago but until now the functions of this domain remained unknown. This protein domain is found in more than 5,000 proteins in UniProt, almost always in association with the c-di-GMP-specific phosphodiesterase (EAL) domain. Both E. coli and Salmonella Typhimurium genomes carry five such genes, which indicates that these proteins must perform some crucial cellular function. The reviewed manuscript finally sheds light on the functions of these proteins and their CSS domains. This is a well-written paper presenting a very impressive work which uncovers the mechanism that allows regulation of biofim formation by the redox status and proteolytic activity in the cell periplasm. That said, this manuscript could benefit from clarification of the following issues.

1. Although the transmembrane segment TM1 of PdeC does not look like a signal peptide, has it been shown experimentally, e.g. by N-terminal sequencing or mass-spectrometry, that it does not get cleaved? In other words, how do we know that the CSS domain is anchored in the membrane from both sides and not just by TM2?
2. The 33-kD proteolytic fragments on Fig. 2 and Fig. 3 form pretty tight bands, rather than smudges often produced by non-specific proteolysis. If, as these gels suggest, proteolysis occurs at a specific point of the PdeC sequence, it would be helpful to define this point (again by N-terminal sequencing or mass-spectrometry), rather than just put it somewhere around amino acid 215 (lines 215-216 of the text).
3. This work deals with the redox regulation of PdeC during the transition to the stationary phase in aerobic conditions. The most physiologically relevant for E.coli would be transition between aerobiosis and anaerobiosis (as in the human gut). It would be important to show that the transition from the oxidized form of PdeC to the reduced form, achieved by the DTT exposure in Fig. 2B, could also occur in vivo, during the switch from an aerobic to anaerobic environment.
4. The data presented on Fig. 4 and S4 show that the TM2-EAL construct localizes to the membrane fraction and is enzymatically active. Clearly, this fragment adopts the correct orientation with respect to the membrane, which allows dimerization of the EAL domain. However, calling TM2 a dedicated dimerization domain is probably a stretch. Its sequence does not contain any residues that would make TM2 likely to form stable dimeric patches. Further, there could be any number of reasons why replacement of TM2 with TM2* did not work, including the possibility that TM2* simply did not properly insert into the membrane. It would be a good idea to soften the language on lines 241 and 658.
5. A very attractive feature of the presented model (Fig. 5) is the inherent temporary response of the CSS-EAL system, which is only active until either the CSS domain gets oxidized or the EAL domain gets proteolytically cleaved from the membrane. This might be worth a brief discussion.
6. The description of Fig. 1 panels B and C is presented in the text but missing in the Figure legend.

Minor comments.
- Line 94. After "proteins", add "respectively".
- Line 143. Change "replaced the functionally important EAL motif by AAL" to "replaced the active site glutamate of the EAL motif by alanine".
- Line 150. Change "AAL mutation" to "EAL to AAL mutation"
- Line 156. Replace "determining" by "monitoring two c-di-GMP-dependent functions:"
- Line 161. Mentioning "the potential to form a DSB" looks a bit premature here, until the presentation of the DTT data. You might want to say that "presence of both Cys75 and Cys106 of the CSS domain results in a lower PDE activity of PdeC, whereas the replacement of one or both of them promotes PdeC activity. This suggested a possible link to the potential of these Cys residues to form a DSB".
- Lines 189-190, 202, and elsewhere in the text. "cys75 and cys106" should be "Cys75 and Cys106"
- Fig. S3. The UniProt entry for PdeC http://www.uniprot.org/uniprot/P32701 lists slightly shorter transmembrane segments (14-34) and (242-262), which is consistent with the alignment on Fig. S1.
- Fig. 5. The presented scheme looks very nice but the positions of the cysteines give the impression that the distance between them (which is 30 aa) is equal or greater than the distance from Cys105 to the TM2 (which is actually 120 aa).

Referee #2:

In bacteria, biofilm formation is promoted by the second messenger c-di-GMP. This molecule is synthesized by diguanylate cyclases and degraded by specific phosphodiesterases. The cellular levels of c-di-GMP depend on the balance between these two classes of enzymes. In this manuscript, Herbst et al investigate the physiological function of a group of five phosphodiesterases expressed by E. coli. These enzymes are characterized by the presence of a periplasmic domain in which two conserved cysteine residues are present. Focusing on one of the five phosphodiesterases, PdeC, the authors demonstrate that formation of a disulfide bond between these two cysteines reduces PdeC phosphodiesterase activity. By contrast, preventing disulfide bond formation by deleting DsbA or DsbD, or reducing the disulfide by adding DTT, activates the enzyme. The authors also show that reduction of the disulfide induces the enzymatic degradation of the periplasmic portion of PdeC by the proteases DegP and DegQ and that the second TM domain of PdeC acts as a dimerization domain promoting phosphiesterase activity. On the basis of these results, they propose an elegant mechanism in which the phosphodiesterase activity of PdeC is controlled through redox regulation and dimerization. Overall this paper is interesting and the data nicely support the proposed mechanism. The detailed mechanistic study is quite impressive and informative.
The conservation of two cysteine residues that form a disulfide in a protein does not necessarily indicate that this protein is redox-regulated. This disulfide might only be required for structural stability. My major concern is that the authors fail to provide data supporting the physiological relevance of their findings. In the discussion, the authors suggest that decreased oxygen concentrations may favor reduction (absence of formation) of the PdeC disulfide and therefore activate the enzyme. Maybe. But, as the authors mention, the DsbA/DsbB system has been shown to function anaerobically, so this might not be the case. I think that some experimental evidence that oxygen limitation is the physiological signal activating PdeC activity is required for publication in a journal like EMBO.
Minor comments:
-E.coli has 4 additional enzymes like PdeC. In this study, they have only tested one. It would be interesting to test the importance of disulfide bond formation on the activity for at least one of the other enzymes (using the macrocolony morphology assay for example).
-The authors could comment more on the interest for E. coli to produce fragments by proteolysis if the full size protein is capable of PDE activity

