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Review Article

REVIEW ARTICLE

Versatility of global transcriptional regulators in alpha-Proteobacteria: from essential cell cycle control to ancillary functions

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One sentence summary: Three transcriptional modules composed of antagonistic regulatory proteins direct cell cycle transcription in *Caulobacter crescentus*, while also often performing non-essential, yet global, regulatory functions in diverse alpha-proteobacterial lineages.

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ABSTRACT

Recent data indicate that cell cycle transcription in many alpha-Proteobacteria is executed by at least three conserved functional modules in which pairs of antagonistic regulators act jointly, rather than in isolation, to control transcription in S-, G2- or G1-phase. Inactivation of module components often results in pleiotropic defects, ranging from cell death and impaired cell division to fairly benign deficiencies in motility. Expression of module components can follow systemic (cell cycle) or external (nutritional/cell density) cues and may be implemented by auto-regulation, ancillary regulators or other (unknown) mechanisms. Here, we highlight the recent progress in understanding the molecular events and the genetic relationships of the module components in environmental, pathogenic and/or symbiotic alpha-proteobacterial genera. Additionally, we take advantage of the recent genome-wide transcriptional analyses performed in the model alpha-Proteobacterium *Caulobacter crescentus* to illustrate the complexity of the interactions of the global regulators at selected cell cycle-regulated promoters and we detail the consequences of (mis-)expression when the regulators are absent. This review thus provides the first detailed mechanistic framework for understanding orthologous operational principles acting on cell cycle-regulated promoters in other alpha-Proteobacteria.

Key words: cell cycle transcription; alpha-Proteobacteria; *Caulobacter crescentus*; global regulator; CtrA; GcrA; CcrM methyltransferase; SciP; MucR; epigenetics

INTRODUCTION

Fifteen years ago, the first evidence was provided that a vast number of transcripts fluctuate as a function of cell cycle progression in the aquatic alpha-Proteobacterium *Caulobacter crescentus* (Laub *et al.*, 2000). The long-awaited matching observations were recently made in the related alpha-proteobacterial plant symbiont *Sinorhizobium meliloti* from the order Rhizobiales (De Nisco *et al.*, 2014). Together these studies provide strong evidence that transcript oscillations may be a general feature of the

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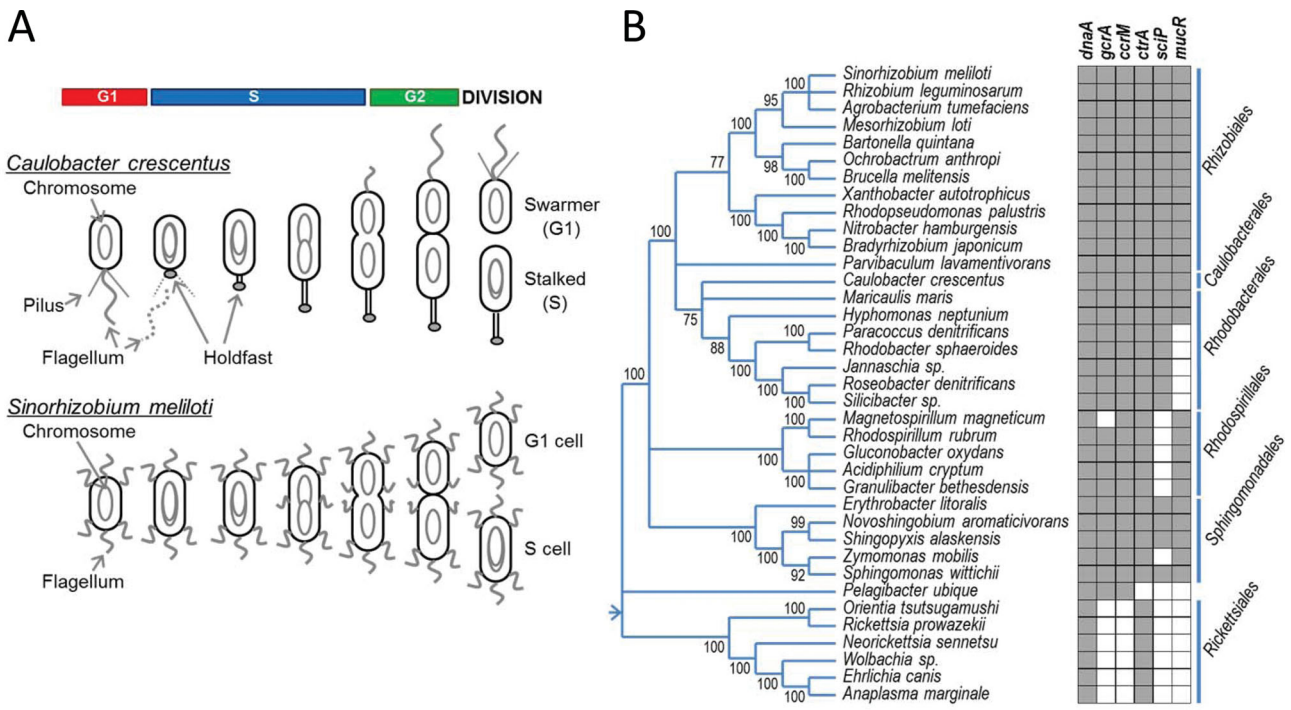

