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The second messenger signaling molecule cyclic di-AMP drives developmental cycle progression in *Chlamydia trachomatis*

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eLife Assessment

In this **useful** study, ectopic expression and knockdown strategies were used to assess the effects of increasing and decreasing Cyclic di-AMP on the developmental cycle in Chlamydia. The authors **convincingly** demonstrate that overexpression of the dacA-ybbR operon results in increased production of c-di-AMP and early expression of the transitionary gene hctA and late gene omcB. Whilst the authors have attempted to revise the submission, the model proposed in the revised manuscript is still not fully supported by the data presented.

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Abstract

The obligate intracellular bacterium *Chlamydia* alternates between two functional forms during its developmental cycle: elementary body (EB) and reticulate body (RB). However, the molecular mechanisms governing the transitions between these forms are unknown. Here, we present evidence cyclic di-AMP (c-di-AMP) is a key factor in triggering the transition from RB to EB (i.e., secondary differentiation) in the chlamydial developmental cycle. By overexpressing or knocking down expression of c-di-AMP synthase genes, we made strains producing different levels of c-di-AMP, which we linked to changes in secondary differentiation status. Increases in c-di-AMP resulted in an earlier increase in transcription of EB-associated genes, and this was further manifested in earlier production of EBs. In contrast, when c-di-AMP levels were decreased, EB production was reduced. Based on these data, we conclude there is a threshold level of c-di-AMP needed to trigger secondary differentiation in *Chlamydia*. This study identifies a mechanism by which secondary differentiation is initiated in *Chlamydia* and reveals a critical role for the second messenger signaling molecule c-di-AMP in this process.

Introduction

Chlamydia species are major pathogens of humans and animals. These obligate intracellular bacteria share one key feature: their unique developmental cycle (see ref. (1)). During this cycle, *Chlamydia* transitions between two different functional and morphological forms: the elementary body (EB), an infectious but non-dividing cell, and the reticulate body (RB), a dividing but non-infectious cell (**Fig. 1A**). A third form, the intermediate body (IB) is a transitional form from the RB to EB. Besides these characteristics, EBs and RBs differ in other ways. For example, EBs are small (~0.3 μm), have a highly disulfide-crosslinked outer membrane (2), and have DNA condensed by histone-like proteins (3). In contrast, RBs are larger (~1 μm), have a Gram-negative cell envelope that lacks peptidoglycan (4-6), and have a dispersed chromosome. RBs divide by an asymmetric polarized division mechanism dependent on MreB-directed peptidoglycan synthesis specifically at the septum (7-10). Not surprisingly, *Chlamydia* expresses genes in a temporally defined manner that corresponds broadly with its developmental cycle (11, 12). “Early” genes (e.g., *euo*) are expressed immediately upon entry into a target host cell and are likely involved in establishing the intracellular niche of *Chlamydia*, the inclusion, and mediating primary differentiation from EB to RB. “Mid” cycle genes (e.g., *mreB, clpPX*) facilitate RB replication and division and inclusion growth. “Late” genes (e.g., *hctA*, *omcB*) are expressed when secondary differentiation is initiated to trigger EB formation. Although developmental gene expression has been characterized for decades, the signals or events that initiate differentiation from one form to the other are not known.

In a 2013 study, another defining characteristic of EBs and RBs was identified: their relative levels of the second messenger signaling molecule cyclic di-AMP (c-di-AMP) (see ref. (13)). Barker et al. studied how IFNβ production is activated in cells infected with *C. trachomatis*. The authors identified a role for the innate immune response protein, STING, which recognizes c-di-AMP. *Chlamydia* encodes a diadenylate cyclase enzyme, DacA, associated with c-di-AMP production, that had not been characterized. As part of their study, the authors determined that c-di-AMP accumulates over the course of the chlamydial developmental cycle, that EBs have high levels of this molecule whereas RBs have low levels, and that DacA is a diadenylate cyclase. It is unlikely that *Chlamydia* produces c-di-AMP only to signal host immune responses. Rather, the parsimonious interpretation is that *Chlamydia* uses this signaling molecule to regulate some aspect of its physiology and that activation of host signaling is “accidental” – similar to activation of NOD2 by chlamydial peptidoglycan (14, 15). However, no direct function of c-di-AMP in chlamydial biology has been defined.

Diverse functions of c-di-AMP have been reported in Gram-positive bacteria. For example, c- di-AMP is implicated in the response to changes in osmotic pressure. When the extracellular solute level is high, bacteria prevent dehydration by importing both extracellular solute molecules and cations (16). In these mechanisms, c-di-AMP binds to proteins associated with K^+ uptake systems such as Ktr/Trk, KimA, Kup, and Kdp and inhibits their activities in *Bacillus subtilis*, *Staphylococcus aureus*, and *Lactococcus lactis* (17-20). Similarly, K^+ export mechanisms are also regulated by c-di-AMP. In *S. aureus*, the cation/proton antiporter A (CpaA) is activated by binding c-di-AMP (21). Moreover, c-di- AMP binds to the riboswitch upstream of the genes encoding the K^+ transporters and controls their transcriptional levels (22). With these mechanisms, osmotic stress is controlled by c-di-AMP. Of note, *Chlamydia* lacks annotated orthologs of K^+ transporters. In addition to osmotic homeostasis, c-di-AMP has been reported to affect DNA replication and sporulation (23). When DNA damage is detected, DisA, a diadenylate cyclase, forms a DNA repair complex with RecA and RadA, resulting in inhibition of diadenylate cyclase activity in *B. subtilis*. Subsequently, c-di-AMP levels decrease, and DNA replication and sporulation are arrested (23-26).

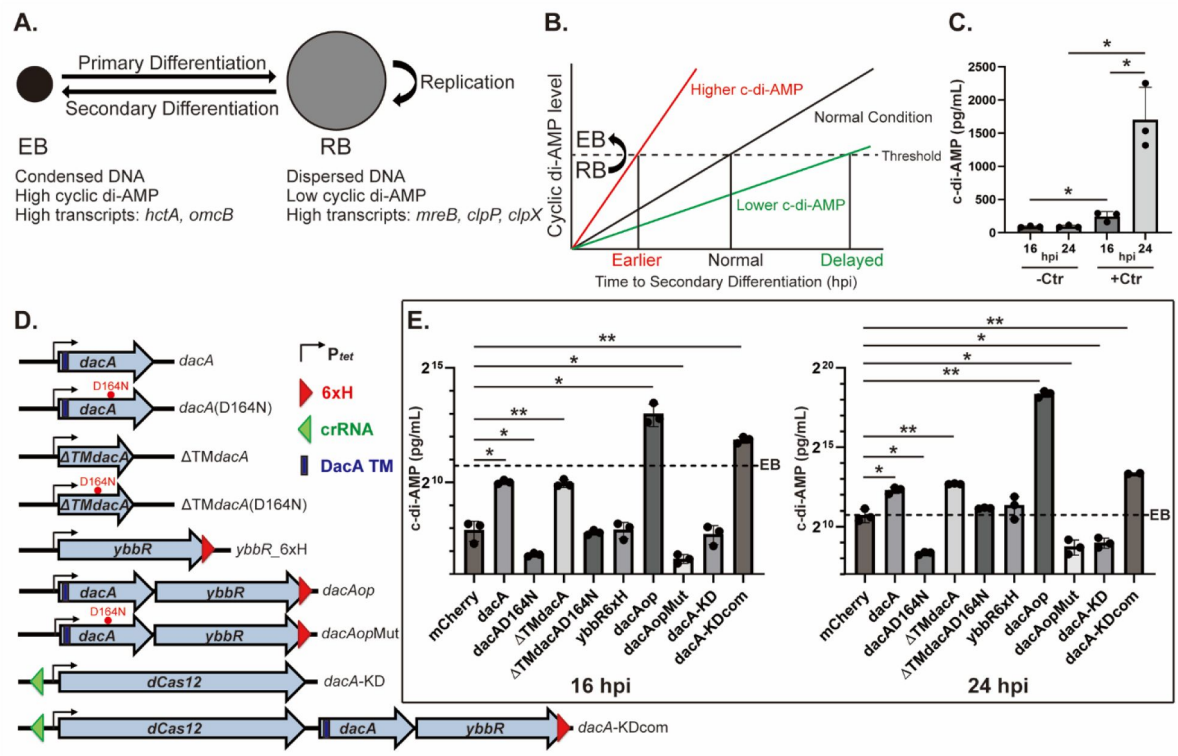

Figure 1.

Cyclic di-AMP accumulation is linked to secondary differentiation in *C. trachomatis*.

A. Defining characteristics of chlamydial EBs and RBs. **B.** A hypothetical model representing the correlation between c-di-AMP levels and the timing of secondary differentiation. The dashed line represents a threshold level of c-di-AMP needed to drive secondary differentiation in a given RB. hpi = hours post-infection. **C.** Measurement of c-di-AMP concentrations in uninfected (-Ctr) and infected (+Ctr) HeLa cell lysates. For infected HeLa cells, *C. trachomatis* serovar L2 (434/Bu) transformed with an mCherry-encoding construct was infected into HeLa cells, and expression of mCherry was induced at 10 hpi with 5 nM anhydrotetracycline (aTc). All samples were harvested at 16 and 24 hpi. **D.** A schematic diagram of the constructs used in this study. All constructs used are aTc-inducible as shown by the P_{tet} promoter. The location of the 6xH tag, the approximate location of the crRNA for the CRISPRi vectors are shown as well as the transmembrane domain of DacA. Diagram is not to scale. **E.** Measurement of c-di-AMP concentrations in infected cell lysates from the strains shown in panel D. *C. trachomatis* serovar L2 (434/Bu) transformed with the indicated constructs was infected into HeLa cells. At 10 hpi, expression of the construct was induced with 5 nM aTc, and the infected cells were harvested at 16 or 24 hpi. Levels of c-di-AMP in the supernatant were measured using ELISA. The left and right panels show the levels of c-di-AMP in the indicated strains at 16 and 24 hpi, respectively, on a log2 scale. For reference, 2^{10} = 1,024, 2^{15} = 32,768, and 2^{20} = 1,048,576. The dashed line in both graphs represents the c-di-AMP level of the 24hpi mCherry-expressing control that is associated with EB production. *: p<0.05, **: p<0.001, NS: Not significant via two sample equal variance t-test compared to the mCherry control.

Given the differences in c-di-AMP levels between EBs and RBs and its function as a diffusible second messenger signaling molecule, we hypothesized that the accumulation of c-di-AMP during the developmental cycle might be a trigger for secondary differentiation in *Chlamydia* (**Fig. 1B**). Once a threshold concentration of c-di-AMP has been reached in a given RB, it will begin the differentiation process to an EB. Importantly, we do not propose that c-di-AMP is necessarily required for RB replication or growth. We predicted that, if our hypothesis were correct, then we should be able to alter the levels of c-di-AMP in the organism and affect its differentiation kinetics accordingly. For example, if we increase c-di-AMP production, then we anticipate prematurely triggering RB-to-EB conversion with a concomitant reduction in overall growth and replication (since only RBs divide). Conversely, if we prevent c-di-AMP production, then we anticipate delaying EB production without impacting growth rate (i.e., normal replication with reduced RB-to-EB conversion).

There are three principal mechanisms to regulate c-di-AMP levels: through synthesis by diadenylate cyclase, through degradation by a phosphodiesterase (PDE), and through secretion by a transporter (27). Interestingly, *C. trachomatis* only encodes the synthesis mechanism as it possesses the genes for diadenylate cyclase (*dacA*) and its regulator (*ybbR*) within a bicistronic operon, but no annotated PDEs or c-di-AMP transporters (28). Both DacA and YbbR are predicted to contain transmembrane domains (3 for DacA and 1 for YbbR) (**Fig. S1**, (13)). To test our hypothesis, we genetically manipulated the levels of DacA and/or YbbR using recently developed strategies in the field. In the present study, we characterized the growth and developmental cycle state of chlamydial strains producing high or low levels of c-di-AMP. Our data show that higher levels of c-di-AMP are directly linked to increased transcript levels of late genes associated with secondary differentiation as well as the concomitant production of EBs at an earlier stage in the developmental cycle. In contrast, in cells with reduced c-di-AMP levels, chlamydial growth was impaired and the developmental cycle was significantly delayed. Based on these data, we conclude that there is a threshold level of c-di-AMP necessary to trigger secondary differentiation in *C. trachomatis*. This is the first study to identify a physiological function for c-di-AMP in *Chlamydia* as well as a signaling mechanism by which secondary differentiation is initiated in these unique bacteria.

Results

Cyclic di-AMP levels increase at later stages of the developmental cycle and in bacteria that overexpress both DacA and YbbR

To verify that EBs have higher levels of c-di-AMP compared to RBs, we infected HeLa cells at a multiplicity of infection (MOI) of 1 with a transformant of *C. trachomatis* L2 carrying a shuttle plasmid with inducible mCherry. This strain serves as a control for subsequent overexpression experiments, and we induced mCherry expression with 5 nM aTc at 10 hours post-infection (hpi). We collected uninfected and infected HeLa cell lysates at 16 and 24 hpi and measured c-di-AMP levels by ELISA (**Fig. 1C**). As expected, only basal amounts (<100 pg/mL) of c-di-AMP were detected in uninfected HeLa cells at either timepoint (**Fig. 1C**). In infected cells, the 16hpi timepoint is characterized by predominantly an RB population whereas the 24 hpi timepoint is characterized by ongoing secondary differentiation and a mixture of RBs, IBs, and EBs. We observed that c-di-AMP levels were significantly higher at 24hpi (~1,700 pg/mL) compared to 16hpi (~250 pg/mL) and increased approximately 7-fold over this timeframe (**Fig. 1C**). These results are in agreement with the Barker et al. study indicating higher levels of c-di-AMP in EBs (13).