-line 174 : a a periplasmic DSB
-line 235 : Fig 3B instead of 4B
-line 377 : limitation instead of limitaton

Referee #3:

Herbst et al. present interesting set of observations suggesting involvement of the periplasmic CSS motif (protein domain) in regulating E. coli c-di-GMP phosphodiesterase (PDE), PdeC. Their study offers genetic evidence (using mutants in the periplasmic disulfide bond formation apparatus) that a disulfide bond is formed between two highly conserved Cys residues in the CSS motif. It further suggests that addition of a strong reducing agent results in the proteolysis of the CSS motif via the periplasmic proteases DegP and DegQ. Proteolysis also takes place when the Cys residues are mutated. Interestingly, a proteolytically processed (truncated) version that lacks the CSS motif but retains a single transmembrane motif (TM2), which is involved in protein dimerization, is catalytically active, and potentially more active than the native PdeC protein.
Overall, this is an interesting story that, however, has some large experimental gaps. Further, it presents a conclusion about the physiological significance of PdeC regulation that contradicts earlier literature reports. Filling the gaps is critical for this story to see the prime time, certainly for a high-profile journal.

MAJOR CONCERNS
1. One key problem with this study is that, while it presents some evidence that an intramolecular disulfide bond is formed within the CSS motif, the evidence is indirect. What is absent is a demonstration and quantification of the disulfide- versus non-disulfide CSS motifs under 'normal' and 'reducing' conditions. The reason why this is critical is because several alternative explanations

exist, e.g. mixed disulfides with proteins that interact with the CSS motif in the periplasm.

2. Physiological assays suggest that the proteolytically processed (truncated) form of PdeC has higher PDE activity than the full-length form. However, this argument is not supported by protein quantification that needs to show that the full-length and truncated forms are present in the same concentrations in cells. What would make the authors' argument even more convincing is their comparing PDE activities of the full-length and truncated proteins in vitro.

3. Another major concern is that the study lacks convincing physiological role of PdeC. (i) All data related to physiology are based on protein overexpression. PdeC deletion makes very little effect. (ii) Since reducing conditions are presented as the signal to activate PdeC, tests of the pdeC mutant need to be run in the presence and absence of reducing agents.
What is troublesome is that the proposed model of increased PDE activity in E. coli under reducing conditions directly contradicts known physiological responses of this bacterium. One involves activation of the diguanylate cyclase (DGC) YfiN via the reduction of its periplasmic partner, YfiR (Hufnagel et al., 2014, J Bacteriol 196:3690). The activated DGC, YfiN, induces curli and cellulose production, which is opposite of the model proposed in this study. The second example involves a pair of oxygen sensing DosC (DGC)-DosP (PDE) enzymes (Tuckerman et al., 2009, Biochemistry 48:9764). These heme-containing enzymes also respond to reducing conditions by increasing c-di-GMP levels and inducing biofilm components. It is possible that each system is unique to some specific media, temperature or other conditions. It is also possible that the authors' predictions here are wrong and that the target for PdeC is different than biofilms. (Perhaps motility?) The contradiction with known physiological responses of E. coli to reducing conditions requires an explanation or further investigation.

4. The last point of concern is a grossly inadequate referencing style of this manuscript.
- The authors neglect to reference in the main text highly relevant sets of papers highlighted in issue #3 that deal with the effects of reducing conditions on E. coli biofilms.
- Further, most references in Introduction are biased toward the senior author's group. There is nothing wrong with self-citations where appropriate, yet this manuscript clearly does so in an unjustifiable way. A reader unfamiliar with the field would be left with an impression that the senior author's laboratory has done all the discovery and mechanistic work on curli and cellulose components in E. coli, as well as their regulation.
- A few additional examples below illustrate negligence in referencing.
143: "...replaced the functionally important EAL motif by AAL, which eliminates PDE activity of EAL domain proteins (Lindenberg et al., 2013)." The EAL-AAL mutations have been known well before Lindenberg et al., 2013.
275: "Structure-function analyses have indicated that PDEs are active as dimers (Robert-Paganin et al., 2012; Sundriyal et al., 2014)". Structure-function analyses have shown PDE dimers well before 2012.
Supplement: "were spotted on salt-free LB or YESCA agar plates (the latter containing casamino acids instead of tryptone as in LB) (Hufnagel et al., 2014)". What is the relevance of this reference; has it established salt-free LB or YESCA agar to measure curli?

ADDITIONAL POINTS
- Replace PdeC(ASS) with PdeC(C106A)
- 138 & elsewhere: sigma70 is more than adequate; no need to talk about vegetative sigma in E. coli
- 149: "These effects were due to enhanced c-di-GMP PDE activity" or due to increased protein abundance.
- 161: same comment as above (line 149)
- 164-5: This has not been established; only PdeC overexpression affect biofilms
- 195: Spell out what "no such effect" means
- 201: "PdeC is highly active when its periplasmic CSS domain is in the free thiol state". No evidence is presented that PdeC contains a reduced thiol in the periplasm for any significant period of time, e.g. it may exist as a disulfide-linked homodimer.
- 214: 2 kD
- 216: "A series of single amino acid exchanges were introduced into this region of PdeCASS217, but none altered its processing pattern (data not shown)." Delete this statement or show the data.
-236: To be trustworthy, this statement needs verification -either via PDE activity measurements or via quantification of various PdeC forms.