Figure 1. Cell cycle progression and conservation of global transcriptional regulators controlling cell cycle transcription in alpha-Proteobacteria. **A.** Schematic of the *C. crescentus* and *S. meliloti* cell cycles. *Caulobacter crescentus* and *S. meliloti* divide asymmetrically at the end of each cell cycle giving rise to two progeny cells with distinct sizes and fates. The smaller G1-arrested daughter cell resides in a quiescent non-replicative (G1-like) state and must differentiate into the S-phase replicative form before proceeding to division. While both *S. meliloti* daughter cells have peritrichous flagella and are motile, only one daughter cell is flagellated and motile in *C. crescentus*. Note that *S. meliloti* has three replicons (a circular chromosome and two large plasmids) but for simplicity its genome is represented here as one circular chromosome as in De Nisco *et al.* (2014). The G1-arrested Caulobacter daughter cell, also known as 'swarmer' cell, is motile and chemotactic. It possesses several adhesive pili and a single flagellum at one pole. During the process of differentiation into the sessile 'stalked' cell, the flagellum and pili are lost and replaced by a tubular stalk structure harboring an adhesive holdfast which is elaborated from the vacated pole. **B.** As discussed in this review, a set of global transcriptional regulators promote the rise and fall of selected transcripts in *C. crescentus* and these cell cycle regulators are conserved among the alpha-Proteobacteria. The phylogenetic tree shows the presence (grey box) or absence (white box) of genes predicted to encode orthologs of DnaA, GcrA, CcrM, CtrA, SciP and MucR. This phylogenetic tree is adapted from Fig. 2 and table S2 of Brilli *et al.* (2010) for DnaA, GcrA, CcrM and CtrA orthologous gene distributions. BLAST analysis tool from NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to determine the distribution of SciP and MucR in the same selected alpha-proteobacterial dataset.

alpha-proteobacterial cell cycle and perhaps the cell cycles of other bacterial phyla, irrespective of the ecological niche that the bacteria occupy. As a plant symbiont, *S. meliloti* lives in a different habitat (soil) than *C. crescentus* (fresh-water), but both lineages rely on conserved systemic cues and effector proteins to orchestrate transcript fluctuations with cell cycle progression (Brilli *et al.*, 2010; Ardissone and Viollier 2012). A cell cycle-regulated polysaccharide capsule permits synchronization of *C. crescentus* cultures by simple density-gradient centrifugation (Ardissone *et al.*, 2014). Owing to this technical advantage along with the genetic tractability of *C. crescentus*, the molecular underpinnings of cell cycle transcription were primarily dissected in this model system, but important recent studies from alpha-proteobacterial symbionts and pathogens lend credence to the idea that the features apply to many other systems (Laub *et al.*, 2000; De Nisco *et al.*, 2014; Deghelt *et al.*, 2014; Fumeaux *et al.*, 2014).

The idea that cell cycle-controlled transcription is likely widespread in alpha-Proteobacteria could be inferred by the observation that cell division in many lineages is morphologically asymmetric (Hallez *et al.*, 2004), yielding daughter cells that reside in different cell cycle stages in which distinct transcriptional programs should be active (Laub, Shapiro and McAdams 2007; De Nisco *et al.*, 2014; Deghelt *et al.*, 2014). The smaller daughter cell is temporarily arrested in a quiescent G1-like phase, unable to replicate its genome or to divide. By contrast, the larger daughter resides in S-phase, progresses into the G2-phase transcriptional program of a pre-divisional cell. Upon compartmentalization of the pre-divisional cell into two unequally sized chambers, the G1-phase transcriptional program is re-instated in the smaller chamber and S-phase transcription resumes in the other (Fig. 1A).

In *C. crescentus*, the larger S-phase cell features a stalk at the old pole and it matures into pre-divisional (G2-phase) cell that has a single flagellum and the pilus secretion machinery at the new cell pole. A flagellated and piliated G1-phase (swarmer) cell and a non-motile S-phase (stalked) cell emerge from each division (Skerker and Laub 2004). Forward genetics was used to unearth several key factors regulating cell cycle transcription (Fig. 2A). Bypass mutants (Murray *et al.*, 2013; Fumeaux *et al.*, 2014) revealed that the identified transcription factors act in antagonistic pairs (defined here as transcriptional modules) that direct cell cycle transcription sequentially to S-, G2- and G1-phase (Fig. 2B). The GcrA transcription factor (Holtzendorff *et al.*, 2004) and the CcrM adenine methyltransferase (Zweiger, Marczynski and Shapiro 1994), whose methylation at the N6 position of adenine (m6A) in the context of 5′-GANTC-3′ recognition sequence in double-stranded DNA (henceforth GANTC), are required for efficient transcription of many S-phase promoters (Fioravanti *et al.*, 2013; Murray *et al.*, 2013; Gonzalez *et al.*, 2014). By contrast, CtrA, a response regulator that also negatively regulates replication initiation (Quon, Marczynski and Shapiro 1996)