To test the link between c-di-AMP levels and chlamydial developmental cycle progression, we made a collection of *C. trachomatis* strains carrying anhydrotetracycline (aTc)-inducible constructs (**Fig. 1D**). These included strains to overexpress a wild-type or a catalytically dead (D164N) DacA

isoform (14), wild-type or mutant isoforms of DacA lacking transmembrane domains (ΔTM), YbbR_6xH, or both wild-type or mutant DacA and YbbR_6xH (*dacA*op or *dacA*opMut). For all of these overexpression strains, we emphasize that the chromosomal expression of wild-type *dacA* and *ybbR* is maintained. In addition to the overexpression constructs, we also made a conditional knockdown construct for *dacA* (*dacA*-KD), targeting its promoter region, to decrease the expression of *dacA-ybbR* using a dCas12/crRNA-based system that our group developed for *Chlamydia* (29). Finally, we made a complementation construct for *dacA*-KD by introducing *dacA-ybbR*_6xH (*dacA*-KDcom) 3' to dCas12 such that induction of dCas12 results in the coexpression of DacA and YbbR_6xH during knockdown of endogenous *dacA-ybbR* transcripts.

To assess whether we could alter c-di-AMP levels in our various strains, we first measured c-di-AMP levels from infected cell lysates at 16 and 24hpi after inducing overexpression or knockdown of the target genes at 10 hpi (**Fig. 1E**). Overexpressing DacA resulted in ~4 fold increase in c-di-AMP levels as compared to the mCherry-expressing strain at 16hpi (~1,000 pg/mL) and ~3-fold increase at 24 hpi (~5,000 pg/mL). Overexpressing YbbR_6xH did not impact c-di-AMP production. When overexpressing the catalytically inactive mutant of DacA(D164N), c-di-AMP levels were ~2-fold lower at 16 hpi (~115 pg/mL) and, at 24 hpi (~320 pg/mL), was similar to the 16hpi mCherry control, indicating a severe reduction in c-di-AMP accumulation. Overexpressing ΔTMDacA phenocopied the effect of overexpressing full-length DacA on c-di-AMP production. Interestingly, the negative effects of overexpressing full-length DacA(D164N) associated with reduced c-di-AMP levels were lost when expressing ΔTMDacA(D164N), which phenocopied the mCherry-expressing control. When DacA and YbbR_6xH were co-overexpressed, c-di-AMP levels increased by approximately 30-fold at 16 hpi (~8,000 pg/mL) and 120-fold at 24 hpi (~340,000 pg/mL) compared to that of the mCherry-expressing control strain. Of note, the levels of c-di-AMP at 16hpi in the *dacA*op strain (~8,000 pg/mL) were higher even than the control strain at 24 hpi (~1,700 pg/mL). To confirm whether DacA enzyme activity is critical for the high levels of c-di-AMP in the *dacA*op strain, we co-overexpressed DacA(D164N) and YbbR_6xH (*dacA*opMut). Here, c-di-AMP levels were reduced compared to the control at 16 (~100 pg/mL) and 24 hpi (~400 pg/mL), suggesting that c-di-AMP synthase activity was blocked in this strain similar to overexpressing the DacA(D164N) alone. For the *dacA*-KD strain, the c-di-AMP level was unchanged at 16 hpi and reduced a statistically significant ~5-fold at 24 hpi (~500 pg/mL; roughly twice the level of the 16h mCherry-expressing control). The loss of c-di-AMP production in this strain was restored in the complemented *dacA*-KD_*dacA*op strain, which showed a phenotype similar to the *dacA*op overexpression strain with c-di-AMP levels above the 24hpi mCherry-expressing strain (~3,700 pg/mL at 16hpi; ~10,000 pg/mL at 24hpi). Based on these data, we conclude that both DacA and YbbR are necessary for optimal c-di-AMP synthesis in *Chlamydia*. Importantly, our collection of strains that are high or low producers of c-di-AMP give us an opportunity to test our overarching hypothesis (**Fig. 1B**).

Overexpression of membrane-localized DacA isoforms, but not YbbR, disrupts chlamydial growth and development

To begin exploring the impact of altering *dacA* and/or *ybbR* expression on chlamydial growth, we performed a series of experiments to assess their protein localization in chlamydiae. We also measured impacts of overexpressing each individually on chlamydial growth and development. To observe the localization of DacA and YbbR, we infected HeLa cells with transformants encoding *dacA* or *ybbR_6xH* alone and induced expression of the constructs at 10 hpi with 5 nM aTc. At 24 hpi, infected cells were fixed, and we performed an indirect immunofluorescence assay (IFA) by labeling the chlamydial major outer membrane protein (MOMP) and DacA or 6xH. We observed that both DacA and YbbR localized at the bacterial membrane as expected (**Fig. 2A** &**S2**). Interestingly, when DacA was overexpressed, the inclusion size was significantly reduced in area (~6 fold) with larger individual organisms (~2 fold) than those in the uninduced control (**Fig. S3A and B**). Similarly, overexpressing the inactive DacA(D164N) mutant resulted in smaller

inclusions similar to overexpressing wild-type DacA (**Fig. 2B**). We did not quantify effects of YbbR_6xH overexpression on inclusion or bacterial size since the measured phenotypes indicated no differences from the uninduced control (see below; **Fig. S4A**).

To investigate the effects of DacA isoforms or YbbR_6xH overexpression on chlamydial growth, we measured EB progeny production using an inclusion forming unit (IFU) assay and genomic DNA copy number (a proxy for total bacteria, i.e., EB+RB) by qPCR. To quantify IFUs, a lysate from a primary infection is prepared and used to infect a fresh monolayer of cells. Any inclusions in the secondary infection are derived from viable EBs present in the primary infection. The vector control strain showed no differences in IFUs when overexpressing mCherry (**Fig. S5**). At 24 hpi, both IFUs and genome copy numbers were significantly decreased in the DacA overexpression strain (**Fig. 2C** and **D**). We obtained similar results when overexpressing wild-type DacA with a 6xHis tag, indicating that a C-terminal tag does not impact these phenotypes in *Chlamydia* (**Fig. S6**). We also performed the same experiments with the inactive isoform of DacA, DacA(D164N). Like overexpression of the wild-type DacA, overexpression of DacA(D164N) also negatively affected IFU production and resulted in reduced genome copy numbers even though c-di-AMP levels were reduced under these conditions (**Fig. 2E** and **F**). YbbR_6xH overexpression did result in a statistically significant, ~2-fold decrease in IFU production (**Fig. S4B**). This reflects one division cycle difference from the uninduced control and, in the absence of any other phenotypic discrepancy, is not considered biologically relevant. No differences were noted in genome copy number when overexpressing YbbR_6xH (**Fig. S4C**).

To further investigate impacts of overexpression of these proteins on developmental cycle progression, we quantified transcripts by RT-qPCR for a canonical early-cycle gene, *euo*, and two late-cycle genes, *hctA* (an "early" late gene (30)) and *omcB* (a canonical late gene (11)). Consistent with the genome copy numbers and IFU data, overexpression of DacA resulted in elevated *euo* transcripts and a reduction in the amounts of the late gene transcripts at 24hpi (**Fig. 2G**). Similar effects on these transcripts were noted when overexpressing DacA(D164N) (**Fig. 2H**) We measured no effect of YbbR_6xH overexpression on transcript levels for *euo*, *hctA*, or *omcB* (**Fig. S4D**).

Given that overexpression of either the wild-type or mutant isoform of DacA gave the same phenotype yet yielded differences in c-di-AMP levels, we next explored the need for DacA or DacA(D164N) to be membrane-localized to effect these changes. Therefore, we evaluated the effects of overexpression of wild-type or mutant DacA lacking its transmembrane domains (ΔTM). As noted in **Figure 1**, overexpression of wild-type ΔTM DacA yielded the same level of c-di-AMP as overexpression of the full-length wild-type DacA. In contrast, c-di-AMP levels measured from chlamydiae overexpressing the mutant ΔTM DacA(D164N) were the same as the mCherry-expressing strain but reduced in chlamydiae overexpressing the full-length DacA(D164N). By IFA, both ΔTM isoforms localized to the cytosol, and there were no observable differences in inclusion or bacterial morphology as compared to the uninduced control (**Fig. 3A** and **B**). We did not quantify effects of overexpression on inclusion or bacterial size since the measured phenotypes indicated no differences from the uninduced control. Overexpression of the ΔTM isoforms resulted in no statistical differences in IFU production or genome copy numbers, and, similarly, there were no differences in transcript levels for *ybbR, euo, hctA*, or *omcB* as compared to the uninduced controls (**Fig. 3C-H**). These data indicate that the negative impacts of DacA overexpression are linked to its membrane localization and are independent of c-di- AMP production.

A low level of c-di-AMP decreases the transcript levels of late genes

As mentioned above, the *dacA*-KD strain displayed a lower c-di-AMP level compared to that of the control strain at 24 hpi whereas the *dacA*-KDcom complemented strain exhibited high levels of c-di- AMP at this timepoint (**Fig. 1E**). The complemented strain encodes not only the *dacA*-knockdown system but also the *dacA-ybbR_6xH* operon as a transcriptional fusion with dCas12. To

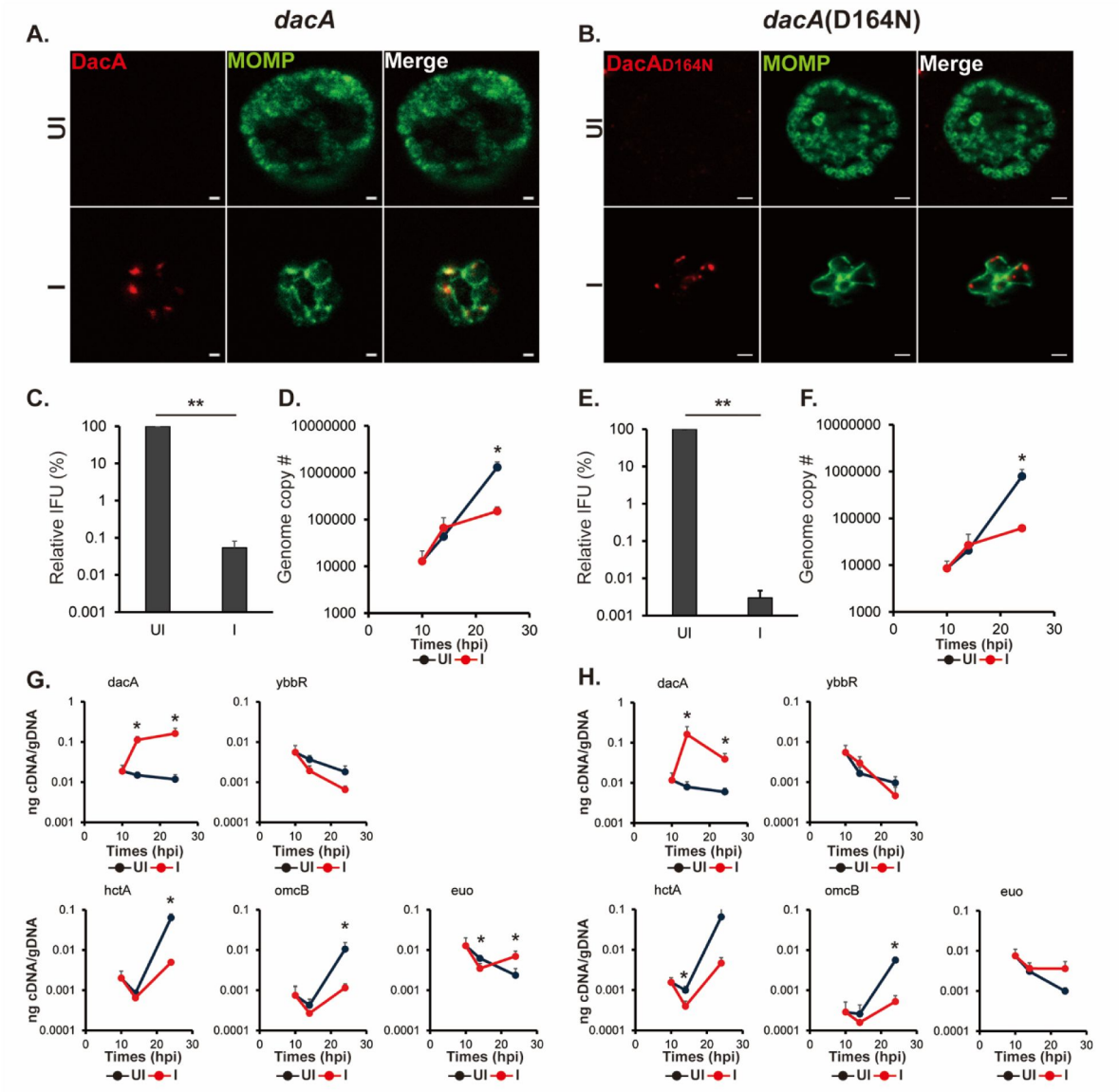

Figure 2.

Overexpression of DacA or DacA(D164N) are detrimental to the chlamydial developmental cycle.

HeLa cells were infected with *C. trachomatis* transformed with a plasmid encoding an anhydrotetracycline (aTc)-inducible DacA or DacA(D164N) (i.e., *dacA* and *dacA*(D164N) respectively; see **Fig. 1D**). At 10 hpi, expression of the construct was induced or not with 5 nM aTc, and DNA and RNA samples were collected at 10, 14, and 24 hpi. Immunofluorescence analysis (IFA) and inclusion forming units (IFU) samples were collected at 24hpi. For IFA images, the green color represents chlamydial major outer membrane protein (MOMP), which shows the chlamydial cell morphology, and the red color represents DacA or DacA(D164N). **A&B.** IFA images of the *dacA*(A) and *dacA*(D164N)(B) strains at 24 hpi. Shown are individual panels of a representative inclusion for the strains with DacA and MOMP labeling as well as the merged image. IFA images were acquired on a Zeiss AxioImager.Z2 equipped with an Apotome2 using a 100X lens objective. Scale bar: 1 μm (A) or 2 μm (B). **C&D.** Quantification of IFUs (C) and genomic DNA copy number (D) from uninduced and induced samples of *dacA* at 24 hpi. **E&F.** Quantification of IFUs (E) and genomic DNA copy number (F) from uninduced and induced samples of *dacA*(D164N) at 24 hpi. **G&H.** Quantification of transcripts by RT-qPCR for *dacA, ybbR*, *euo*, *hctA*, and *omcB* from uninduced and induced samples of *dacA* (G) and *dacA*(D164N) (H). UI = uninduced (i.e., -aTc); I = induced (i.e., +aTc) for all sample types. *: p<0.05; **: p<0.001 via two sample equal variance t-test.