- Figs. 2 & 3A: Fig. 3 shows presumed PdeC dimers formed due to the disulfide bonds via the Cys residues of the CSS domain. Seeing such dimers is not uncommon if protein samples are incompletely reduced before SDS-PAGE. Have they been incompletely reduced? Do they disappear if reduced completely (if not, are they disulfides)? What is the status of disulfide dimers in Fig. 2? Show the upper part of the gel (~100 kD) in Fig. 3A to compare to Fig. 2.
- Fig. 4C: The toxicity of overexpression of the TM2-EAL construct in the BTH assay is highly problematic. If TM2-EAL protein is toxic, then it is possible that nonspecific toxicity, instead of higher PDE activity, is responsible for the observed phenotypes. Therefore, showing that TM2-EAL dimerize in the "wild-type" background is essential. The simplest solution is to change the multicopy BTH vector to a low-copy vector.

1st Revision - authors' response 11 December 2017

EMBOJ-2017-97825
Herbst et al.: Transmembrane redox control and proteolysis of PdeC, a novel type of c-di-GMP phosphodiesterase

Response to Reviewers

In response to the reviewers´ comments, we performed a substantial number of new experiments and the manuscript and supplementary information were extensively and thoroughly worked over. The new experiments led to three additional new figures and in short are the following:

• PdeC expressed at **physiological levels** (from its chromosomal gene, labeled with a C-terminal Flag tag) is now shown to show the same response to DTT, redox control by DsbA and proteolysis by DegP/DegQ (new Fig. 5A).
• The same is now shown for a **second CSS domain PDE (PdeB)** and also for PdeB, the non-DSB variant PdeBASS is demonstrated to be more active than PdeBwt (new Fig. 5A and B).
• We **purified detergent-solubilized PdeC and reconstituted it into nanodiscs** (in the absence of reducing agent). Reconstituted PdeC not only showed PDE activity in vitro, but above all the addition of **DTT stimulated PDE activity**. Since PdeCwt also contains three cytoplasmic cysteine residues (in the EAL domain), which may possibly form illegitimate DSBs during preparation that are likely to interfere with PDE activity, we also generated a PdeC variant with these 3 cytoplasmic cysteines replaced by serines as well as a chimeric protein consisting of the TM1+CSS+TM2 region of PdeC fused to the naturally cysteinefree EAL domain of PdeN (which is one of the other four CSS domain PDEs). When purified and nanodisc-reconstituted, also these two constructs show higher PDE activity in the presence of DTT. All this (as well as in vivo activity in macrocolonies of these constructs) is now shown in the new Fig. 6.
• Finally, we show a **physiological activity of PdeC** (chromosomally encoded; shown in comparison to mutants lacking PdeC or all five CSS domain PDEs) directly *in situ* in biofilms: PdeC and additional CSS domain PDEs are involved in controlling the production and supracellular architecture of the extracellular matrix specifically in the deeper layers of mature macrocolony biofilms (new Fig. 7). This finding and its relationship to the steep oxygen gradient found in this type of thick biofilm is now discussed in detail in a new section on the physiological function of PdeC and CSS domain PDEs in the Discussion.

Furthermore, we have dealt with all comments made by the referees – thank you for their constructive comments and the significant amount of work they have put into this!

Here are the responses to all comments in detail:

Referee #1:

General comment

The "CSS-motif" domain entry in the Pfam database has been created several years ago but until now the functions of this domain remained unknown. This protein domain is found in more than 5,000 proteins in UniProt, almost always in association with the c-di-GMP-specific phosphodiesterase (EAL) domain. Both E. coli and Salmonella Typhimurium genomes carry five such genes, which indicates that these proteins must perform some crucial cellular function. The reviewed manuscript finally sheds light on the functions of these proteins and their CSS domains. This is a well-written paper presenting a very impressive work which uncovers the mechanism that allows regulation of biofim formation by the redox status and proteolytic activity in the cell periplasm. That said, this manuscript could benefit from clarification of the following issues.

1. Although the transmembrane segment TM1 of PdeC does not look like a signal peptide, has it been shown experimentally, e.g. by N-terminal sequencing or mass-spectrometry, that it does not get cleaved? In other words, how do we know that the CSS domain is anchored in the membrane from both sides and not just by TM2?

A: The following evidence argues against cleavage of TM1:
• TM1 does not end with a signal peptidase cleavage site (A/GxA);
• PdeCΔperi (= TM1+TM2+EAL) is clearly of larger size than TM2+EAL; this construct actually contains the 9 amino acids just following TM1 (K34 to R43) as a small periplasmic loop that allows TM1 and TM2 to adopt their natural positions and functions; if there were a signal peptide cleavage site at the end of TM1, it would be intact;
• we now also show that PdeC does not accumulate as a larger precursor protein in a *secA(ts)* mutant at non-permissive temperature (see new Fig. S8)

2. The 33-kD proteolytic fragments on Fig. 2 and Fig. 3 form pretty tight bands, rather than smudges often produced by non-specific proteolysis. If, as these gels suggest, proteolysis occurs at a specific point of the PdeC sequence, it would be helpful to define this point (again by N-terminal sequencing or mass-spectrometry), rather than just put it somewhere around amino acid 215 (lines 215-216 of the text).

A: DegP and DegQ are processive proteases, i.e. they recognize unfolded regions of substrate proteins and then processively degrade from this initial recognition site (Clausen et al, 2011). The point in the sequence of PdeC where they stop is determined by the fact that PdeC enters the membrane with TM2 and DegP/DegQ cannot 'pull' it out of the membrane (as periplasmic proteases, they do not have an ATPase function). That this stop is at a certain distance upstream from the beginning of TM2 reflects the geometry of DegP and DegQ, which have relatively large quaternary structures with the catalytic/proteolytic site inside the protease complexes. In other words, the position in PdeC where the two proteases stop to degrade is not a specific recognition/cleavage site but it is at a certain distance from the beginning of TM2. All this is discussed on p. 8, top paragraph.