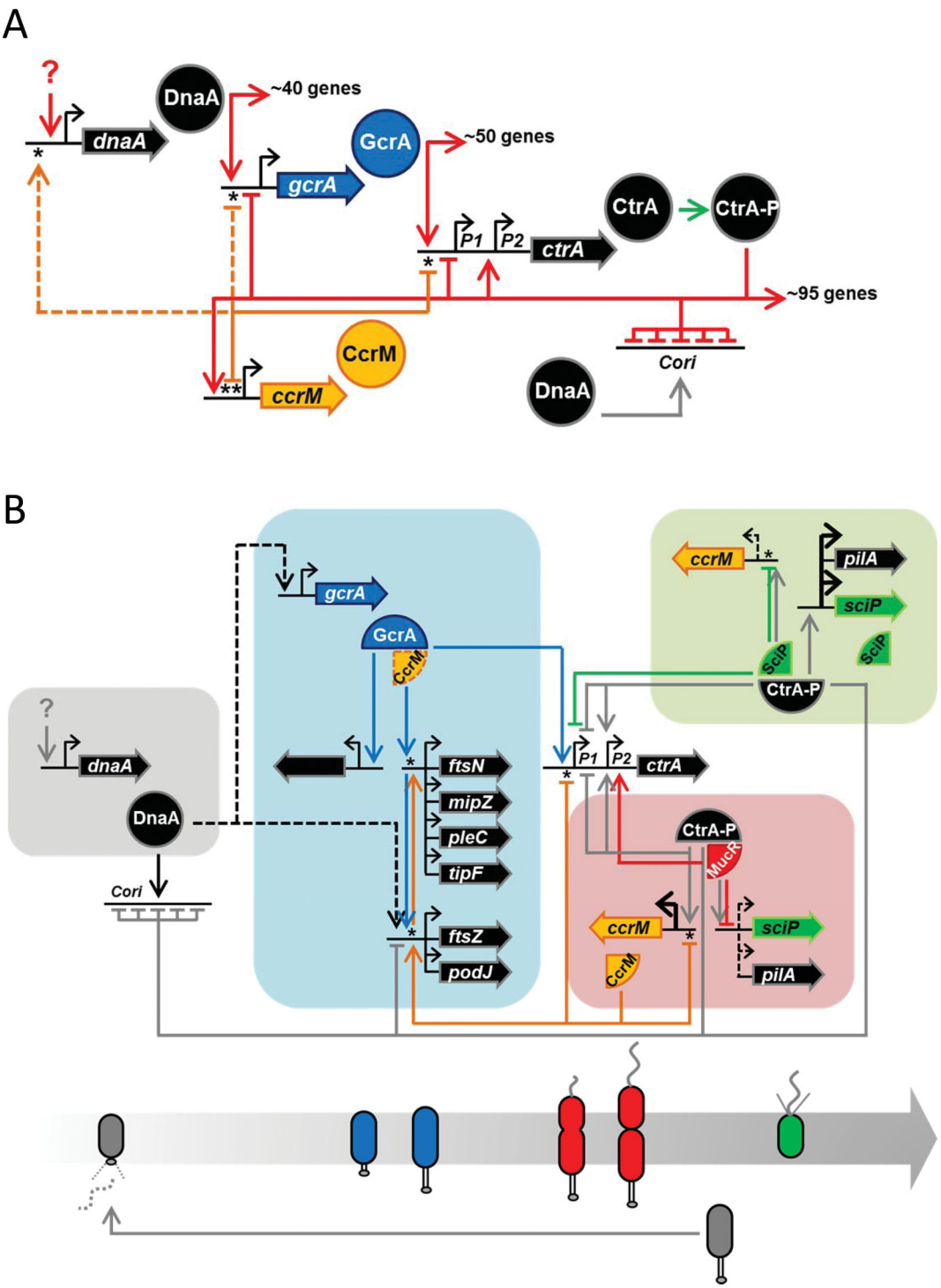

Figure 2. Refinement of the *C. crescentus* regulatory network orchestrated by three transcriptional modules acting on cell cycle-regulated target promoters. **A.** Representation of the genetic regulatory network controlling cell cycle transcription as detailed previously (Brilli *et al.*, 2010; Collier 2012; Mohapatra, Fioravanti and Biondi 2014) describing transcriptional regulators (DnaA, GcrA, CtrA and CcrM) acting sequentially and in isolation. Red lines represent transcriptional (activation or repression) controls by the regulators. Orange lines represent methylation control by CcrM on promoters. The asterisk (*) represents the methylation site (5′-GANTC-3′). The green line represents the phosphorylation of CtrA (CtrA-P) by the CckA phosphorelay. Broken lines correspond to connections that remain controversial and question marks indicate elements of the circuit that are not understood. **B.** New three-tiered modular network showing each module: S-phase (boxed in blue) controlled by GcrA and CcrM, G2-phase (boxed in red) instated by CtrA and SciP, and G1-phase (green) dictated by CtrA and MucR. The scheme below the panel shows the corresponding time in the cell cycle when the target promoters of each module fire. The fluctuation of transcripts from these target genes during the cell cycle as determined by microarray (Laub *et al.*, 2000; McGrath *et al.*, 2007; Fang *et al.*, 2013) and RNA-Seq analysis is shown in Fig. 3. DnaA control at the promoter of the *gcrA*, *podJ* and *ftsZ* genes is depicted as dotted lines because the occupancy of this promoter by DnaA has only demonstrated *in vitro*, but not yet been observed *in vivo*.