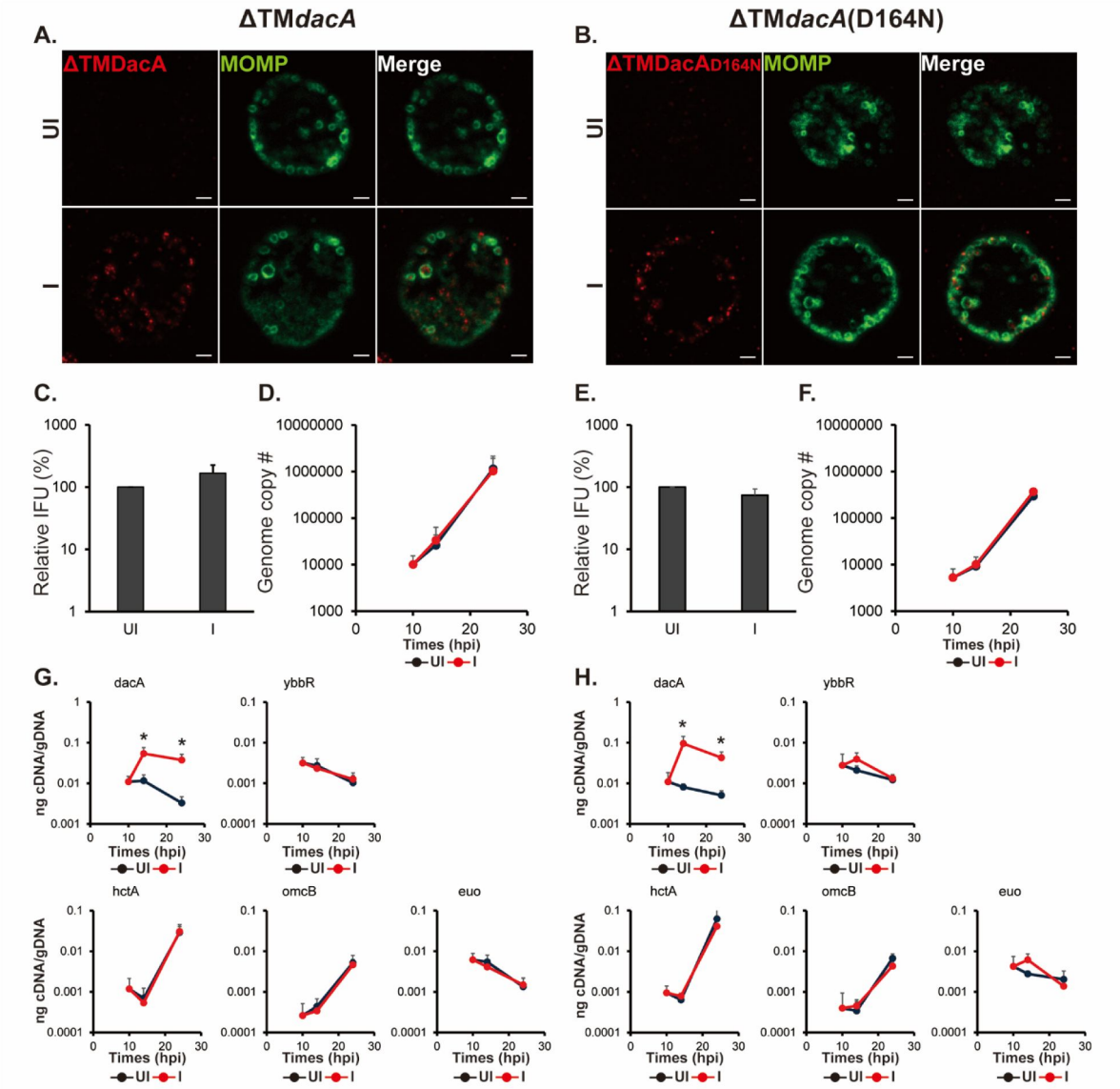

Figure 3.

Overexpression of ΔTMDacA or ΔTMDacA(D164N) does not alter the chlamydial developmental cycle.

HeLa cells were infected with *C. trachomatis* transformed with a plasmid encoding an anhydrotetracycline (aTc)-inducible ΔTMDacA or ΔTMDacA(D164N) (i.e., ΔTM*dacA* and ΔTM*dacA*(D164N); see **Fig. 1D**). At 10 hpi, expression of the construct was induced or not with 5 nM aTc, and DNA and RNA samples were collected at 10, 14, and 24 hpi. Immunofluorescence analysis (IFA) and inclusion forming units (IFU) samples were collected at 24 hpi. For IFA images, the green color represents chlamydial major outer membrane protein (MOMP), which shows the chlamydial cell morphology, and the red color represents ΔTMDacA or ΔTMDacA(D164N). **A&B.** IFA images of ΔTM*dacA*(A) and ΔTM*dacA*(D164N)(B) strains at 24 hpi. Shown are individual panels of a representative inclusion for the strains with DacA and MOMP labeling as well as the merged image. IFA images were acquired on a Zeiss AxioImager.Z2 equipped with an Apotome2 using a 100X lens objective. Scale bar: 2 μm. **C&D.** Quantification of IFUs (C) and genomic DNA copy number (D) from uninduced and induced samples of ΔTM*dacA* at 24 hpi. **E&F.** Quantification of IFUs (E) and genomic DNA copy number (F) from uninduced and induced samples of ΔTM*dacA*(D164N). **G&H.** Quantification of transcripts by RT-qPCR for *dacA, ybbR*, *euo*, *hctA*, and *omcB* from uninduced and induced samples of ΔTM*dacA* (G) and ΔTM*dacA*(D164N) (H). UI = uninduced (i.e., -aTc); I = induced (i.e., +aTc) for all sample types. *: p<0.05 via two sample equal variance t-test.

observe effects of knockdown or complementation on the developmental cycle, we first examined inclusion and bacterial morphology by IFA. When the knockdown system was induced, bacterial cell size was enlarged (~2 fold), but we observed no change in inclusion area as compared to that of the uninduced control (**Fig. 3A**, **S7A&B**). In the complemented strain, induction of dCas12 and YbbR_6xH was confirmed by IFA under inducing conditions (**Fig. 4B**). We expected that co-expressing DacA and YbbR_6xH would complement the phenotype of *dacA*-KD. However, the inclusion area was reduced (~3 fold) with slightly larger organisms (~1.4 fold) as compared to the uninduced control (**Fig. S7C&D**).

In addition, we also measured the amount of EB progeny (IFUs) and genome copy numbers. Although genome copy numbers were the same, IFUs decreased by approximately 80% compared to that of the uninduced sample at 24 hpi in the knockdown strain (**Fig. 4C** &D). IFUs and genome copies showed an approximate 2-fold reduction at 24 hpi in the complemented strain (**Fig. 4E** &F). The data for the knockdown strain suggest that low levels of c-di-AMP are detrimental for secondary differentiation. To further explore this, we performed RT-qPCR to quantify transcripts of relevant gene targets. When dCas12 was induced, both *dacA* and *ybbR* transcript levels decreased (**Fig. 4G**). Since *dacA* and *ybbR* are transcribed in an operon, this result is not surprising. Consistent with reduced EB yields, transcripts of the late genes *omcB* and *hctA* were decreased at 24 hpi. In contrast, transcripts of the early gene *euo* were slightly elevated at 24 hpi. We have previously observed no effects on genome levels or transcription of these genes when overexpressing the dCas12 gene alone (29), indicating these effects are specific to *dacA*-KD. For the complemented strain, both *dacA* and *ybbR* transcripts were increased compared to the *dacA*-KD strain (**Fig. 4H**), and transcript levels for these genes were increased beyond the “wild-type” uninduced control levels. Nonetheless, transcripts for the early gene *euo* were indistinguishable to levels measured in the uninduced strain (**Fig. 4H**). Surprisingly, *hctA* transcripts were increased ~10-fold at 14 hpi compared to that of the uninduced sample, and *omcB* transcripts were slightly, but not significantly, increased at 14 and 24 hpi (**Fig. 4H**). These data suggest that overexpressing DacA and YbbR_6xH may alter the timing of secondary differentiation.

High levels of c-di-AMP induce late gene expression

To clarify the effect of DacA and YbbR_6xH overexpression on secondary differentiation, we next evaluated the phenotype of the *dacA*op strain in contrast with the *dacA*opMut strain, in which the active site residue of DacA has been mutated. When we induced expression of the wild-type constructs, we confirmed the induction of DacA and YbbR_6xH and their colocalization at the membrane (**Fig. 5A**; Pearson correlation coefficient of 0.713 ± 0.109 from 20 inclusions measured by JACoP Plugin of ImageJ; values near 1 indicate colocalization (31, 32)). This is not surprising as both proteins are critical for c-di-AMP synthesis based on our c-di-AMP measurements (**Fig. 1E**). Organism and inclusion morphology were similar to the *dacA*-KDcom complemented strain (**Fig. 4B&5A**; **Fig. S8A&B**). The bacterial morphology of the *dacA*opMut strain indicated larger organisms (~1.8 fold) in smaller inclusions (~2.5 fold) after inducing expression (**Fig. 5B**; **S8C&D**). We next assessed whether the overexpressed DacA and YbbR_6xH affected IFU production and replication. IFUs were reduced roughly 2-fold for the *dacA*op strain and 1,000-fold in the *dacA*opMut strain after inducing overexpression (**Fig. 5C** &E). Again, the genome copy data for *dacA*op overexpression closely phenocopied the complemented knockdown strain, showing a decrease at 24 hpi (**Fig. 5D**). Similarly, genome copy numbers for the *dacA*opMut strain showed a significant drop at 24 hpi after inducing expression (**Fig. 5F**).

We then quantified transcripts for the developmentally regulated genes *euo*, *hctA*, and *omcB* as well as for *dacA* and *ybbR* (**Fig. 5G** &H). Not surprisingly, *dacA* and *ybbR* transcripts were elevated at the timepoints assessed under inducing conditions for both the *dacA*op and *dacA*opMut strains. Transcripts for *euo* were not statistically changed but trended higher at the 24 hpi timepoint whereas *omcB* transcripts were slightly, but not significantly, increased at 14 and 24 hpi during *dacA*op overexpression. Once again, we observed that *hctA* transcripts were increased over

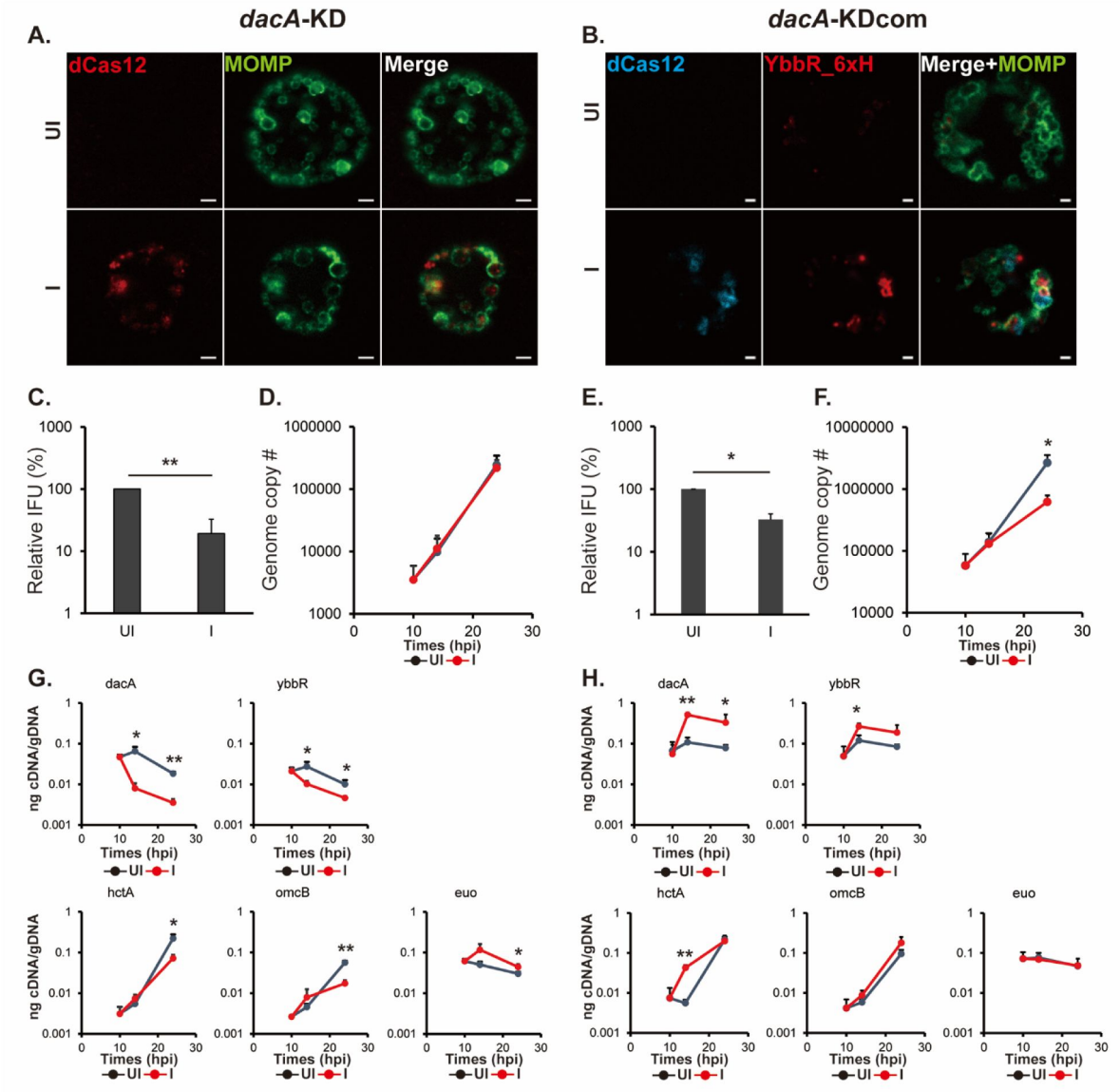

Figure 4.