3. This work deals with the redox regulation of PdeC during the transition to the stationary phase in aerobic conditions. The most physiologically relevant for E.coli would be transition between aerobiosis and anaerobiosis (as in the human gut). It would be important to show that the transition from the oxidized form of PdeC to the reduced form, achieved by the DTT exposure in Fig. 2B, could also occur in vivo, during the switch from an aerobic to anaerobic environment.

A: We now show that PdeC and additional CSS domain PDEs (expressed from their genes on the chromosome) are active in vivo in the *deeper* layers of mature macrocolony biofilms, where they contribute to determine production and supracellular architecture of the extracellular matrix (see new Fig. 7). Since these biofilms are characterized by steep oxygen gradients (Dietrich et al., 2013), this indeed suggests that the redox state and activity of PdeC and other CSS domain PDEs change when cells transit from aerobic to microaerobic/anaerobic conditions (for a more detailed discussion, see p. 17).

4. The data presented on Fig. 4 and S4 show that the TM2-EAL construct localizes to the membrane fraction and is enzymatically active. Clearly, this fragment adopts the correct orientation with respect to the membrane, which allows dimerization of the EAL domain. However, calling TM2 a dedicated dimerization domain is probably a stretch. Its sequence does not contain any residues that would make TM2 likely to form stable dimeric patches. Further, there could be any number of reasons why replacement of TM2 with TM2 did not work, including the possibility that TM2* simply did not properly insert into the membrane. It would be a good idea to soften the language on lines 241 and 658.*

A: We agree that the finding that the presence of TM2 promotes dimerisation formally does not prove that it is a 'dimerisation domain' per se. Alternatively, it may affect the structure of the EAL domain in a manner that allows the EAL domain to dimerize. Membrane association alone does not seem to promote dimerisation, since the PdeCTM2* construct did properly localize to the membrane fraction. To reflect this, we rephrased from TM2 being 'a dimerisation domain' to 'promotes dimerisation of PdeC'.

5. A very attractive feature of the presented model (Fig. 5) is the inherent temporary response of the CSS-EAL system, which is only active until either the CSS domain gets oxidized or the EAL domain gets proteolytically cleaved from the membrane. This might be worth a brief discussion.

A: This is now discussed more clearly both in the first part (p. 14) as well as on p. 16 by pointing out that PdeC has several 'options': (i) being oxidized by DsbA/DsbB, (ii) staying reduced/more active and/or (iii) being processed by DegP/DegQ); the latter activates in a non-reversible manner, which therefore calls for further proteolysis as a terminal inactivation mechanism. Which of these options 'wins' or dominates, depends mainly on the activity of the DsbA/DsbB system, which in turn is affected by oxygen as a terminal electron acceptor. An interesting side observation of the new temperature shift experiment with wildtype and *secA(ts)* strains (Fig. S8) is that at non-permissive temperature (42oC) processing of PdeCwt is stimulated, which is likely to reflect increased levels of DegP (which is a heat shock protein) as well as increased activity of DegP as a protease (DegP acts both as a chaperone and as a protease, with the latter activity being stimulated at higher temperatures; see Krojer et al, 2002).

6. The description of Fig. 1 panels B and C is presented in the text but missing in the Figure legend.
A: Sorry, we had overlooked this and it is of course included now.

Minor comments.
- Line 94. After "proteins", add "respectively".
A: Done.

- Line 143. Change "replaced the functionally important EAL motif by AAL" to "replaced the active site glutamate of the EAL motif by alanine".
A: Done.

- Line 150. Change "AAL mutation" to "EAL to AAL mutation"".
A: Done.

- Line 156. Replace "determining" by "monitoring two c-di-GMP-dependent functions:"
A: Done.

- Line 161. Mentioning "the potential to form a DSB" looks a bit premature here, until the presentation of the DTT data. You might want to say that "presence of both Cys75 and Cys106 of the CSS domain results in a lower PDE activity of PdeC, whereas the replacement of one or both of them promotes PdeC activity. This suggested a possible link to the potential of these Cys residues to form a DSB".
A: Done.

- Lines 189-190, 202, and elsewhere in the text. "cys75 and cys106" should be "Cys75 and Cys106"
A: Done.

- Fig. S3. The UniProt entry for PdeC http://www.uniprot.org/uniprot/P32701 lists slightly shorter transmembrane segments (14-34) and (242-262), which is consistent with the alignment on Fig. S1.
A: The algorithm used by UniProt seems very stringent. Amino acids 8-13 are QLLALP, which I think qualifies as hydrophobic. Amino acids 235-241 are LITHFYN, which is of intermediate hydrophobicity. For our TM2+EAL constructs we just decided to leave these amino acids in the construct in order to be on the safe side and not to cut away too much.

- Fig. 5. The presented scheme looks very nice but the positions of the cysteines give the impression that the distance between them (which is 30 aa) is equal or greater than the distance from Cys105 to the TM2 (which is actually 120 aa).
A: This is not drawn to scale, but we agree and have changed it in the figure.

Referee #2:

In bacteria, biofilm formation is promoted by the second messenger c-di-GMP. This molecule is synthesized by diguanylate cyclases and degraded by specific phosphodiesterases. The cellular levels of c-di-GMP depend on the balance between these two classes of enzymes. In this manuscript, Herbst et al investigate the physiological function of a group of five phosphodiesterases expressed by E. coli. These enzymes are characterized by the presence of a periplasmic domain in which two conserved cysteine residues are present. Focusing on one of the five phosphodiesterases, PdeC, the authors demonstrate that formation of a disulfide bond between these two cysteines reduces PdeC phosphodiesterase activity. By contrast, preventing disulfide bond formation by deleting DsbA or DsbD, or reducing the disulfide by adding DTT, activates the enzyme. The authors also show that reduction of the disulfide induces the enzymatic degradation of the periplasmic portion of PdeC by the proteases DegP and DegQ and that the second TM domain of PdeC acts as a dimerization domain promoting phosphiesterase activity. On the basis of these results, they propose an elegant mechanism in which the phosphodiesterase activity of PdeC is controlled through redox regulation and dimerization. Overall this paper is interesting and the data nicely support the proposed mechanism. The detailed mechanistic study is quite impressive and informative.
The conservation of two cysteine residues that form a disulfide in a protein does not necessarily indicate that this protein is redox-regulated. This disulfide might only be required for structural stability.