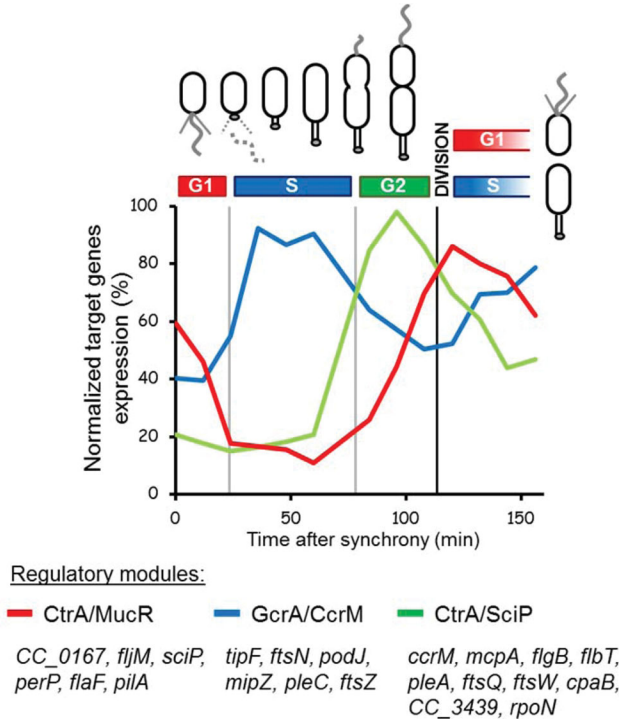

Figure 3. GcrA/CcrM, CtrA/SciP and CtrA/MucR transcriptional modules respectively control sequential waves of transcription in S-, G2- and G1-phase. For each module, a list of validated target genes was generated based on the ChIP-seq data from Holtzendorff *et al.* (2004), Gora *et al.* (2010), Tan *et al.* (2010), Fioravanti *et al.* (2013), Murray *et al.* (2013), Fiebig *et al.* (2014) and Fumeaux *et al.*, 2014). Prerequisites to be select are: (i) expression of the target gene is dependent of regulators of the selected module (published microarrays or promoter reporter assays, see citations above) and (ii) this regulation must be direct as the promoter region of the target gene is bind (at least) by the considered regulators of the module (published ChIP, ChIP-seq, footprinting or EMSA experiments). For each transcriptional module, an average value of the temporal expression profile [from McGrath *et al.* (2007)] of listed genes was calculated and normalized to maximal expression. This average temporal expression profile is depicted as sequential waves of activities of GcrA/CcrM, CtrA/SciP and CtrA/MucR transcriptional modules.

either directly and/or indirectly (Quon *et al.*, 1998; Bastedo and Marczynski 2009), binds and induces the accumulation of G2- and G1-phase-specific transcripts. CtrA acts with different negative regulators to restrict promoter firing to the correct cell cycle phase (Laub *et al.*, 2000, 2002; Fiebig *et al.*, 2014; Fumeaux *et al.*, 2014). The G2-phase transcriptional module comprises CtrA and the helix-turn-helix domain protein SciP that is only present in G1-phase (Gora *et al.*, 2010; Tan *et al.*, 2010) and targets the G2-class of CtrA-activated promoters (Fumeaux *et al.*, 2014) (Fig. 3). By contrast, the MucR paralogs (henceforth MucR1/2) resemble ancestral Zinc-finger proteins (Close, Tait and Kado 1985; Malgieri *et al.*, 2007; Baglivo *et al.*, 2009) and bind the promoters of G1-phase genes (Fumeaux *et al.*, 2014). Thus, MucR1/2 and CtrA control the G1-phase transcriptional module.

GcrA, CcrM, SciP and CtrA have each been described as essential master cell cycle regulators in recent reviews (McAdams and Shapiro 2011; Collier 2012). Here, we instead refer to these cell cycle protein as 'global regulators' on the grounds of recent genetic experiments showing (i) that GcrA, CcrM, SciP or MucR1/2 are dispensable in *C. crescentus* (Gora *et al.*, 2010; Gonzalez and Collier 2013; Murray *et al.*, 2013; Fumeaux *et al.*, 2014), (ii) that CtrA can be inactivated in several alpha-proteobacterial lineages including the *Rhodobacterales* and the *Rhodospirillales* (Mercer *et al.*, 2010; Bird and MacKrell 2011; Greene *et al.*, 2012; Zan *et al.*, 2013; Wang *et al.*, 2014) and (iii) that these regulators bind a vast number of developmental promoters and regulatory sites *in vivo* (CtrA targets ca. 283 sites, GcrA ca. 218, SciP ca. 51 and MucR1/2 ca. 113/134) (Fioravanti *et al.*, 2013; Fumeaux *et al.*, 2014). For the essential replication initiator DnaA, also classified previously as a master transcriptional regulator (McAdams and Shapiro 2011; Collier 2012), firm conclusions on its direct role in cell cycle transcription (as opposed to indirect effects stemming from its essential role in replication initiation), must await molecular or genetic dissection of the two activities of DnaA and/or the determination of DnaA-bound promoters *in vivo*. On the basis of this limitation, we put the contribution of DnaA provisionally aside in this review, although we acknowledge *in vitro* experiments supporting the notion that DnaA acts concurrently with or before the GcrA/CcrM module (Hottes, Shapiro and McAdams 2005).

The GcrA/CcrM module activates S-phase promoters

The GcrA/CcrM module is the first epigenetic regulatory pair reported to have a global role in cell cycle transcription in bacteria. Phylogenetic analysis reveals that coding sequences for GcrA and CcrM generally co-occur in alpha-proteobacterial genomes (Brilli *et al.*, 2010; Murray *et al.*, 2013) (Fig. 1B). GcrA is induced with the onset of DNA replication in *C. crescentus* cells (Hottes, Shapiro and McAdams 2005; Collier, Murray and Shapiro 2006) and binds S-phase promoters *in vivo* (Fioravanti *et al.*, 2013; Murray *et al.*, 2013). Most of the preferred targets of GcrA harbor m6A GANTC marks introduced by CcrM (Zweiger, Marczynski and Shapiro 1994) (Fig. 4). However, as CcrM only accumulates after these S-phase promoters fire (i.e. in G2-phase), GANTC methylation must occur in the G2-phase of the previous cell cycle, facilitating the recruitment of GcrA to these promoters in the S-phase of the ensuing cell cycle (Fioravanti *et al.*, 2013).