CRISPRi-mediated *dacA-ybbR* knockdown displays reduced levels of transcripts for late genes.

HeLa cells were infected with *C. trachomatis* transformed with a plasmid encoding an anhydrotetracycline (aTc)-inducible CRISPRi-dCas12 system targeting the *dacA* promoter (*dacA*-KD) or *dacA*-KD system and DacA/YbbR_6xH (i.e., *dacA*-KDcom; see **Fig. 1D**). At 10 hpi, knockdown was induced or not with 5 nM aTc, and DNA and RNA samples were collected at 10, 14, and 24 hpi. Immunofluorescence analysis (IFA) and inclusion forming units (IFU) samples were collected at 24hpi. **A&B.** IFA images of the *dacA*-KD (A) and *dacA*-KDcom (B) strains at 24 hpi. Shown are individual panels of a representative inclusion for the strains for dCas12, YbbR_6xH, and major outer membrane protein (MOMP) labeling as well as the merged image. IFA images were acquired on a Zeiss AxioImager.Z2 equipped with an Apotome2 using a 100X lens objective. Scale bar: 2(A) or 1(B) μm. **C&D.** Quantification of IFUs (C) and genomic DNA copy number (D) from uninduced and induced samples of *dacA*-KD at 24 hpi. **E&F.** Quantification of IFUs (E) and genomic DNA copy number (F) in uninduced and induced samples of *dacA*-KDcom. **G&H.** Quantification of transcripts by RT-qPCR for *dacA, ybbR*, *euo*, *hctA*, and *omcB* from uninduced and induced samples of *dacA*-KD (G) and *dacA*- KDcom (H). UI = uninduced (i.e., -aTc); I = induced (i.e., +aTc) for all sample types. *: p<0.05; **: p<0.001 via two sample equal variance t-test.

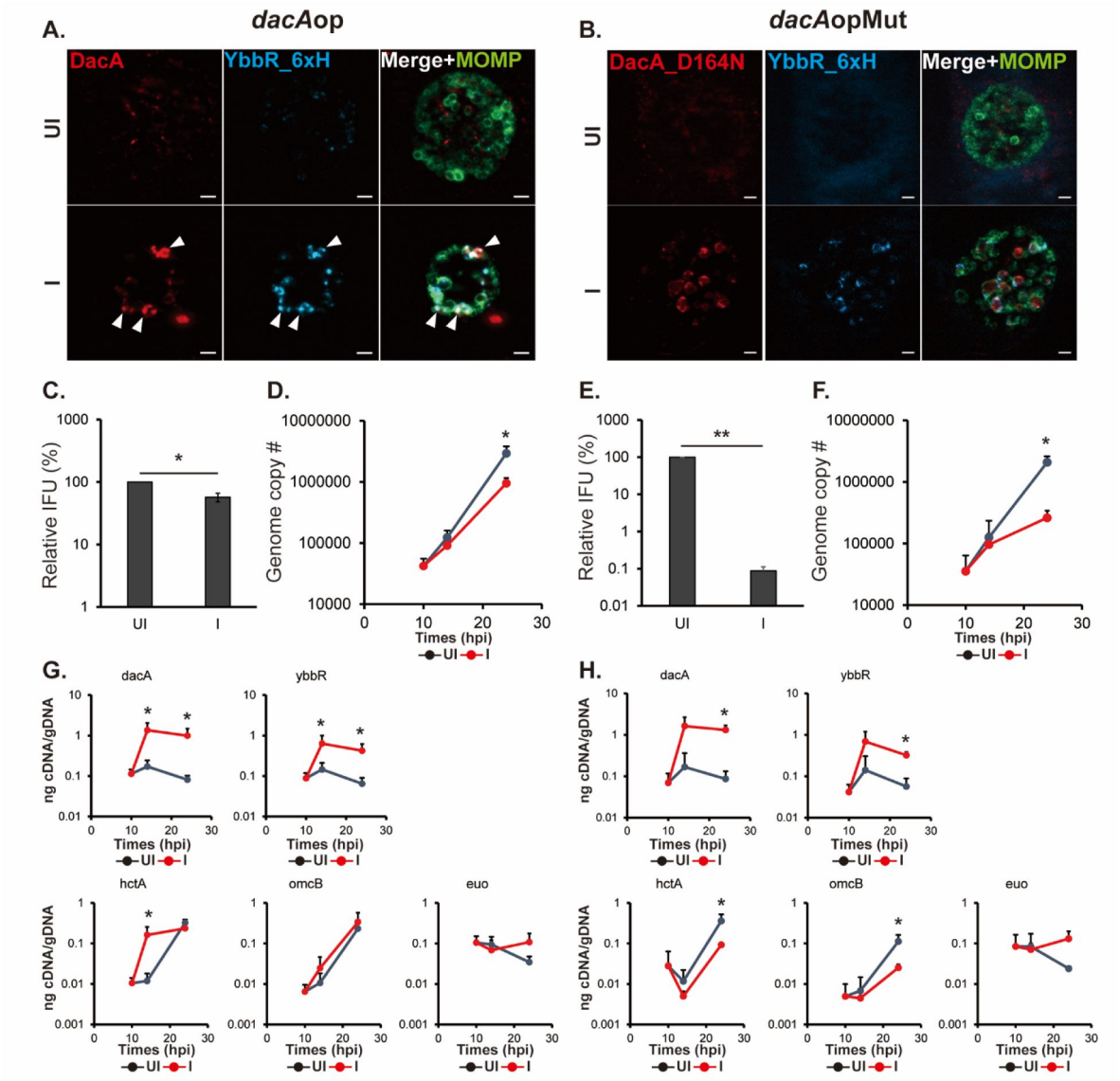

Figure 5.

Overexpression of DacA and YbbR_6xH prematurely increases *hctA* transcript levels.

HeLa cells were infected with *C. trachomatis* transformed with an aTc-inducible plasmid encoding wild- type DacA/YbbR_6xH or DacA(D164N)/YbbR_6xH (i.e., *dacA*op and *dacA*opMut, respectively; see **Fig. 1D**). At 10 hpi, expression of the constructs were induced or not with 5 nM aTc, and DNA and RNA samples were collected at 10, 14, and 24 hpi. Immunofluorescence analysis (IFA) samples were collected at 24 hpi. **A&B.** IFA images of the *dacA*op (A) and *dacA*opMut (B) at 24 hpi. Shown are individual panels of a representative inclusion for the strains for DacA and YbbR_6xH as well as the merged image with major outer membrane protein (MOMP) labeling. The arrowheads represent the co-localization of DacA and YbbR. IFA images were acquired on a Zeiss AxioImager.Z2 equipped with an Apotome2 using a 100X lens objective. Scale bar: 2 μm. **C&D** Quantification of IFUs (C) and genomic DNA copy number (D) from uninduced and induced samples of *dacA*op at 24 hpi. **E&F.** Quantification of IFUs (E) and genomic DNA copy number (F) from uninduced and induced samples of *dacA*opMut. **G&H.** Quantification of transcripts by RT-qPCR for *dacA, ybbR, euo, hctA,* and *omcB* from uninduced and induced samples of *dacA*op (G) and *dacA*opMut (H). UI = uninduced (i.e., -aTc); I = induced (i.e., +aTc) for all sample types. *: p<0.05 two sample equal variance t-test.

10-fold at 14hpi in the developmental cycle (**Fig. 5G**), similar to what we measured for the complemented knockdown strain (**Fig. 4H**). These data reinforce that elevated c-di-AMP levels (**Fig. 1E**) in these strains lead to increased expression of the late gene *hctA* at an earlier timepoint (14 hpi) in the developmental cycle. As further validation of this *dacA*op overexpression strain that was constructed in a beta-lactamase producing background, we also generated a spectinomycin-resistant *dacA*op overexpression strain and validated that induction of the *dacA* operon resulted in earlier accumulation of *hctA* transcripts (**Fig. S9**). In assessing developmentally-regulated transcripts for the *dacA*opMut strain, *euo* levels were maintained, albeit not significantly so, at 24 hpi (**Fig. 5H**). In contrast to the c-di-AMP overproducing strains, transcripts for *hctA* and *omcB* were decreased at 24 hpi (**Fig. 5H**). The transcriptional results of the developmentally regulated genes in the *dacA*opMut strain were very similar to the *dacA*-KD strain (**Fig. 4G**), suggesting that blocking c-di-AMP accumulation interferes with developmental cycle progression.

Elevated c-di-AMP levels result in increased transcript levels of genes necessary for secondary differentiation

Given the surprising finding that, in strains overproducing c-di-AMP, *hctA* transcripts were 10-fold higher at a timepoint not associated with secondary differentiation, we asked the question whether *all* genes related to secondary differentiation were increased after inducing production of c-di-AMP. Conversely, we wanted to explore whether reducing c-di-AMP levels would delay expression of genes related to secondary differentiation. Thus, to further investigate how c-di-AMP affects transcription of such genes, we performed RNA sequencing on both the *dacA*op overexpression and *dacA*-KD strains and compared the transcriptome between uninduced and induced samples within the given strain at the given timepoint. HeLa cells were infected with these transformants, and overexpression or knockdown was induced or not at 10 hpi with 5 nM aTc. For the *dacA*op strain, RNA was collected at 16 hpi, a time at which late genes are beginning to be expressed (as opposed to 14hpi) but remain near a basal level of transcription(11). The rationale for this was to determine whether high c-di-AMP levels result in increased late gene transcripts at this timepoint *above and beyond* the levels of the control, uninduced condition. For the *dacA*-KD strain, RNA was collected at 24 hpi, a time at which late genes are peaking in their expression. The rationale for this was to determine whether late gene transcription was decreased, which could not otherwise be assessed at the 16 hpi timepoint.

RNA sequencing results were statistically analyzed by the UNMC Bioinformatics Core (Table S2&3). **Figure 6** shows a volcano plot of the results for the *dacA*op overexpression and *dacA*-KD strains. Of note, many late genes were evident in the upregulated quadrant for the *dacAop* strain whereas these genes were present in the downregulated quadrant for the *dacA*-KD strain. We further characterized the upregulated or downregulated gene sets for the *dacA*op overexpression and *dacA*-KD strains, respectively, based on significant difference ($p<0.05$) and fold-change (>1.5). A summary of these results is presented in **Table 1** (all data are presented in Table S3). We grouped the differentially expressed genes into five categories: 1) canonical late genes for which the literature has associated them with EB function, 2) outer membrane associated, 3) gene regulation associated, 4) glycogen synthesis associated, and 5) type III secretion system associated. Recent work from our group and the Hefty group to define the regulons of the alternative sigma factors in *Chlamydia* demonstrated that these sigma factors regulate some late gene expression associated with outer membrane remodeling, type III secretion, and other processes (33, 34). Consistent with our RT-qPCR data, we observed that all canonical late genes as well as all the other genes listed in these categories showed an increase in expression after c-di-AMP production was induced. In contrast, all the genes, and particularly the canonical late genes, showed a decrease in expression under conditions where c-di-AMP production was impaired. Overall, these RNA sequencing data confirm the direct influence of c-di-AMP on expression of genes related to secondary differentiation.

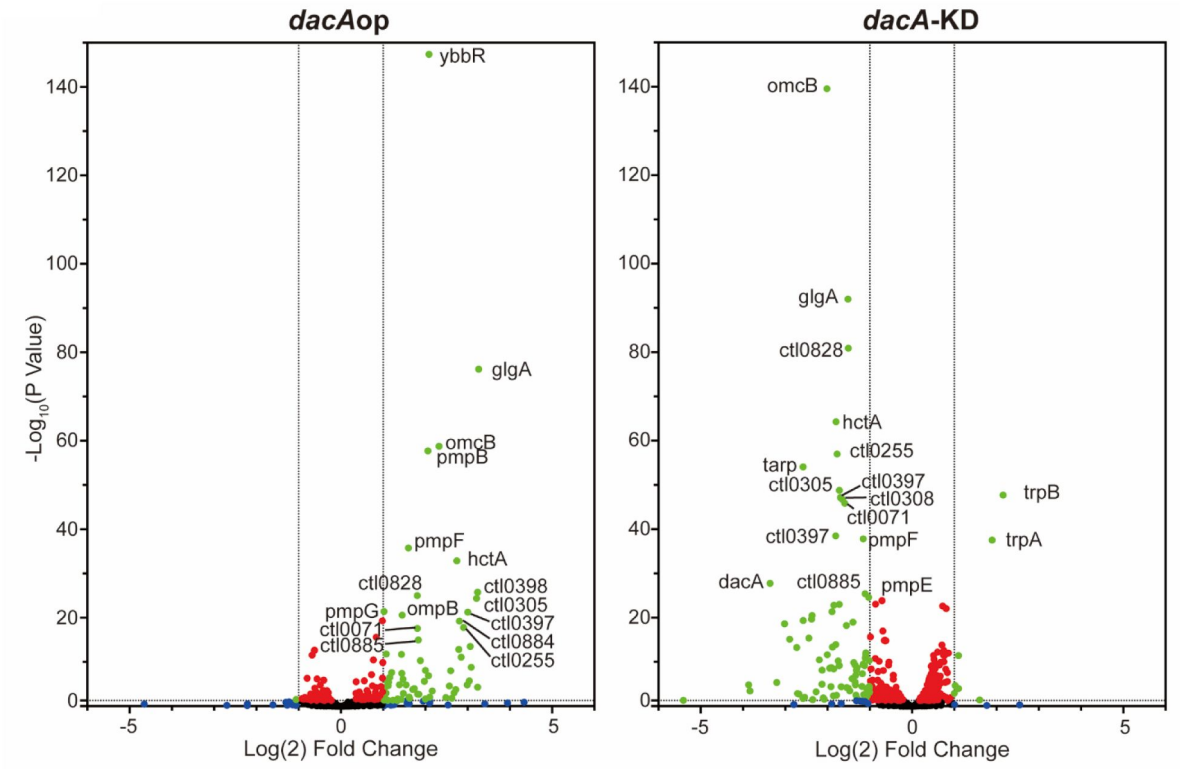

Figure 6.