A: We agree that DSBs can have either a structural or a regulatory role (Wouters et al. 2010). The former is the case for many periplasmic proteins, for which DSB formation is required to fold into the native and active conformation. However, in the case of PdeC (and now also shown for PdeB), it is the reduced form that is more active, which already argues against a structural role of DSB formation.

My major concern is that the authors fail
to provide data supporting the physiological relevance of their findings. In the discussion, the authors suggest that decreased oxygen concentrations may favor reduction (absence of formation) of the PdeC disulfide and therefore activate the enzyme. Maybe. But, as the authors mention, the DsbA/DsbB system has been shown to function anaerobically, so this might not be the case. I think that some experimental evidence that oxygen limitation is the physiological signal activating PdeC activity is required for publication in a journal like EMBO.

A: While this study originally focused on the redox control of PdeC as an example of a new class of signaling proteins, i.e. the CSS domain PDEs (and this functional aspect of

the *protein* is now further elaborated by also showing redox control of the purified protein reconstituted into nanodiscs; see new Fig. 6), we now also provide evidence for the physiological function of PdeC and other CSS domain PDEs: these c-di-GMP signaling enzymes are involved in controlling the production and supracellular architecture of the extracellular matrix in the deeper, oxygen-poor layers of mature macrocolony biofilms (see new in-situ data now shown as Fig. 7; see also comment to Reviewer #1 above). Macrocolony biofilms exhibit steep oxygen gradients (Dietrich et al, 2013) and microaerobic conditions in the deeper layers should slow down the activity of the DsbA/DsbB system (there are also no alternative electron acceptors for anaerobic respiration under the growth conditions in our experiments). Overall, this physiological function of PdeC and other CSS domain PDEs is really exciting since it shows that the elaborate matrix architecture in this type of biofilm is the result of a complex regulatory interplay of many DGCs and PDEs that differentially respond to nutritional cues (which mainly affect the σ70/σS dependency of expression of many DGCs and PDEs as previously summarized by Serra & Hengge, 2014) and the oxygen gradient (affecting redox controlled CSS domain PDEs).

Minor comments:
-E.coli has 4 additional enzymes like PdeC. In this study, they have only tested one. It would be interesting to test the importance of disulfide bond formation on the activity for at least one of the other enzymes (using the macrocolony morphology assay for example).
A: We now show DsbA/redox control of activity and DegP/DegQ-dependent proteolysis also for a second CSS domain PDE, PdeB, which behaves just as PdeC (new Fig. 5).

-The authors could comment more on the interest for E. coli to produce fragments by proteolysis if the full size protein is capable of PDE activity
A: Proteolysis by DegP/DegQ may be seen as a by-product of partial unfolding of the CSS domain that comes along with opening of the DSB; however, it is also a way of achieving slow turnover of PdeC and other CSS domain PDEs. Notably, 4 of the 5 CSS domain PDEs are under σS control, i.e. they are mainly expressed in slowly growing and/or stationary phase cells, in which proteins are no longer subject to divisionassociated dilution. Thus, in stationary phase the only way to eventually reduce or even get rid of a cellular protein is by proteolysis.

-line 174 : a a periplasmic DSB
A: Done.

-line 235 : Fig 3B instead of 4B
A: Done.

-line 377 : limitation instead of limitaton
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Referee #3:

Herbst et al. present interesting set of observations suggesting involvement of the periplasmic CSS motif (protein domain) in regulating E. coli c-di-GMP phosphodiesterase (PDE), PdeC. Their study offers genetic evidence (using mutants in the periplasmic disulfide bond formation apparatus) that a disulfide bond is formed between two highly conserved Cys residues in the CSS motif. It further suggests that addition of a strong reducing agent results in the proteolysis of the CSS motif via the periplasmic proteases DegP and DegQ. Proteolysis also takes place when the Cys residues are mutated. Interestingly, a proteolytically processed (truncated) version that lacks the CSS motif but retains a single transmembrane motif (TM2), which is involved in protein dimerization, is catalytically active, and potentially more active than the native PdeC protein.
Overall, this is an interesting story that, however, has some large experimental gaps. Further, it presents a conclusion about the physiological

significance of PdeC regulation that contradicts earlier literature reports. Filling the gaps is critical for this story to see the prime time, certainly for a high-profile journal.

MAJOR CONCERNS

1. One key problem with this study is that, while it presents some evidence that an intramolecular disulfide bond is formed within the CSS motif, the evidence is indirect. What is absent is a demonstration and quantification of the disulfideversus non-disulfide CSS motifs under 'normal' and 'reducing' conditions. The reason why this is critical is because several alternative explanations exist, e.g. mixed disulfides with proteins that interact with the CSS motif in the periplasm.

A: In all our SDS-PAGE/Western blot experiments, there is no DTT or any other reducing agent in the samples. Any mixed disulfides of PdeC with other proteins should therefore become visible as bands of larger size. However, we did not detect any such bands with PdeCwt. Only when one of the two cysteines in the CSS domain was eliminated (in PdeCC75A or PdeCASS), a larger band became detectable (Fig. S5). This bands corresponds in size to a PdeC dimer and again completely disappeared when also the second cysteine was eliminated (in PdeCC75A/ASS), i.e. these bands correspond to a DSB-linked homodimer of PdeCC75A or PdeCASS, respectively. That no such homodimer is observed with PdeCwt also indicates that the formation of the *intra*molecular DSB is favoured (most likely kinetically) over the formation of the *inter*molecular DSB required to generate this dimer (or any mixed disulfide with other proteins).