GANTC methylation clearly enhances GcrA binding to its target promoter *in vitro* and *in vivo* (Fioravanti *et al.*, 2013). *In vitro* GcrA binds its target promoters even in the absence of methylation, but the binding is enhanced *in vivo* and *in vitro* when the overlapping GANTC motif(s) carries an m6A methylation mark (Fioravanti *et al.*, 2013). Such methylation-dependent binding *in vitro* has also been observed for GcrA from other alpha-Proteobacteria (Fioravanti *et al.*, 2013), suggesting that this mechanism of GcrA-dependent promoter recruitment and presumably activation is conserved in many alpha-Protebacteria. However, not all GcrA target promoters harbor GANTC sequences (Fioravanti *et al.*, 2013; Murray *et al.*, 2013). Conversely, GANTC methylation by CcrM is clearly not sufficient for the recruitment of GcrA to its targets, as indicated by the fact that GcrA has a clear preference for certain sites carrying m6A GANTC marks *in vivo*, but several other GANTC sites are also methylated and are not efficiently bound by GcrA *in vivo* (Fioravanti *et al.*, 2013; Murray *et al.*, 2013). Thus, additional determinants or possibly other methylases (Kozdon *et al.*, 2013) must also contribute to the recruitment of GcrA to its targets *in vivo*.

The methylation state of GANTC sites change sequentially as the DNA replication machinery progressively copies the chromosome and in doing so converts fully methylated GANTC to the hemi-methylated form (before CcrM is present, Fig. 4A). Thus upon replication, these genomic GANTC sites reside in a hemi-methylated state until re-methylated by CcrM once it accumulates in G2-phase. By contrast, *Cori*-distal sites (near the replication terminus) remain fully methylated for most of the cell

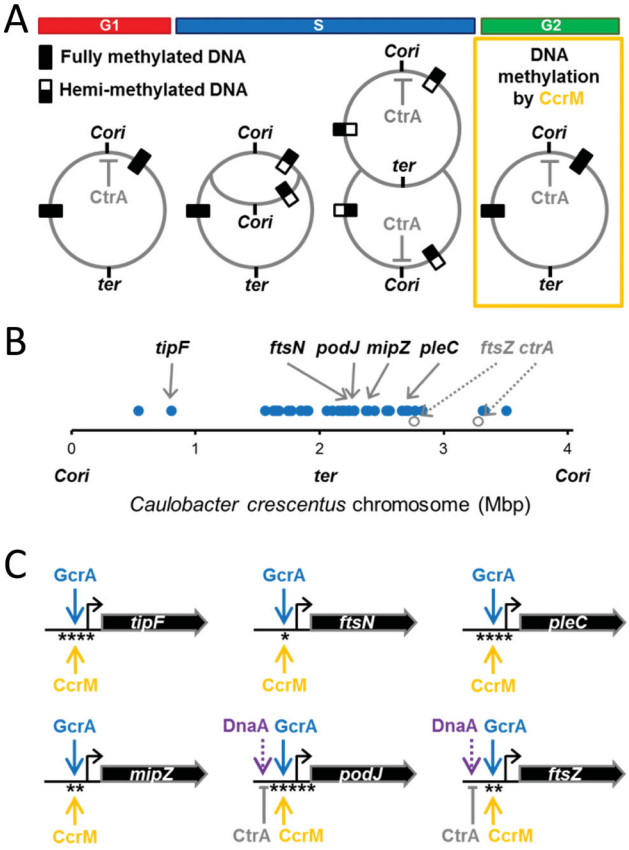

Figure 4. Target genes of the GcrA/CcrM transcriptional module and their position on the *C. crescentus* genome. **A.** Replication control and temporally regulated (re-)methylation of the *C. crescentus* chromosome at 5′-GANTC-3′ sites during cell cycle progression. *Caulobacter crescentus* has a 4.01 Mbp chromosome that is replicated only once during the cell cycle and at different times in the daughter cells after division. This replicative asymmetry is negatively controlled in G1 by CtrA. Following the degradation of CtrA during the G1→S transition, DnaA assembles on *Cori* to promote the initiation of chromosome replication. CtrA re-accumulates during the middle of S-phase and prevents re-initiation (multi-fork replication). Concurrently, with progression of the replication fork during S-phase, the GANTC sequences are converted from a full-methylated state to a hemi-methylated state. DNA re-methylation only occurs in G2-phase once the CcrM-specific methylase (methylates GANTC at the N6-position of adenine) accumulates. **B.** Many validated target genes of the GcrA/CcrM transcriptional module localize near the *ter* region. A putative gene list directly controlled by the GcrA/CcrM module was generated by combining the gene expression data of GcrA-depleted cells (Holtzendorff *et al.*, 2004), GcrA promoter occupancy as determined by ChIP-seq analysis (Fioravanti *et al.*, 2013; Murray *et al.*, 2013) and m6A-methylation data as determined by ChIP-seq analysis and global methylome analysis (Fioravanti *et al.*, 2013; Kozdon *et al.*, 2013). In agreement with published data on GcrA/CcrM dependence using transcriptional reporter strains (Fioravanti *et al.*, 2013; Murray *et al.*, 2013), *tipF*, *ftsN*, *podJ*, *mipZ* and *pleC* genes were retained in this selection of 37 putative controlled genes. Unfortunately, *ctrA* and *ftsZ* are not included in the list as they were already absent in the GcrA-regulated genes list from Holtzendorff *et al.* (2004). The position of the selected genes as a function of their location on the *C. crescentus* chromosome is depicted. **C.** Summary of all known interactions of the transcriptional regulators at the promoters of validated genes controlled by the GcrA/CcrM transcriptional module. Asterisks (*) represent the methylation site (5′-GANTC-3′) in the different promoters. DnaA control at the *podJ* and *ftsZ* promoters is depicted as dotted arrow because the occupancy at this promoter by DnaA interaction has only demonstrated *in vitro* (Hottes *et al.*, 2005), but not yet been observed *in vivo*.