The levels of c-di-AMP are correlated with late gene transcripts.

RNA sequencing was performed from HeLa cells infected with either the *dacA*op or *dacA*-KD strains. RNA samples were collected at 16hpi for *dacA*op and 24hpi for *dacA*-KD after inducing expression of the relevant constructs at 10hpi. Shown is a volcano plot of the RNA sequencing results with the vertical dashed lines indicating a two-fold change in transcript levels as compared to the respective uninduced control for the given strain and the horizontal lines indicating a p-value of 0.05. The plot was made using GraphPad Prism software. Green spots represent genes demonstrating a statistically significant two-fold change in transcription levels between uninduced and induced samples. Red dots represent genes with significant changes in transcript levels less than two-fold. Blue dots represent genes not significantly different but more than two-fold changed between the conditions. Black dots represent genes not significantly different and less than two-fold changed between the conditions. See also **Table 1** and Supplemental Tables 2 and 3 for more details.

			Canonical late genes	Fold change		
Gene ID	Ctr D ORF	Name	Protein Names	*dacA*op_OE	*dacA*-KD	Ref.
CTL0112	CT743	*hctA*	Histone H1-like protein HC1	6.70	-3.47	(11, 56)
CTL0302	CT046	*hct2*	Histone H1-like protein HC2	3.52	-5.96	(11, 57)
CTL0336	CT080	*ltuB*	Late transcription unit B protein	4.00	-3.61	(11, 56)
CTL0700	CT441	*tsp*	Carboxy-terminal processing protease	6.18	-6.62	(57)
CTL0702	CT443	*omcB*	Large cysteine-rich periplasmic protein	5.00	-4.04	(11, 34)
CTL0703	CT444	*omcA*	Small cysteine-rich outer membrane protein	5.93	-3.71	(11, 34)
CTL0716	CT456	*tarp*	Translocated actin-recruiting phosphoprotein	6.06	-5.97	(33, 34)
			Membrane organization associated	Fold change		
Gene ID	Ctr D ORF	Name	Protein Names	*dacA*op_OE	*dacA*-KD	Ref.
CTL0082	CT713	*ompB*	Outer membrane protein B	2.73	-2.15	(58)
CTL0248	CT869	*pmpE*	Polymorphic outer membrane protein	1.99	-2.04	(33, 58)
CTL0249	CT870	*pmpF*	Polymorphic outer membrane protein	3.03	-2.23	(33, 58)
CTL0250	CT871	*pmpG*	Polymorphic outer membrane protein	2.03	-1.55	(11, 58)
CTL0429	CT177	*dsbA*	Disulfide bond chaperone	2.79	-2.47	(33)
CTL0610	CT356	*dsbH*	Thioredox_DsbH domain-containing protein	3.07	-2.11	(11, 58)
CTL0670	CT413	*pmpB*	Polymorphic outer membrane protein	4.17	-1.74	(33, 58)
			Gene Regulation associated	Fold change		
Gene ID	Ctr D ORF	Name	Protein Names	*dacA*op_OE	*dacA*-KD	Ref.
CTL0044	CT675	*mcsB*	Protein-arginine kinase	3.52	-2.21	(33)
CTL0045	CT676	*mcsA*	UVR domain-containing protein	2.53	-2.92	(33)
CTL0727	CT467	*atoS*	Two component regulator, histidine kinase	3.33	-1.80	(33)
CTL0728	CT468	*atoC*	Two component system response regulator	7.95	-2.78	
CTL0894	CT630	*chxR*	Atypical response regulator protein ChxR	3.68	-2.93	(59)
			Glycogen synthesis	Fold change		
Gene ID	Ctr D ORF	Name	Protein Names	*dacA*op_OE	*dacA*-KD	Ref.

Table 1.

Genes impacted by cyclic di-AMP levels.

CTL0167	CT798	*glgA*	Glycogen synthase	9.58	-2.86	(11, 58)
CTL0342	CT087	*malQ*	4-alpha-glucanotransferase	9.43	-4.29	(42)
CTL0500	CT248	*glgP*	Alpha-1,4 glucan phosphorylase	2.70	-1.71	(58)
			Type III Secretion System	Fold change		
Gene ID	Ctr D ORF	Name	Protein Names	*dacA*op_OE	*dacA*-KD	Ref.
CTL0041	CT672	*sctQ*	Type III secretion component, basal body	1.93	-1.76	(42)
CTL0043	CT674	*cdsC*	Type III secretion structural protein	1.79	-1.56	(42)
CTL0343	CT088	*scc1*	Type III secretion chaperone	4.48	-3.78	(42)
CTL0824	CT561	*sctL*	Type III secretion system protein	2.18	-1.75	(33)

OE = overexpression; KD = knockdown

Table 1. (continued)

Alterations in c-di-AMP levels impact the timing of EB production

The phenotypic effect of increased late gene transcription should be an increase in EB production. However, if EB production is initiated at an earlier time in the developmental cycle when there are fewer non-infectious RBs to convert, then overall EB production should be decreased with a concomitant decrease in genome copies since only the RB replicates DNA. Conversely, delayed expression of late genes should be associated with a delay in EB production. Therefore, to test these predictions, we quantified EB production at 2h intervals from 18 to 24hpi to assess EB production during earlier phases of the developmental cycle, as well as at 32 and 48hpi to assess overall EB yields (**Fig. 7**). We did not detect any EBs at 16hpi or earlier (not shown). Cells were infected with the *dacA*op, *dacA*opMut, and *dacA*-KD strains and induced or not at 10 hpi with 5 nM aTc as previously noted. Consistent with the transcriptional data, we measured higher EB yields at 18 and 20 hpi during *dacA*op overexpression that quickly plateaued by 24 hpi (**Fig. 7A**). Conversely, when c-di-AMP accumulation was blocked by reducing the expression of DacA and YbbR (*dacA*-KD), we observed delayed EB production (**Fig. 7B**). We also measured lower EB yields when c-di-AMP levels were decreased by overexpression of the DacA(D164N) isoform and YbbR (*dacA*opMut; **Fig. 7C**). The uninduced control conditions for each strain showed a steady accumulation of EBs throughout the course of the experiment, as expected. The vector control strain expressing mCherry showed no differences as previously noted (**Fig. S5**). From these data, we conclude that earlier production of c-di-AMP levels results in earlier production of EBs.

Discussion

Secondary differentiation is essential for the propagation and survival of *Chlamydia* species. However, the mechanisms governing this essential process are largely unknown. It is possible that both internal and external environmental changes might serve as signals to trigger the shift from the non-infectious RB to the infectious EB. It is also possible, and even likely, that *Chlamydia* integrates multiple signal inputs during the differentiation process. For example, we recently described two post-translational processes that impact secondary differentiation: the activity of the unfoldases ClpX and ClpC([35], [36]) and the redox status of *Chlamydia*([37]). As secondary differentiation is asynchronous, any mechanism must account for the stochasticity within the population of RBs "considering" differentiating to EBs. Here, we provide the first evidence of a signaling molecule that can directly activate EB-associated gene expression with a concomitant early production of EBs.

Several reports have proposed mechanisms by which *Chlamydia* triggers secondary differentiation. For example, Thompson et al. investigated the non-canonical regulation of the major sigma factor, σ^{66}, by the Rsb phosphoregulatory system([38]). The authors provided evidence that altering the levels of RsbW or RsbV1 impacted the expression of σ^{66}-controlled genes. This led them to propose a model whereby sensing of ATP by the Rsb system controls the availability of σ^{66}, implying that low levels of ATP lead to initiation of secondary differentiation. Further support from this came from work by Kuwabara et al. who showed effects of ATP, GTP, and glucose on the activity of different Rsb components([39]). Finally, work from Soules et al. identified TCA intermediates as ligands for RsbU, linking the TCA cycle and ATP synthesis to the Rsb system([40]). However, no effect was noted on premature late gene expression in these experimental systems.

Sequestration/inactivation of the major sigma factor under low ATP conditions would presumably render RNA polymerase free to interact with the alternative sigma factors, σ^{54} and σ^{28}. These alternative sigma factors have been linked to late gene expression with studies from Soules et al. and Hatch and Ouellette indicating that σ^{54} is associated with increased transcription of outer

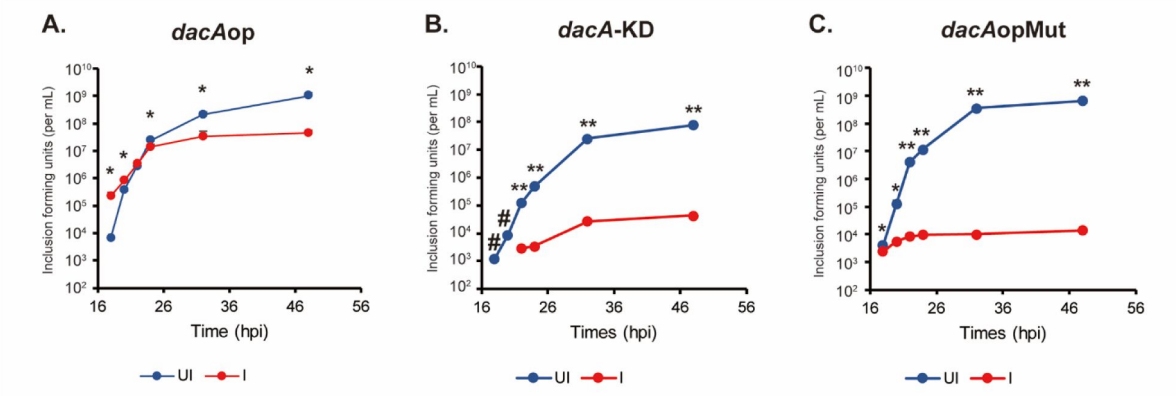

Figure 7.

EB production is induced by high levels of c-di-AMP.

(A-C) HeLa cells were infected with the chlamydial transformants *dacA*op (A), *dacA*-KD (B), and *dacA*opMut (C). Expression of the constructs was induced or not with 5 nM aTc at 10hpi. At 18, 20, 22, 24, 32, and 48 hpi, infected cell lysates were harvested for IFU quantification. UI = uninduced (i.e., -aTc); I = induced (i.e., +aTc) for all sample types. #: Non-detected from the induced samples. *: $p<0.05$; **: $p<0.001$ via two sample equal variance t-test.

membrane components, type III secretion (T3S) system components, and other genes typically expressed late in development (33, 34). However, some of these changes are likely indirect and downstream to the σ^{54} regulon itself since some affected genes have been shown to have σ^{66} promoter sequence elements (41, 42). Significant remodeling of the outer membrane occurs as the RB transitions to the EB (43, 44), and, similarly, the EB is prepackaged with T3S effectors, like TarP (45), that facilitate EB invasion into a target host cell. σ^{28} was shown to control expression of two canonical late genes: *hctB* and *tsp* (33). Each of the encoded proteins is highly toxic if overexpressed (46, 47), suggesting that the additional layer of regulation by σ^{28} is necessary to ensure EBs are formed at the correct time. Hatch and Ouellette proposed that these genes may be the last to be activated precisely because of this (33). Overall, these data clearly link transcriptional regulation, via the sigma factors, to developmental cycle progression and secondary differentiation.

Despite the clear changes in transcription that occur during the chlamydial developmental cycle, activation of late gene expression alone does not guarantee secondary differentiation. We recently explored the function of the Clp protease systems in chlamydial growth and development (36). Interestingly, we observed that the inability to degrade SsrA-tagged products prevented functional secondary differentiation with a severe defect in EB production. This was demonstrated in strains unable to degrade SsrA products either by preventing their recognition by ClpX or by altering the SsrA tag to a version that is not degraded efficiently. Nonetheless, late gene transcription was activated in these strains. Therefore, post-translational regulation of secondary differentiation is also critical to the process.

Assuming a post-translational gene regulation model for secondary differentiation, we were interested in exploring known differences between EBs and RBs as a clue to what factors could be serving as a signal for this process. EBs and RBs differ in a number of characteristics, from their function to their morphology to their gene expression. In 2013, a study from Barker et al. added another difference – their relative levels of c-di-AMP with RBs having lower levels than EBs. Although the study from Barker et al. was focused on understanding how IFNβ is activated in *Chlamydia*-infected cells, we were intrigued by this relative difference in c-di-AMP levels. We hypothesized that c-di-AMP production might be a signal for secondary differentiation. We compared chlamydial growth in normal HeLa or STING-KO HeLa cells but could not detect any differences in growth in these cell types (**Fig. 4G** &**S6 vs Fig. S10**), indicating that c-di-AMP production by *Chlamydia* is unlikely used as means of activating the host cell. Rather, STING activation is an “accidental” outcome during chlamydial developmental cycle progression. Using recently developed genetic tools for *Chlamydia*, we demonstrated that we could manipulate the levels of c-di-AMP in the bacterium to either block or stimulate its production. Excitingly, we observed that elevated c-di-AMP was linked to earlier secondary differentiation whereas blocking c-di-AMP production prevented EB production (**Fig. 7**). Importantly, c- di-AMP production resulted in an increase in late gene transcripts as noted above for the function of the alternative sigma factors (**Fig. 6**). Therefore, c-di-AMP may act as a post-translational mechanism to trigger secondary differentiation. We can also directly conclude from our experiments that the threshold level of c-di-AMP necessary to trigger secondary differentiation in our culture conditions is greater than 1,000 pg/mL and less than 2,000 pg/mL. We base this on the fact that the 16hpi level of c-di-AMP in the DacA-overexpressing strain (1,000 pg/mL) did not trigger secondary differentiation whereas, during a normal infection (e.g., mCherry expressing strain) when EBs are being produced at 24hpi, the c-di-AMP level is ~2,000pg/mL. To our knowledge, this is the first study to identify and provide experimental evidence for a signaling factor involved in differentiation of an obligate intracellular bacterium.