2. Physiological assays suggest that the proteolytically processed (truncated) form of PdeC has higher PDE activity than the full-length form. However, this argument is not supported by protein quantification that needs to show that the full-length and truncated forms are present in the same concentrations in cells. What would make the authors' argument even more convincing is their comparing PDE activities of the full-length and truncated proteins in vitro.

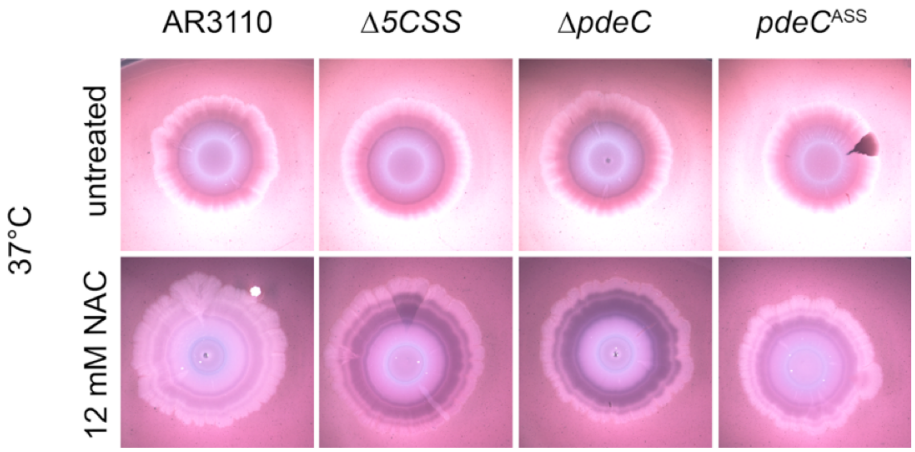
A: We do not intend to claim that the proteolytically processed form of PdeC has higher activity than the non-DSB form of full size PdeC. That the TM2+EAL construct shows higher activity in vivo than PdeCASS is indeed most likely due to the higher expression of the former (a simple consequence of generating TM2+EAL as well as EAL alone, since these constructs needed new Shine-Dalgarno/start codon configurations and we also paid attention to generate stable constructs according to the N-end rule).
The point we actually want to make is that PdeC activity depends on the redox state of its CSS domain. Besides providing genetic evidence, we now also show this with the purified protein reconstituted in nanodiscs (new Fig. 6).

A: We now also show similar effects of DTT and knockout mutations in *dsbA*/*degP*/*degQ* for single copy, i.e. chromosomally encoded PdeC (as well as for chromosomally encoded PdeB; see new Fig. 5). In addition we show that *pdeC* deletion as well as deletion of all five genes encoding CSS domain PDEs affect matrix production and architecture specifically in deeper layers in mature macrocolony biofilms (new Fig. 7; see also comments to both other Reviewers above)

(ii) Since reducing conditions are presented as the signal to activate PdeC, tests of the pdeC mutant need to be run in the presence and absence of reducing agents.

A: Physiological activity in wildtype compared to *pdeC* mutant strains requires growth of macrocolonies which takes time (at least 3d; usually we let them grow for 5 d to reach the fully developed morphology). We have grown macrocolonies on agar plates also containing DTT, but DTT is too unstable under these aerobic growth conditions and did not make a difference. However, we also tested the reducing agent N-acetyl-cysteine (NAC; this is actually given to cystic fibrosis patients to apparently reduce biofilm formation of pathogens colonizing the CF lung). NAC seems more stable and we

observed effects with macrocolonies grown at 37oC, where biofilm formation is reduced (no more wrinkles are formed since matrix production is lower than at 28oC, but the residual matrix still stains with Congo red): in the presence of NAC, the wildtype strain AR3110 (as well as the strain that chromosomally expresses the highly active/nonredox-regulated PdeCASS variant) shows clearly less CR staining than the strains that lack PdeC or all five CSS domain PDEs indicating that NAC activates PdeCwt.



A: As discussed now on p. 18, we don´t think that this is not a contradiction but may reflect an interesting physiological relationship. c-di-GMP levels are generally 'negotiated' by both DGCs and PDEs being present at certain levels at the same time. Thus, the equally membrane-associated YfiN (now DgcN) may actually be the antagonistic DGC partner for PdeC (or CSS domain PDEs in general). In addition, the control of expression of DgcN is different from CSS domain PDEs: YfiN/DgcN is under σ70 control, i.e. it is present in rapidly growing as well as in stationary phase cells, whereas 4/5 CSS domain PDEs (all except PdeN) are under σS control, i.e. accumulate in stationary phase cells (see Sarenko et al, 2017). As a result, a dominance of DgcN (over CSS domain PDEs) in growing cells could be a means to allow these cells to generate a biofilm matrix (in particular cellulose) when exposed to reducing stress – while in stationary phase cells exposed to reducing stress, YfiN/DgcN activity would be neutralized by CSS domain PDEs, with matrix production taking place anyway and being dependent on the σS/CsgD/DgcC pathway (with DgcC activating cellulose synthesis specifically). This is consistent with the study by Hufnagel et al. (2014) which showed that matrix synthesis when driven by YfiN/DgcN is CsgD-independent. In any case, we´ll study this interesting functions of YfiN/DgcN and its antagonistic relationship to CSS domain PDEs further, but this is beyond the scope of the present study. Furthermore, we have also tested *dosC* (dgcO) and *dosP* (*pdeO*) mutants for any alterations in the extracellular matrix in vertical cryosections of macrocolony biofilms. Unlike for the mutants with alterations in CSS domain PDEs, we did not observe any differences in matrix production and architecture, which indicates that this antagonistic oxygen-regulated DGC/PDE pair does not play a role in matrix formation in this type of biofilm. This suggests that DosC/DosP are either not expressed (although we expect them to be expressed, since they are induced in stationary phase in a strictly σSdependent manner; Sommerfeldt et al, 2009 & Sarenko et al. 2017) or that they may actually control some other c-di-GMP-regulated function (probably in conjunction with the degradosome to which they localize according to Tuckerman et al, 2011).