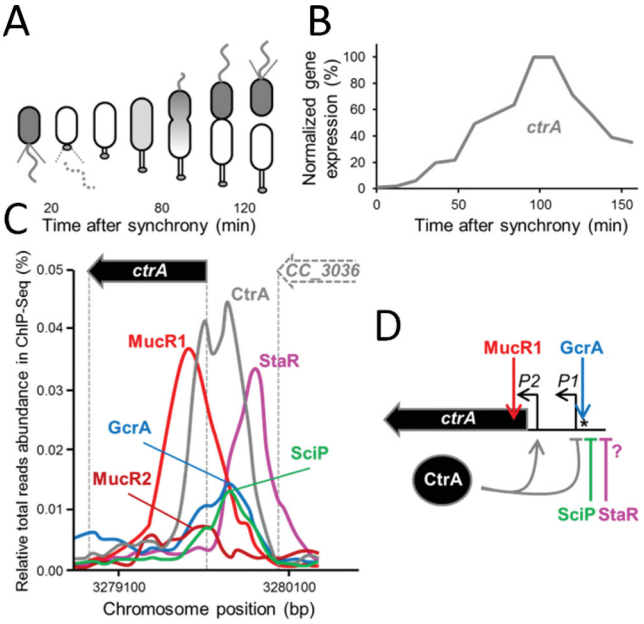

Figure 5. Complex regulation of the *C. crescentus ctrA* promoter by multiple cell cycle transcriptional regulators. **A.** The schematic shows the spatio-temporal changes of CtrA during the cell cycle. **B.** The graph shows the fluctuation of the *ctrA* transcript during the cell cycle, computed from the global transcriptional profiling data (McGrath *et al.*, 2007) normalized to maximal expression for each gene. **C.** ChIP-Seq traces showing the occupancy of various transcriptional regulators at the *ctrA* promoter based on data from Fioravanti *et al.* (2013), Murray *et al.* (2013), Fiebig *et al.* (2014) and Fumeaux *et al.* (2014). **D.** Regulation of the two promoters of the *ctrA* gene, P1 and P2. The weaker P1 promoter fires first and is activated by GcrA, repressed by SciP and CtrA, and silenced by full DNA methylation (* represents methylation site). The stronger P2 promoter is activated by CtrA in a positive feedback loop. MucR1/2 bind to the *ctrA* promoter and were also recently shown to influence *ctrA* expression (Fumeaux *et al.*, 2014). This interconnected regulation allows progressive *ctrA* expression throughout S- and G2-phase (see Fig. 5A). The *ctrA* promoter is also bound by the StaR transcription factor as described by Fiebig *et al.* (2014).

cycle as they are replicated only later (Fig. 4B). Such gradual change in methylation state during the replication cycle offers a simple and appealing way by which cells can coordinate transcription of selected genes with the cell cycle (also known as the 'methylation ratchet' model) (Collier, McAdams and Shapiro 2007). Such a mechanism may well dictate the timing of transcription of the *ctrA* gene during the cell cycle (Domian, Reisenauer and Shapiro 1999). The *ctrA* gene is located at a proximal position to the *C. crescentus* origin of replication (*Cori*) on the circular chromosome and the *ctrA* P1 promoter fires early in S-phase, coincident with the passage of the DNA replication fork past the *ctrA* locus. By contrast, most of the preferred GcrA target promoters reside at *Cori*-distal positions (Nierman *et al.*, 2001) (Fig. 4A–C), yet the transcripts of these genes are induced concurrently with *ctrA* (Laub *et al.*, 2000). The fact that GcrA-dependent promoters at both of these chromosomal locations fire in S-phase implies that the timing of promoter activation by GcrA is not directly coupled to the change in methylation state of the promoter induced by passage of the DNA replication fork past the corresponding locus (i.e. not correlated with the change in methylation state from full- to hemi-methylation). It therefore seems that the model that the replication fork passage is tied to the temporal activation of promoters is not a general feature for GcrA/CcrM-regulated S-phase promoters. One possible exception to this could to be the P1 promoter of the *ctrA* gene (henceforth *ctrA*_P1, Fig. 5D) and perhaps the promoter of

the long non-coding RNA *CCNA_R0116*, originally annotated as hypothetical gene *CCNA_00697* (Marks *et al.*, 2010). Unlike most other GcrA-dependent promoters, mutation of GANTC sites in these two promoters does not cripple promoter activity, provided that the mutation itself does not inadvertently increase the affinity of RNA polymerase (RNAP) for the mutant promoter (Reisenauer and Shapiro 2002; Fioravanti *et al.*, 2013). By contrast, *ctrA*_P1 is less active when it is moved from its normal *Cori*-proximal position to a *Cori*-distal site on the chromosome (Reisenauer and Shapiro 2002), suggesting that for this promoter there might indeed be a correlation between *ctrA*_P1 activity and the time it resides in the hemi-methylated state. GcrA binding to this promoter is enhanced by GANTC methylation *in vitro* and *in vivo* (Fioravanti *et al.*, 2013). However, GcrA exhibited the highest affinity for the fully methylated *ctrA*_P1 promoter versus the hemi-methylated promoter (and more affinity for the hemi-methylated than the non-methylated promoter) *in vitro* (Fioravanti *et al.*, 2013). These results may be reconciled with the notion that GcrA can act either positively or negatively on the *ctrA* promoter as a function of the methylation state, resulting in an apparently neutral effect on promoter activity in the absence of methylation. Two lines of evidence support this notion. First, *in vitro* footprinting revealed the different extent of binding at methylated versus hemi-methylated *ctrA*_P1 (Fioravanti *et al.*, 2013), with the former yielding a larger protection that covers the entire promoter, while the latter is confined to the −35 site and the juxtaposed GANTC site. Second, mutational analysis in GcrA depletion strains suggest that GcrA acts negatively in the absence of methylation (of the *ftsN* promoter, see below), while it acts positively when the site is methylated (Murray *et al.*, 2013).