How might c-di-AMP function in *Chlamydia*? This is not readily apparent because *Chlamydia* lacks orthologs of proteins that have been characterized in other bacterial systems to bind c-di-AMP. For example, *Chlamydia* lacks annotated K^+ transporters, and it is unknown how these bacteria maintain their osmolarity. However, a recent study suggested that changes in K^+ levels affect the

chlamydial developmental cycle (48). Therefore, c-di-AMP may function as a K^+ homeostasis coordinator in *Chlamydia* through as-yet unknown pathways. We did observe altered chlamydial morphology in some of our strains in which we altered c-di-AMP levels, and this may indicate changes in osmostability or effects on cell division. In regard to the latter possibility, in *S. aureus*, DacA activity is inhibited by interacting with GlmM (phosphoglucosamine mutase) (49). GlmM produces UDP-GlcNAc that is a precursor in both peptidoglycan and LPS synthesis. Given that *Chlamydia* uses peptidoglycan for cell division, we tested whether DacA and YbbR were localized to the division septum. However, their localization was not associated with the division septum (**Fig. S10**). In addition, we did not detect an interaction between DacA and GlmM using the Bacterial Adenylate Cyclase-based Two Hybrid (BACTH) system (data not shown), and the chlamydial GlmM lacks the residues required for DacA interaction. Therefore, we conclude that DacA and YbbR are unlikely to directly impact cell division. However, based on the cell morphological changes in DacA overexpression, we cannot exclude a function for c-di-AMP on cell wall metabolism. Future studies will focus on identifying c-di-AMP binding proteins and characterizing their function in *Chlamydia*. We also cannot exclude a function for c-di-AMP in directly manipulating gene expression by riboswitches (50), and further work is necessary to understand how c-di-AMP directly controls chlamydial development.

The use of c-di-AMP as a signal for secondary differentiation presents a “chicken-or-egg” quandary. How is the activity and expression of DacA, the diadenylate cyclase, regulated? Our transcript analyses indicate that *dacA-ybbR* transcripts peak during the RB phase of growth, similar to most genes in *Chlamydia*. One possibility not easily tested due to the obligate intracellular nature of *Chlamydia*, is that c-di-AMP production depletes ATP levels, which then reduce σ^{66} activity as described above. Our data indicate YbbR is necessary to activate DacA function since expressing membrane-localized or ΔTM isoforms of DacA alone only modestly increased c-di-AMP levels (**Fig. 1E**). However, overexpressing DacA alone did negatively impact chlamydial growth, suggesting that the balance between DacA and YbbR levels is important, with YbbR being the limiting factor for c-di-AMP production. For example, too much DacA insertion into the membrane without binding to YbbR may disrupt the membrane biology of the RB. This is supported by the fact that we observed no detrimental effects of overexpressing ΔTM isoforms of either wild-type or mutant (D164N) DacA. Indeed, overexpressing ΔTMDacA(D164N) did not alter c-di-AMP levels. Therefore, YbbR itself may be a target for regulation. As a monotopic transmembrane protein, YbbR may be degraded by proteases such as the inner membrane-associated FtsH or the periplasmic proteases HtrA or Tsp. This would presumably shut down c-di-AMP synthesis. Alternatively, DacA may interact with other membrane proteins, and overexpressing it alone may impair the function of this binding partner(s), resulting in the observed phenotypes. However, this possibility contradicts the effects of overexpressing the D164N isoform, which blocked c-di-AMP production. These experiments were conducted in the presence of the chromosomally expressed copy of DacA, and we suggest that the mutant isoform acts as a dominant negative by binding and/or interfering with the wild-type DacA or by titrating away YbbR from the wild-type DacA. This is also supported by the fact that overexpressing the ΔTMDacA(D164N) isoform had no impact on c-di-AMP levels or chlamydial growth. In contrast, knocking down *dacA-ybbR* transcripts will reduce, but not completely eliminate, DacA and YbbR proteins, which may result in residual c-di-AMP levels as compared to overexpressing the DacA mutant (either alone or with YbbR in *dacA*opMut) as well as the phenotypic differences between these strains. Further work is needed to test these possibilities. However, our data are clear in linking c-di-AMP levels to chlamydial developmental progression.

Even though diverse functions of c-di-AMP in other bacteria have been reported previously, this is the first time c-di-AMP has been described as a checkpoint in chlamydial development. It is possible that the levels of c-di-AMP act as a de facto means for monitoring the bacterial population within the inclusion or for their overall developmental status. Canonical bacteria can use quorum sensing to detect bacteria at the population level and to communicate with other species (51). However, *C. trachomatis* lacks homologues of genes related to quorum sensing (28). In addition,

secondary differentiation occurs asynchronously, and this feature is different from a quorum sensing mechanism. Our model accounts for the asynchronicity of secondary differentiation. As RBs accumulate c-di-AMP and divide, it is likely the c-di-AMP will be distributed unevenly between the mother and daughter cell since *Chlamydia* divides through an asymmetric budding mechanism (7) (**Fig. 8**). This will lead to two cells with different levels of c-di-AMP, one of which may then accumulate enough c-di-AMP to trigger secondary differentiation while the other continues to divide (**Fig. 8**). This is consistent with a recent model proposing that a population of division-competent RBs is maintained throughout the developmental cycle (52). Similar uneven distribution of post-translational factors between asymmetrically dividing mother and daughter cells would also impact other recently described signals for differentiation, including the levels of ClpC protein or overall redox via AhpC antioxidant levels(35, 37).

Materials and Methods

Organisms and Cell Culture

Wild-type (ATCC, Manassas, VA) and STING KO (Dr. Frank van Kuppeveld; Utrecht University) HeLa, human cervical epithelial-derived, and McCoy (kind gift of Dr. Harlan Caldwell, NIH), mouse fibroblast-derived, cell lines were cultured at 37°C with 5% CO_2 in Dulbecco's Modified Eagle Medium (DMEM; Invitrogen, Waltham, MA) containing 10% fetal bovine serum (FBS; Hyclone, Logan, UT) and 10 µg/mL gentamicin (Gibco, Waltham, MA). *C. trachomatis* serovar L2 (434/Bu) lacking the endogenous plasmid (-pL2; kind gift of Dr. Ian Clarke, Univ. Southampton) was used for transformation. All cell cultures and chlamydial stocks were routinely tested for Mycoplasma contamination using the Mycoplasma PCR detection kit (Sigma, St. Louis, MO). For *E. coli*, NEB10β competent cells (New England Biolabs, Ipswich, MA) were used for the amplification of pBOMB-derivative vectors. *E. coli* was grown at 30°C in LB media. All chemicals and antibiotics were obtained from Sigma unless otherwise noted.

Cloning

The list of the vectors and primers used in this study is detailed in Supplemental Table 1. Target genes were amplified by PCR with Phusion DNA polymerase (NEB) using 10 ng *C. trachomatis* L2 genomic DNA or appropriate vectors as a template. Some DNA segments were directly synthesized as a gBlock fragment (Integrated DNA Technologies, Coralville, IA). If plasmids were used as a template, then we treated the PCR product with DpnI enzyme to remove templates. The PCR products were purified using a PCR purification kit (Qiagen, Hilden, Germany). The HiFi Assembly reaction master mix (NEB) was used following the manufacturer's manual in conjunction with plasmids pBOMBL(29) linearized with EagI and KpnI or pBOMBL12CRia (empty vector) linearized with BamHI. The linearized plasmids were also dephosphorylated with FastAP (ThermoFisher). The products of the HiFi reaction were transformed into NEB10β competent cells (NEB) and plated on LB agar with appropriate antibiotics. Plasmids were subsequently isolated using a mini-prep kit (Qiagen) and verified by plasmid digest and sequencing from individual colonies grown overnight in LB broth with appropriate antibiotic selection.

Transformation of Chlamydia trachomatis

McCoy cells were plated in a six-well plate the day before beginning the transformation procedure. *C. trachomatis* serovar L2 without plasmid (-pL2) resuspended with Tris-$CaCl_2$ buffer (10 mM Tris-Cl pH 7.5, 50 mM $CaCl_2$) was incubated with 2 µg plasmid at room temperature for 30 min. During this step, McCoy cells were washed with 2 mL Hank's Balanced Salt Solution (HBSS) media containing Ca^{2+} and Mg^{2+} (Gibco). After that, McCoy cells were infected with the transformants in 2 mL HBSS per well. The plate was centrifuged at 400 x g for 15 min at room temperature and incubated at 37°C for 15 min. The inoculum was aspirated, and 2 mL 1X DMEM containing 10% FBS and 10 µg/mL gentamicin was added per well. At 8 hours post infection (hpi), 1 µg/mL

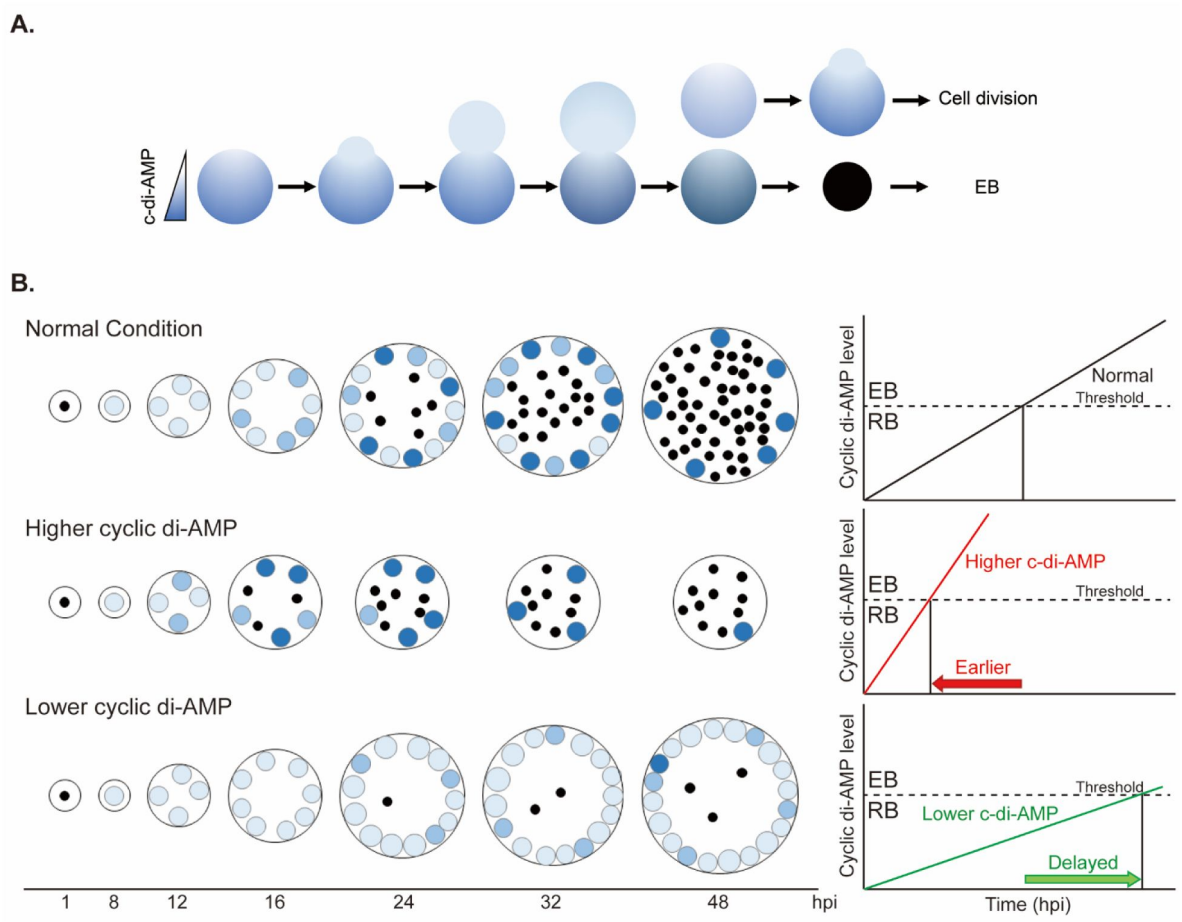

Figure 8.

A working model illustrating how c-di-AMP impacts the onset of secondary differentiation in *C. trachomatis*.

A. During RB division, there is a gradient of c-di-AMP that forms between the mother and daughter cell. The lighter blue represents lower levels of c-di-AMP, the darker blue represents higher levels of c-di-AMP. In the depicted scenario, the daughter cell is able to divide again, having not reached a critical threshold of c-di-AMP. In contrast, the mother cell accumulates sufficient c-di-AMP to trigger secondary differentiation to an EB. **B.** A model illustrating how c-di-AMP levels impact secondary differentiation progression. Shown is the "normal" condition of wild-type bacteria, bacteria overexpressing DacA and YbbR (higher cyclic di-AMP), and bacteria expressing the CRISPRi system targeting the *dacA* promoter (lower cyclic di-AMP). Depending on the amount of c-di- AMP produced, secondary differentiation can either be triggered earlier or later as shown. The black dots represent EBs; the bigger and blue-colored circles show the RB cells. The brightness of blue reflects the c-di-AMP level.

cycloheximide and either 1 or 2 U/mL penicillin G or 500 µg/mL spectinomycin were added, and the plate was incubated at 37°C until 48 hpi. At 48 hpi, the transformants were harvested and infected onto a new McCoy cell monolayer. These harvest and infection steps were repeated every 44-48 hpi until fluorescent, antibiotic-resistant inclusions were observed.