4. The last point of concern is a grossly inadequate referencing style of this manuscript.
- The authors neglect to reference in the main text highly relevant sets of papers highlighted in issue #3 that deal with the effects of reducing conditions on E. coli biofilms.
A: We cite the above mentioned papers on YfiN and DosC/DosP. We don´t know what 'issue #3' is supposed to be.

- Further, most references in Introduction are biased toward the senior author's group. There is nothing wrong with self-citations where appropriate, yet this manuscript clearly does so in an unjustifiable way. A reader unfamiliar with the field would be left with an impression that the senior author's laboratory has done all the discovery and mechanistic work on curli and cellulose components in E. coli, as well as their regulation.
A: We did not mean to generate this impression – sorry for this. We hope to have this correcting by now citing additional studies.

- A few additional examples below illustrate negligence in referencing.

143: "...replaced the functionally important EAL motif by AAL, which eliminates PDE activity of EAL domain proteins (Lindenberg et al., 2013)." The EAL-AAL mutations have been known well before Lindenberg et al., 2013.
A: We now also cite Schmidt et al. (2005) and Tchigvintsev et al. (2010) at this position (on the first page of Results)

275: "Structure-function analyses have indicated that PDEs are active as dimers (Robert-Paganin et al., 2012; Sundriyal et al., 2014)". Structure-function analyses have shown PDE dimers well before 2012.
A: We now also cite Barends et al., Minasov et al. and Rao et al. (all 2009).

ADDITIONAL POINTS
- Replace PdeC(ASS) with PdeC(C106A)
A: We decided to continue to use PdeCASS as we also use PdeCAAL because these designations (which are explained when introduced) are more readily intuively understood than just numbered aminoacids since they refer to the relevant CSS and EAL motifs.

- 138 & elsewhere: sigma70 is more than adequate; no need to talk about vegetative sigma in E. coli
A: Done.

- 149: "These effects were due to enhanced c-di-GMP PDE activity" or due to increased protein abundance.
A: the point was not PDE activity vs. abundance, but PDE activity vs. some other effects acting on matrix production. To make this clear, we rephrased the text.

- 161: same comment as above (line 149)
A: Done as above.

- 164-5: This has not been established; only PdeC overexpression affect biofilms
A: We now show data for PdeC overexpression as well as for physiological expression levels (wildtype vs. knockout mutants; see new Fig. 7)

- 195: Spell out what "no such effect" means
A: Done.

- 201: "PdeC is highly active when its periplasmic CSS domain is in the free thiol state". No evidence is presented that PdeC contains a reduced thiol in the periplasm for any significant period of time, e.g. it may exist as a disulfide-linked homodimer.
A: This relates to comment #1 again. We would have seen DSB-linked homodimers, since our SDS-PAGE buffer did not contain DTT or any other reducing agents (in fact we see them for PdeC variants that have only one cysteine residue in their CSS domains; see reply to comment #1 above).

- 214: 2 kD
A: Changed throughout the manuscript.

- 216: "A series of single amino acid exchanges were introduced into this region of PdeCASS217, but none altered its processing pattern (data not shown)." Delete this statement or show the data.
A: now shown as Fig. S6.

-236: To be trustworthy, this statement needs verification -either via PDE activity measurements or via quantification of various PdeC forms.
A: This repeats comment #2; see reply above.

- Figs. 2 & 3A: Fig. 3 shows presumed PdeC dimers formed due to the disulfide bonds via the Cys residues of the CSS domain. Seeing such dimers is not uncommon if protein samples are incompletely reduced before SDS-PAGE. Have they been incompletely reduced? Do they disappear if reduced completely (if not, are they disulfides)? What is the status of disulfide dimers in Fig. 2? Show the upper part of the gel (~100 kD) in Fig. 3A to compare to Fig. 2.
A: As already stated several times above, our SDS-PAGE samples are not reduced (no DTT or any other reducing agent present), exactly because we wanted to see putative DSB-linked homodimers or mixed disulfides, if present. The only instance where such larger bands (dimers) were detected, was with the PdeC variants that have only one Cys in their CSS domains. These bands did disappear when DTT is added to the sample buffer. Disulfide dimers were also seen on the gel shown in Fig. 2. However, since this is a side aspect of this experiment that occurs only with the one-Cys mutant versions of PdeC, these dimers are mentioned somewhat later and we therefore prefer to show them seperately in Fig. S5.

- Fig. 4C: The toxicity of overexpression of the TM2-EAL construct in the BTH assay is highly problematic. If TM2-EAL protein is toxic, then it is possible that nonspecific toxicity, instead of higher PDE activity, is responsible for the observed phenotypes. Therefore, showing that TM2-EAL dimerize in the "wild-type" background is essential. The simplest solution is to change the multicopy BTH vector to a low-copy vector.
A: We did not mean to say that this toxicity of TM2+EAL overexpression from both vectors is something 'specific' – it may be unspecific in the sense that too much of TM2+EAL (which also has a highly efficient translational start region; see comment above) in the membrane is just harmful for the cell. However, while the standard protocol for this 2H system involves induction of hybrid proteins with IPTG, we now also tried without IPTG addition and it worked just fine at basal expression levels – which also indicates that these interactions are quite specific – and nothing was toxic anymore. Thus, Figs. 4C and D were replaced by new interaction panels obtained without IPTG induction.