Life and division without GcrA and/or CcrM

The target promoters of GcrA/CcrM in *C. crescentus* include the promoters of dispensable genes encoding polarity factors and regulators (e.g. *podJ*, *pleC*, *tipF* and *flaEY*; Figs 2B and 4B and C) that promote flagellum assembly and the extrusion of pili at the new pole (opposite the stalk) (Davis *et al.*, 2013; Fioravanti *et al.*, 2013; Murray *et al.*, 2013). GcrA/CcrM also regulates a number of other target promoters that promote expression of essential cell division genes, such as *ftsN* and *ftsZ* (Moll and Thanbichler 2009; Gonzalez and Collier 2013; Murray *et al.*, 2013), indicating that GcrA/CcrM controls other essential functions in addition to CtrA.

Interestingly, the GcrA/CcrM module was recently shown to be dispensable in several alpha-Proteobacteria (Fig. 1B), at least when cells grow slowly (Murray *et al.*, 2013; Curtis and Brun 2014). The fact that no orthologs of GcrA and CcrM are encoded in the sequenced genomes of *Wolbachia* and *Rickettsia* sp. (Brilli *et al.*, 2010) indicates that GcrA/CcrM is not required for survival within eukaryotic host cells, possibly reflecting growth at a reduced rate. Disruption of either *gcrA* or *ccrM* in *Brevundimonas subvibrioides*, a slow growing species featuring a doubling time 4-fold longer than that of wild-type *C. crescentus*, is apparently possible without adverse effects (Curtis and Brun 2014). Moreover, *C. crescentus* cells lacking either CcrM or GcrA grow slowly and are very filamentous owing to a shortage in division proteins (Gonzalez and Collier 2013; Murray *et al.*, 2013). It is conceivable that when growing slowly these mutants have sufficient time to accumulate enough division proteins required to assemble a complete circumferential cytokinetic structure to divide (de Boer 2010). By contrast, cell division seems insupportable under fast growth resulting in death due to a failure to divide. Thus, the GcrA/CcrM regulatory pair together may serve to coordinate fast and efficient growth and division in the free-living alpha-proteobacterial lineages (Fig. 1B).

Unexpectedly, joint inactivation of *gcrA* and *ccrM* in *C. crescentus* did not accentuate the division defect of the single mutants and render them non-viable, but instead had the opposite effect: it *improved* growth and *elevated* steady-state levels of several division proteins compared to the single mutants (Murray *et al.*, 2013). This indicates that in isolation GcrA or CcrM affect cell cycle progression in an adverse way in the absence of the other and that it is more beneficial for cells to have both or to lack both, rather than having only one of the two. This trend is recapitulated by the phylogenetic analysis shown in Fig. 1B (with the caveat that methylases of restriction modification systems can be erroneously annotation as CcrM orthologs). The antagonistic genetic relationship (where growth defects from a loss-of-function mutation in one gene can be partially compensated by a loss-of-function mutation in the second gene), along with the aforementioned biochemical dependencies, could explain the loss of *gcrA* and *ccrM* in the obligate intracellular alpha-Proteobacteria or their acquisition by the free-living lineages to permit rapid advancement through the S-phase program when needed.

What is the molecular basis for the adverse effects of GcrA on growth and division when CcrM is absent and *vice versa*? Since GcrA can interact with RNAP and can cover a large area of the promoter in a methylation-dependent manner (Fioravanti *et al.*, 2013), it is conceivable that GcrA plays a role in the proper positioning of RNAP at the promoter for activation or, when it is improperly regulated, interferes with transcriptional activation, for example by tethering RNAP to the promoter and preventing clearance of RNAP on a fully methylated promoter. Indeed, depletion of GcrA cripples the wild-type *ftsN* promoter, but causes an increase in activity of the *ftsN* 'GANTC mutant' promoter, likely due to de-repression (Murray *et al.*, 2013) (Fig. 4C). While promoter methylation by CcrM appears to convert GcrA from a negative regulator of transcription into an activator, it is unclear why CcrM acts negatively in the absence of GcrA. It is possible that CcrM performs functions independently of methylation or that methylation serves a critical but unknown role in alpha-proteobacterial cell physiology other than cell cycle transcription (Gonzalez *et al.*, 2014). Alternatively, the mere presence (binding) of CcrM at the promoter during methylation of the GANTC motif might simply interfere with residual transcription from a crippled promoter when GcrA is absent (Murray *et al.*, 2013). In this case, elimination of such an interfering activity on transcription with a *ccrM* mutation could explain the fairly modest (2-fold) increase in the steady-state levels of the cell division proteins FtsN and MipZ seen in the *C. crescentus gcrA/ccrM* double mutant versus the *gcrA* single mutant (Murray *et al.*, 2013). On the scale of an individual cell division protein, the increase in abundance in cell division proteins such as FtsN or MipZ is quite small; however, the cumulative small increase in all GcrA/CcrM-regulated cell divisions proteins could easily amount to a strong beneficial effect, explaining why the selective pressure was sufficiently strong to unearth a *ccrM* transposon (*ccrM*::Tn) mutation that improves growth of GcrA-deficient *C. crescentus* cells. On the other hand, experiments show that elevated expression of FtsN alone is sufficient to ameliorate the growth defects of *gcrA* or *ccrM* mutant cells (Murray *et al.*, 2013). It is known that overexpression of critical late division components such as FtsN can overcome shortages in other division proteins in *Escherichia coli* simply because overexpression can

stabilize the division machine or can confer sufficient critical cell wall biosynthetic activity to promote constriction (Bernard *et al.*, 2007; Gerding *et al.*, 2009).