(RT-)qPCR

HeLa cells were infected with chlamydial transformants at an MOI of 0.5. At 10 hpi, 5 nM anhydrotetracycline (aTc) was added or not to the culture medium. Total RNA and DNA were harvested at this time point from duplicate wells not treated with aTc. At 14 and 24 hpi, total RNA and DNA were collected using Trizol (Invitrogen) and DNeasy Tissue (Qiagen) kit, respectively, as described elsewhere(33). After DNase treatment of total RNA, cDNA was synthesized using Superscript III reverse transcriptase (Invitrogen). After diluting the cDNA 10-fold, 5 µl of the diluted cDNA was used as a template for qPCR. Equal masses of genomic DNA were used from each of the samples to quantify chlamydial genomes, which were used to normalize transcript data as described(41). For both cDNA and gDNA samples, qPCR reactions were prepared using 2X SYBR Green (ThermoFisher) in a total volume of 25 uL per well. Standard cycling conditions were used with a melting curve analysis to verify products. Transcripts and genome copies were assessed from at least three biological replicates.

Indirect immunofluorescence assay (IFA)

HeLa cells were infected with chlamydial transformants as above. At 10 hpi, 5 nM aTc was added or not, and the infected cells were fixed with fixing solution (3.2% formaldehyde and 0.022% glutaraldehyde in 1X DPBS) for 2 min and permeabilized with 90% MeOH for 1 min at 10.5 hpi or 24 hpi. The fixed cells were labeled with primary antibodies including rabbit anti-DacA (custom anti-peptide antibody targeting the C-terminal TRNERKTNPIISWMRKK prepared by Pacific Immunology, Ramona, CA), goat anti-major outer-membrane protein (MOMP; Meridian, Memphis, TN), and rabbit anti-six histidine tag (Genscript, Piscataway, NJ and Abcam, Cambridge, UK, respectively). To visualize the primary antibodies, donkey anti-goat antibody (488), donkey anti-mouse (405), or donkey anti-rabbit antibody (594) were used as secondary antibodies. The secondary antibodies were obtained from Invitrogen or Jackson Immunology (West Grove, PA). Coverslips were observed using a Zeiss AxioImager.Z2 with Apotome2 as noted in the figure legends.

Inclusion forming unit (IFU) measurement

HeLa cells were infected with chlamydial transformants as above. At 10 hpi, 5 nM aTc was added or not to the culture medium. At the indicated times, infected cells were harvested in 1 mL 2SP media then frozen at -80°C. After thawing the lysates, the samples were serially 1:10 diluted and used to infect HeLa cells seeded in 24-well plates. At 24 hpi, the number of GFP expressing inclusions was counted from 30 fields of view to calculate the IFUs from the original sample. Three biological replicates were performed.

Cyclic di-AMP measurement

HeLa cells were infected with the indicated chlamydial transformants as above. At 10 hpi, expression of the constructs was induced or not with 5 nM aTc, and, at 16 and 24 hpi, samples were prepared for measuring the level of cyclic di-AMP. After aspirating the media, the infected cell monolayers were washed with 1X PBS and resuspended with B-Per Bacterial Cell Lysis Buffer (Thermo). Samples were vortexed for 1 min and then centrifuged at 13,300xg for 15 min at 4 . The levels of cyclic di-AMP from the supernatants of the cell lysates were quantified using the Cyclic di-AMP ELISA kit (Cayman) following the manufacturer's instructions(53).

Preparation of RNA sequencing samples

Samples were prepared as reported previously(33). Briefly, the transformants of *dacA*op and *dacA*-KD were infected into HeLa cells. At 10 hpi, the constructs were induced or not with 5 nM aTc. RNA samples were prepared from *dacA*op-infected cells and *dacA*-KD-infected cells at 16 hpi and 24 hpi, respectively. 20 µg RNA samples were treated with DNase to remove DNA contamination using DNA-free Turbo kit (Thermo) according to the manufacturer's instruction. Ribosomal RNA was depleted from samples using the MICROBEnrich (Thermo) and MICROBExpress kits (Thermo) following the manufacturer's instructions. RNA samples were processed for sequencing by the UNMC Genomics Core Facility. The resultant libraries from the individual samples were multiplexed and subjected to 100-bp paired-read sequencing to generate approximately 60 million pairs of reads per sample on an Illumina NovaSeq6000 sequencer in the UNMC Genomics Core facility. The original fastq format reads were trimmed by fqtirm tool (*https://ccb.jhu.edu/software/fqtrim*) to remove adapters, terminal unknown bases (Ns) and low quality 3' regions (Phred score < 30). The trimmed fastq files were processed by FastQC (54) for quality control . *Chlamydia trachomatis* 434/Bu bacterial reference genome and annotation files were downloaded from Ensembl (*http://bacteria.ensembl.org/Chlamydia_trachomatis_434_bu/Info/Index*). Sequencing data were analyzed by the Bioinformatics and Systems Biology Core (BSBC). The trimmed fastq files were mapped to *Chlamydia trachomatis* 434/Bu by CLC Genomics Workbench 23 for RNAseq analyses.

Statistical Analysis

To analyze the statistical significance between uninduced and induced samples of qPCR and IFU data, we used two sample equal variance Student's t-test. For the levels of cyclic di-AMP data, we used two sample equal variance Student's t-test.

Data availability

The raw and processed RNA sequencing reads in fastq format have been deposited in the Gene Expression Omnibus (GEO; *www.ncbi.nlm.nih.gov/geo/*) under accession no. GSE252732. All other data are available in the main text or the supplementary materials.

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Additional information

Author contributions

Junghoon Lee, Methodology, Investigation, Visualization, Writing – original draft, and Writing-review & editing

Scot P. Ouellette, Conceptualization, Methodology, Investigation, Visualization, Supervision, and Writing-review & editing

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Additional files

Supplemental Figure 1

Supplemental Figure 2

Supplemental Figure 3

Supplemental Figure 4

Supplemental Figure 5

Supplemental Figure 6

Supplemental Figure 7

Supplemental Figure 8

Supplemental Figure 9

Supplemental Figure 10

Supplemental Table S1

Supplemental Figure Legends

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Reviewer #2 (Public review):

This manuscript describes the role of the production of c-di-AMP on the chlamydial developmental cycle. The main findings remain the same. The authors show that overexpression of the dacA-ybbR operon results in increased production of c-di-AMP and early expression of transitionary and late genes. The authors also knocked down the expression of the dacA-ybbR operon and reported a modest reduction in the expression of both hctA and omcB. The authors conclude with a model suggesting the amount of c-di-AMP determines the fate of the RB, continued replication, or EB conversion.

Overall, this is a very intriguing study with important implications however the data is very preliminary and the model is very rudimentary. The data support the observation that dramatically increased c-di-AMP has an impact on transitionary gene expression and late

gene expression suggesting dysregulation of the developmental cycle. This effect goes away with modest changes in c-di-AMP (detaTM-DacA vs detaTM-DacA (D164N)). However, the model predicts that low levels of c-di-AMP delays EB production is not not well supported by the data. If this prediction were true then the growth rate would increase with c-di-AMP reduction and the data does not show this. The levels of of c-di-AMP at the lower levels need to be better validated as it seems like only very high levels make a difference for dysregulated late gene expression. However, on the low end it's not clear what levels are needed to have an effect as only DacAopMut and DacAopKD show any effects on the cycle and the c-di-AMP levels are only different at 24 hours.

The data still do not support the overall model.

In Figure 1 the authors show at 24 hpi.

DacA overexpression increases cdiAMP to ~4000 pg/ml

DacAmut overexpression reduces cdiAMP dramatically to ~256 pg/ml)

DacATM overexpression increases cdiAMP to ~4000 pg/ml.

DacAmutTM overexpression does not seem to change cdiAMP ~1500 pg/ml .

dacAKD decreases cdiAMP to ~300 pg/ml .

dacAKDcom increased cdiAMP to ~8000 pg/ml.

DacA-ybbRop overexpression increased cdiAMP to ~500,000 pg/ml.

DacA-ybbRopmut ~300 pg/ml.

However in Figure 2 the data show that overexpression of DacA (cdiAMP ~4000 pg/ml) did not have a different phenotype than over expression of the mutant (cdiAMP ~256 pg/ml). HctA expression down, omcB expression down, euo not much change, replication down, and IFUs down. Additionally, Figure 3 shows no differences in anything measured although cdiAMP levels were again dramatically different. DacATM overexpression (~4000 pg/ml) and DacAmutTM (~1500). This makes it unclear what cdiAMP is doing to the developmental cycle.

In Figure 4 the authors knockdown dacA (dacA-KD) and complement the knockdown (dacA-KDcom) dacAKD decreases cdiAMP (~300) while DacA-KDcom increases cdiAMP much above wt (~8000).
KD decreased hctA and omcB at 24hpi. Complementation resulted in a moderate increase in hctA at a single time point but not at 24 hpi and had no effect on euo or omcB expression. Importantly, complementation decreased the growth rate. Based on the proposed model, growth rate should increase as the chlamydia should all be RBs and replicating and not exiting the cell cycle to become EBs (not replicating). Interestingly reducing cdiAMP levels by over expressing DacAmut (~256 pg/ml) did not have an effect on the cycle but the reduction in cdiAMP by knockdown of dacA (~300 pg/ml) did have a moderate effect on the cycle.

For Figure 5 DacA-ybbRop was overexpressed and this increased cdiAMP dramatically ~500,000 pg/ml as compared to wt ~1500. This increased hctA only at an early timepoint and not at 24hpi and again had no effect on omcB or euo. Overexpression of the operon with the mutation DacA-ybbRopmut reduced cdiAMP to ~300 pg/ml and this showed a reduction in growth rate similar to dacAmut but a more dramatic decrease in IFUs.

Overall:

DacA overexpression increases cdiAMP to ~4000 pg/ml (decreased everything except euo)

DacAmut overexpression reduces cdiAMP dramatically (~256 pg/ml). (decreased everything except euo)

DacATM overexpression increases cdiAMP to ~4000 pg/ml (no changes noted)

DacAmutTM overexpression does not seem to change cdiAMP ~1500 pg/ml (no changes noted)

dacAKD decrease cdiAMP to ~300 pg/ml (decreased everything except euo)

dacAKDcom increased cdiAMP to ~8000 pg/ml (decreases growth rate, increase hctA a little but not omcB)

DacA-ybbRop overexpression increased cdiAMP to ~500,000 pg/ml (decreases growth rate, increase hctA a little but not omcB)

DacA-ybbRopmut ~300 pg/ml (decreased everything except euo)

Overall, the data show that increasing cdiAMP only has a phenotype if it is dramatically increased, no effect at 4000 pg/ml. Decreasing cdiAMP has a consistent effect, decreased growth rate, IFU, hctA expression and omcB expression. However, if their proposed model was correct and low levels of cdiAMP blocked EB conversion then more chlamydial cells would be RBs (dividing cells) and the growth rate should increase. Conversely, if cdiAMP levels were dramatically raised then all RBs would all convert and the growth rate would be very low. When cdiAMP was raised to ~4000 pg/ml there was no effect on the growth rate. However, an increase to ~8000 pg/ml resulted in a significant decrease but growth continued. Increasing cdAMP to ~500,000 pg/ml had less of an impact on the growth rate. Overall, the data does not cleanly support the proposed model.

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Author response:

The following is the authors' response to the current reviews

> ***Reviewer #2 (Public review):***
>
> *This manuscript describes the role of the production of c-di-AMP on the chlamydial developmental cycle. The main findings remain the same. The authors show that overexpression of the dacA-ybbR operon results in increased production of c-di-AMP and early expression of transitionary and late genes. The authors also knocked down the expression of the dacA-ybbR operon and reported a modest reduction in the expression of both hctA and omcB. The authors conclude with a model suggesting the amount of c-di-AMP determines the fate of the RB, continued replication, or EB conversion.*
>
> *Overall, this is a very intriguing study with important implications however the data is very preliminary and the model is very rudimentary. The data support the observation that dramatically increased c-di-AMP has an impact on transitionary gene expression and late gene expression suggesting dysregulation of the developmental cycle. This effect goes away with modest changes in c-di-AMP (detaTM-DacA vs detaTM-DacA (D164N)). However, the model predicts that low levels of c-di-AMP delays EB production is not not well supported by the data. If this prediction were true then the growth rate would increase with c-di-AMP reduction and the data does not show this. The levels of of c-di-AMP at the lower levels need to be better validated as it seems like only very high levels make a difference for dysregulated late gene expression. However, on the low end it's not clear what levels are needed to have an effect as only DacAopMut and DacAopKD show any effects on the cycle and the c-di-AMP levels are only different at 24 hours.*

These appear to be the same comments the reviewer presented last time, so we will reiterate our prior points here and elsewhere. We do not think and nor do we predict that low c-di-AMP levels should increase growth rate (as measured by gDNA levels), and this conclusion cannot be drawn from our data. Rather, we predict that the inability to accumulate c-di-AMP should delay production of EBs, and this is what the data show. The reviewer has applied their own subjective (and erroneous) interpretation to the model. The asynchronicity of the normal developmental cycle means RBs continue to replicate as EBs are forming, so gDNA levels cannot be used as the sole metric for determining RB levels. We show that reduced c-di-AMP levels reduce EB levels as well as transcripts associated with late stages of development. The parsimonious interpretation of these data support that low c-di-AMP levels delay progression through the developmental cycle consistent with our model.

> *The data still do not support the overall model.*

We disagree. We have presented quantified data that include appropriate controls and statistical tests, and the reviewer has not disputed that or pointed to additional experiments that need to be performed. The reviewer has imposed a subjective interpretation of our model based on their own biases. A reader is free, of course, to disagree with our model, but a reviewer should not block a manuscript based on such a disagreement if no experimental flaws have been identified.