2nd Editorial Decision 9 January 2018

Thank you for submitting a revised version of your manuscript. The manuscript has now been seen by all original referees, who find that their main concerns have been addressed. There remain only a few minor editorial issues that have to be dealt with before formal acceptance of the manuscript.

1. Please implement the textual changes requested by reviewer #1.
2. Figure 1A and Table S1 are not referred to in the text.
3. We generally encourage the publication of source data for electrophoretic gels and blots, with the aim of making primary data more accessible and transparent to the reader. We would need one file per figure (which can be a composite of source data from several panels) in jpg, gif or PDF format, uploaded as "Source data files". The gels should be labeled with the appropriate figure/panel number, and should have molecular weight markers; further annotation would clearly be useful but is not essential. These files will be published online with the article as supplementary "Source Data". Please let me know if you have any questions about this policy.
4. Finally, papers published in The EMBO Journal include a 'Synopsis' to further enhance discoverability. Synopses are displayed on the html version of the paper and are freely accessible to all readers. The synopsis includes a short paragraph - written by the handling editor - as well as 2-5

one-sentence bullet points that summarise the paper and are provided by the authors. I would therefore ask you to include your suggestions for bullet points. Please also send us a synopsis image. This image should provide a rapid overview of the question addressed in the study, but still needs to be kept fairly modest, since the image size cannot exceed 550x400 pixels.

Please let me know if you have any further questions regarding any of these points. You can use the link below to upload the revised version.

Thank you again for giving us the chance to consider your manuscript for The EMBO Journal. I am looking forward to receiving the final version.

REFEREE REPORTS

Referee #1:

In the revised version of the manuscript by Herbst et al., all my concerns have been addressed. In my opinion, the manuscript is now suitable for publication but would need some copy-editing, as described below.

Minor suggestions.
L. 36. Remove 'i.e.'
L. 39, 110, 245, 285, 294, 303, 305, 314, etc. 'dimerization' is spelled in this text in both 'z' and 's' versions, often on the adjacent lines (ll. 303 and 305). Please use either British or American spelling throughout the text.
L. 49. Remove 'i.e.'
L. 52. Change 'tolerant against antibiotics and evade' to 'more tolerant against antibiotics and better evade'
L. 143. Change 'CSS into ASS' to 'the CSS sequence motif into ASS'
L. 219. Change 'ser231 after a starting methionine' to 'Ser231'
L. 221. Change 'aminoacid exchanges' to 'amino acid changes'
L. 321. Change 'occurs' to 'occur'
L. 436. Change 'terminal acceptor to deliver electrons' to 'terminal electron acceptor'
L. 442. Change 'the state of activity of' either to 'the state of' or 'the activity of'
L. 450. Remove 'i.e.'
L. 453. Change 'shown' to e.g. 'manifested'
L. 467. Change 'C-terminal periplasmic DSB-forming domains' to 'C-terminal DSB-forming domains in the periplasm'
L. 534. Change 'primordially' to 'initially aimed at'
L. 550. Change 'respirative' to 'respiratory'
L. 560. Either remove 'i.e.' or change it to 'namely'
L. 562. Same as l. 442
L. 604. Change 'these' to 'this'
L. 890. Change 'leucin' to 'leucine'

Referee #2:

I think that the authors have done an excellent work in addressing the many comments that were raised. I find this version much improved compared to the first one. This paper will be interesting to a large audience.

Referee #3:

This is a significantly improved revision in regard to the strength of evidence for redox regulation of the phosphodiesterase activity, physiological role of PdeC and reference style. My concerns about the original version have been adequately addressed.

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Regine Hengge
Journal Submitted to: EMBO Journal
Manuscript Number: EMBOJ-2017-97825

Reporting Checklist For Life Sciences Articles (Rev. June 2017)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	As good practice for the type of experiments performed and hroughout the study, all experiments were done in three complete biological replicates, except for the in-situ analysis of bacterial biofilms (Fig. 7), which was done in two complete biological replicates because this type of experiment requires a lot of time and effort. See also Statistics statement in the M&M section.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	NA
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	NA
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	NA
For animal studies, include a statement about randomization even if no randomization was used.	NA
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	NA
4.b. For animal studies, include a statement about blinding even if no blinding was done	NA
5. For every figure, are statistical tests justified as appropriate?	see 1a
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	NA
Is there an estimate of variation within each group of data?	NA
Is the variance similar between the groups that are being statistically compared?	NA (there is no statistical comparison of groups)

C- Reagents

USEFUL LINKS FOR COMPLETING THIS FORM

http://www.antibodypedia.com
http://1degreebio.org
http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-repo

http://grants.nih.gov/grants/olaw/olaw.htm
http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals/index.htm
http://ClinicalTrials.gov
http://www.consort-statement.org
http://www.consort-statement.org/checklists/view/32-consort/66-title

http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tun

http://datadryad.org

http://figshare.com

http://www.ncbi.nlm.nih.gov/gap

http://www.ebi.ac.uk/ega

http://biomodels.net/

http://biomodels.net/miriam/
http://jjj.biochem.sun.ac.za
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html
http://www.selectagents.gov/

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	Origin of all antibodies (commercial or custom-made) used is given in the Supplementary M&M in the Appendix
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	NA

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	NA
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	NA
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	NA

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	NA
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	NA
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	NA
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
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F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	Our study does not include data of the types a-e. All our figures show original data (either in the main manuscript or the Appendix/Supplement).
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	All relevant original data that could not be shown in the main manuscript, are shown in the Appendix/Supplement.
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	NA
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	NA

G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	NA