> *In Figure 1 the authors show at 24 hpi.*

We also showed data from 16hpi, which is a more relevant timepoint for assessing premature transition to EBs. In contrast, the 24hpi is more important for assessing developmental effects of reduced c-di-AMP levels.

> *DacA overexpression increases cdiAMP to ~4000 pg/ml*
>
> *DacAmut overexpression reduces cdiAMP dramatically to ~256 pg/ml)*
>
> *DacATM overexpression increases cdiAMP to ~4000 pg/ml.*
>
> *DacAmutTM overexpression does not seem to change cdiAMP ~1500 pg/ml .*
>
> *dacAKD decreases cdiAMP to ~300 pg/ml .*
>
> *dacAKDcom increased cdiAMP to ~8000 pg/ml.*
>
> *DacA-ybbRop overexpression increased cdiAMP to ~500,000 pg/ml.*
>
> *DacA-ybbRopmut ~300 pg/ml.*
>
> *However in Figure 2 the data show that overexpression of DacA (cdiAMP ~4000 pg/ml) did not have a different phenotype than over expression of the mutant (cdiAMP ~256 pg/ml). HctA expression down, omcB expression down, euo not much change, replication down, and IFUs down. Additionally, Figure 3 shows no differences in anything measured although cdiAMP levels were again dramatically different. DacATM overexpression (~4000 pg/ml) and DacAmutTM (~1500). This makes it unclear what cdiAMP is doing to the developmental cycle.*

As we have explained in the text and in response to reviewer comments on previous rounds of review, overexpressing the full-length WT or mutant DacA is detrimental to developmental cycle progression for reasons that have nothing to do with c-di-AMP levels (likely disrupting membrane function), since, as the reviewer notes, the WT DacA deltaTM strain had similar c-di-AMP levels but no negative effects on growth/development. If we had not presented the

effects of overexpressing the individual isoforms, then a reviewer would surely have requested such, which is why we present these data even though they don't seem to support our model. This is an honest representation of our findings. The reviewer seems intent on nitpicking a minor datapoint that seems to contradict the rest of the manuscript while ignoring or not carefully reading the rest of the manuscript.

> *In Figure 4 the authors knockdown dacA (dacA-KD) and complement the knockdown (dacA-KDcom)*
>
> *dacAKD decreases cdiAMP (~300) while DacA-KDcom increases cdiAMP much above wt (~8000).*
>
> *KD decreased hctA and omcB at 24hpi. Complementation resulted in a moderate increase in hctA at a single time point but not at 24 hpi and had no effect on euo or omcB expression.*

By 24hpi, late gene transcripts are being maximally produced during a normal developmental cycle. It is unclear why the reviewer thinks that these transcripts should be elevated above this level in any of our strains that prematurely transition to EBs. There is no basis in the literature to support such an assumption. As we noted in the text, the dacA-KDcom strain phenocopied the dacAop OE strain, and we showed RNAseq data and EB production curves for the latter that support our conclusions of the effect of increased c-di-AMP levels on developmental progression.

> *Importantly, complementation decreased the growth rate.*

Yes, since the c-di-AMP levels breached the "EB threshold" at 16hpi, it causes premature transition to EBs, which do not replicate their gDNA, at an earlier stage of the cycle when fewer organisms are present. Therefore, the gDNA levels are decreased at 24hpi, which is consistent with our model.

> *Based on the proposed model, growth rate should increase as the chlamydia should all be RBs and replicating and not exiting the cell cycle to become EBs (not replicating).*

This is a spurious conclusion from the reviewer. As we clearly showed, the dacA-KDcom did not restore a wild-type phenotype and instead mimicked the dacAop OE strain. This was commented on in the text.

> *Interestingly reducing cdiAMP levels by over expressing DacAmut (~256 pg/ml) did not have an effect on the cycle but the reduction in cdiAMP by knockdown of dacA (~300 pg/ml) did have a moderate effect on the cycle.*

This is again a spurious conclusion from the reviewer. The dacAMut and dacA-KD strains are distinct. As noted in the text and above for DacA WT OE, overexpressing the DacAMut similarly disrupts organism morphology, which is different from dacA-KD. These strains should not be directly compared because of this. This point has been previously highlighted in the text (in Results and Discussion).

> *For Figure 5 DacA-ybbRop was overexpressed and this increased cdiAMP dramatically ~500,000 pg/ml as compared to wt ~1500. This increased hctA only at an early timepoint and not at 24hpi and again had no effect on omcB or euo.*

As we explained in prior reviews, our RNAseq data more comprehensively assessed transcripts for the dacAop OE strain. These data show convincingly that late gene transcripts (not just hctA and omcB) are elevated earlier in the developmental cycle. Again, it is not clear

why the reviewer should expect that late gene transcripts should be higher in these strains than they are during a normal developmental cycle. This is not part of our model and appears to be a bias that the reviewer has imposed that is not supported by the literature.

> *Overexpression of the operon with the mutation DacA-ybbRopmut reduced cdiAMP to ~300 pg/ml and this showed a reduction in growth rate similar to dacAmut but a more dramatic decrease in IFUs.*

As we described in the text, in earlier revisions, and above, the dacAMut OE strain has distinct effects unrelated to c-di-AMP levels and, therefore, should not be compared to other strains in terms of linking its c-di-AMP levels to its phenotype.

> *Overall:*
>
> *DacA overexpression increases cdiAMP to ~4000 pg/ml (decreased everything except euo)*
>
> *DacAmut overexpression reduces cdiAMP dramatically (~256 pg/ml). (decreased everything except euo)*
>
> *DacATM overexpression increases cdiAMP to ~4000 pg/ml (no changes noted)*
>
> *DacAmutTM overexpression does not seem to change cdiAMP ~1500 pg/ml (no changes noted)*
>
> *dacAKD decrease cdiAMP to ~300 pg/ml (decreased everything except euo)*
>
> *dacAKDcom increased cdiAMP to ~8000 pg/ml (decreases growth rate, increase hctA a little but not omcB)*
>
> *DacA-ybbRop overexpression increased cdiAMP to ~500,000 pg/ml (decreases growth rate, increase hctA a little but not omcB)*
> *DacA-ybbRopmut ~300 pg/ml (decreased everything except euo)*
>
> *Overall, the data show that increasing cdiAMP only has a phenotype if it is dramatically increased, no effect at 4000 pg/ml.*

Yes, this clearly shows there is a threshold - as we hypothesize! However, these thresholds are more important at the 16hpi timepoint not 24hpi (which the reviewer is referencing) when assessing premature transition to EBs. We specifically highlighted in our prior revision in Figure 1E this EB threshold to make this point clearer for the reader. Once the threshold is breached, then the overall c-di-AMP levels become irrelevant as the RBs have begun their transition to EBs.

> *Decreasing cdiAMP has a consistent effect, decreased growth rate, IFU, hctA expression and omcB expression. However, if their proposed model was correct and low levels of cdiAMP blocked EB conversion then more chlamydial cells would be RBs (dividing cells) and the growth rate should increase.*

The only effect should be normal gDNA levels, which is what we see in the dacA-KD. Given the asynchronicity of a normal developmental cycle in which RBs continue to replicate as EBs are still forming, there is no basis to assume gDNA levels should increase under these conditions for the dacA-KD strain at 24hpi.

> *Conversely, if cdiAMP levels were dramatically raised then all RBs would all convert and the growth rate would be very low.*

We agree. This is what is reflected by the dacAop OE and dacA-KDcom strains, with reduced gDNA levels at 24hpi since organisms have transitioned to EBs at an earlier time post-infection.

> *When cdiAMP was raised to ~4000 pg/ml there was no effect on the growth rate.*

Yes, because it had not breached the EB threshold at 16hpi – consistent with our model! The reviewer is confusing effects of elevated c-di-AMP at 24hpi when they should be assessed at the 16hpi timepoint for strains overproducing this molecule.

> *However, an increase to ~8000 pg/ml resulted in a significant decrease but growth continued.*

If the reviewer is referring to the dacA-KDcom strain, then this is not accurate. gDNA levels were decreased in this strain at 24hpi when the c-di-AMP levels were increased compared to the WT (mCherry OE) control at 16hpi, indicating this strain had breached the "EB threshold" and initiated conversion to EBs at an earlier timepoint post-infection when fewer organisms were present.

> *Increasing cdAMP to ~500,000 pg/ml had less of an impact on the growth rate.*

It is not clear what this conclusion is based on and what the reviewer is comparing to. This is a subjective assessment not based on our data.

> *Overall, the data does not cleanly support the proposed model.*

It is an unfortunate aspect of biology, particularly for obligate intracellular bacteria – a challenging experimental system on which to work, that the data are not always "clean". The overall effects of increased c-di-AMP levels on chlamydial developmental cycle progression we have documented support our model, and we think the reader, as always, should make their own assessment.

The following is the authors' response to the original reviews.

> ***Reviewer #2 (Public review):***
>
> *This manuscript describes the role of the production of c-di-AMP on the chlamydial developmental cycle. The main findings remain the same. The authors show that overexpression of the dacA-ybbR operon results in increased production of c-di-AMP and early expression of transitionary and late genes. The authors also knocked down the expression of the dacA-ybbR operon and reported a modest reduction in the expression of both hctA and omcB. The authors conclude with a model suggesting the amount of c-di-AMP determines the fate of the RB, continued replication, or EB conversion.*
>
> *Overall, this is a very intriguing study with important implications however, the data is very preliminary, and the model is very rudimentary. The data support the observation that dramatically increased c-di-AMP has an impact on transitionary gene expression and late gene expression suggesting dysregulation of the developmental cycle. This effect goes away with modest changes in c-di-AMP (detaTM-DacA vs detaTM-DacA (D164N)). However, the model predicts that low levels of c-di-AMP delays EB production is not not well supported by the data. If this prediction were true then the growth rate would increase with c-di-AMP reduction and the data does not show this.*

Thank you for the comments. We have apparently not adequately communicated our predictions and the model. We do not think and nor do we predict that low c-di-AMP levels should increase growth rate, and there is no basis in any of our data to support that. Rather, we predict that the inability to accumulate c-di-AMP should delay production of EBs, and this is what the data show. We have clarified this in the text (line 89 paragraph).

> *The levels of c-di-AMP at the lower levels need to be better validated as it seems like only very high levels make a difference for dysregulated late gene expression. However, on the low end it's not clear what levels are needed to have an effect as only DacAopMut and DacAopKD show any effects on the cycle and the c-di-AMP levels are only different at 24 hours.*

Our hypothesis is that increasing concentrations of c-di-AMP within a given RB is a signal for it to undergo secondary differentiation to the EB, and the data support this as noted by the reviewers. Again, we stress that low levels of c-di-AMP are irrelevant to the model. We have revised Figure 1E to indicate the level of c-di-AMP in the control strain at the 24hpi timepoint that coincides with increased EB levels. We hope this will further clarify the goals of our study. That a given strain might be below the EB control is not relevant to the model beyond indicating that it has not reached the necessary threshold for triggering secondary differentiation.

> *The authors responded to reviewers' critiques by adding the overexpression of DacA without the transmembrane region. This addition does not really help their case. They show that detaTM-DacA and detaTM-DacA (D164N) had the same effects on c-di-AMP levels but the figure shows no effects on the developmental cycle.*

As it relates directly to the reviewer's point, the delta-TM strains did not show the same level of c-di-AMP. It may be that the reviewer misread the graph. The purpose of testing these strains was to show that the negative effects of overexpressing full-length WT DacA were due to its membrane localization. Both the FL and deltaTM-DacA (WT) overexpression had equivalent c-di-AMP levels even though the delta-TM overexpression looked like the mCherry-expressing strain based on the measured parameters. This shows that the c-di-AMP levels were irrelevant to the phenotypes observed when overexpressing these WT isoforms. For the mutant isoforms, the delta-TM looked like the mCherry-expressing control while the FL isoform was negatively impacted for reasons we described in the Discussion (e.g., dominant negative effect). In addition, at 16hpi, neither delta-TM strain had c-di-AMP levels that approached the 24h control as denoted in Figure 1E (dashed line) and in the text, which explains why these strains did not show increased late gene transcripts at an earlier timepoint like the dacAop and dacA-KDcom strains.

> *Describing the significance of the findings:*
>
> *The findings are important and point to very exciting new avenues to explore the important questions in chlamydial cell form development. The authors present a model that is not quantified and does not match the data well.*

We respectfully disagree with this assessment as noted above in response to the reviewer's critique. All of our data are quantified and support the hypothesis as stated.

> *Describing the strength of evidence:*
>
> *The evidence presented is incomplete. The authors do a nice job of showing that overexpression of the dacA-ybbR operon increases c-di-AMP and that knockdown or overexpression of the catalytically dead DacA protein decreases the c-di-AMP levels.*

> *However, the effects on the developmental cycle and how they fit the proposed model are less well supported.*
>
> *Overall this is a very intriguing finding that will require more gene expression data, phenotypic characterization of cell forms, and better quantitative models to fully interpret these findings.*

It is not clear what quantitative models the reviewer would prefer, but, ultimately, it is up to the reader to decide whether they agree or not with the model we present. The data are the data, and we have tried to present them as clearly as possible. We would emphasize that, with the number of strains we have analyzed, we have presented a huge amount of data for a study with an obligate intracellular bacterium. As a comparison, most publications on Chlamydia might use a handful of transformant strains, if any. Given the cost and time associated with performing such studies, it is prohibitive to attempt all the time points that one might like to do, and it is not clear to us that further studies will add to or alter the conclusions of the current manuscript.

> ***Reviewer #2 (Recommendations for the authors):***
>
> *Minor critiques*
>
> *The graphs have red and blue lines but the figure legends are red and black. It would be better if these matched.*

Changed.

> *For Figure 1C. The labels are not very helpful. It's not clear what is HeLa vs mCherry. I believe it is uninfected vs Chlamydia infected.*

Changed.

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