Molecular basis of phenotypic differences between *gcrA* and *ccrM* mutants

Despite the many commonalities between GcrA and CcrM, there are also important phenotypic differences in the mutant strains that provide valuable clues about the regulation of key target genes. For example, *ccrM*-deleted cells are elongated without obvious constrictions, while *gcrA*-deleted cells are impaired at a late stage of division and show constrictions. This difference pointed to a defect in FtsZ accumulation in the former, but not in the latter (Murray *et al.*, 2013; Gonzalez *et al.*, 2014). While DnaA is known to bind the *ftsZ* promoter (and others such as the *podJ* promoter) *in vitro* (Hottes, Shapiro and McAdams 2005), it is possible that CcrM-dependent control of DnaA at the *ftsZ* promoter underlies the differences in the cell division phenotype between the *gcrA* and the *ccrM* mutants.

Alternatively, differences in CtrA levels could account for the lower levels of FtsZ accumulation in the *ccrM*::Tn mutant compared to the Δ*gcrA* or Δ*gcrA ccrM*::Tn strains (Murray *et al.*, 2013; Gonzalez *et al.*, 2014). CtrA directly represses the *ftsZ* (and *podJ*) promoter (Kelly *et al.*, 1998; Crymes, Zhang and Ely 1999; Fiebig *et al.*, 2014; Fumeaux *et al.*, 2014) (Fig. 4C). While Δ*gcrA* mutants exhibit reduced CtrA abundance, *ccrM*::Tn and Δ*gcrA ccrM*::Tn mutants accumulate CtrA to near wild-type levels (Murray *et al.*, 2013), suggesting that *ftsZ* is more strongly repressed in the *gcrA/ccrM* double mutant compared to the Δ*gcrA* single mutant (Murray *et al.*, 2013). The *ctrA* transcript levels are at best slightly upregulated in the absence of CcrM (Gonzalez *et al.*, 2014), consistent with the notion that DNA methylation does not affect *ctrA* promoter activity quantitatively (see above), in contrast to the known down-regulation when GcrA is depleted (Holtzendorff *et al.*, 2004). In support of this, the down-regulation of *ctrA*_P1 in the *gcrA* mutant resembles that of strain in which *ctrA*_P1 was inactivated by a five base pair (bp) insertion between the −35 and −10 sites, in the middle of the GcrA-binding region (Schredl *et al.*, 2012). If the reduction in CtrA is solely due to the inability of mutant *ctrA*_P1 to be activated by GcrA, then CcrM should also still act negatively on this mutant promoter akin to the wild-type promoter in *gcrA* mutant cells, as suggested by the fact that inactivation of *ccrM* in *gcrA* cells restores CtrA levels (Murray *et al.*, 2013). Indeed, the GANTC remains intact in this *ctrA*_P1 mutant (Schredl *et al.*, 2012), suggesting that GcrA binding is disturbed at the mutant promoter and that CcrM can still act negatively on this mutant promoter.

CcrM is a distributive, not a processive, DNA methylase

The negative role of CcrM in the absence of GcrA calls for further structure-function and biochemical studies of CcrM, especially in light of the unusual ca. 80-residue C-terminal extension of unknown function that is present only in CcrM-type alpha-proteobacterial methylases. It has been proposed that CcrM might depend on an accessory factor that helps to load the methylase onto the DNA (Albu, Jurkowski and Jeltsch 2012), as the biochemical mechanism of action of CcrM is very distinct from that of Dam from *E. coli* and coliphage T4. While *C. crescentus* CcrM is now known to be distributive enzyme *in vitro*, the aforementioned Dam enzymes feature a processive mode of action (Berdis *et al.*, 1998; Zinoviev *et al.*, 2003; Peterson and Reich 2006; Albu, Jurkowski and Jeltsch 2012).

Why different bacteria rely on methylases with different kinetic properties for methylation control is an interesting question not previously discussed. Owing to the distributive mode of action, the dwell time of CcrM on DNA is likely short and may limit target interactions. As CcrM is a dimeric enzyme (Shier, Hancey and Benkovic 2001) and monomeric CcrM can bind and methylate DNA, dimerization could endow CcrM with the capacity to methylate two hemi-methylated target sites simultaneously (one per CcrM monomer, for example juxtaposed sites on the same chromosome or the same loci on the two sister chromatids), concomitantly increasing its dwell time on the supercoiled DNA *in vivo* at promoters with different numbers of GANTC sites. Despite a 5-fold higher number of Dam methylation sites (ca. 20 000) than CcrM (4542) in *C. crescentus*, the cellular concentration of Dam in *E. coli* is more than 23-fold lower than that of CcrM (ca. 130 molecules versus 3000 molecules per cell, respectively) (Shier, Hancey and Benkovic 2001) and may be even further accentuated by the multi-fork replication in *E. coli* compared to the non-overlapping one in *C. crescentus*. Proteolytic degradation of a distributive enzyme might ensure a rapid drop in steady-state levels below the threshold needed for methylation, while a low number of processive methylase molecules that escape degradation or arise from stochastic synthesis might suffice to keep the genome fully methylated at all times.

In *C. crescentus*, CcrM degradation does not seem to occur at a specific time in the cell cycle (Wright *et al.*, 1996), but a burst in *ccrM* transcription in G2-phase dictates when the enzyme accumulates (Stephens, Zweiger and Shapiro 1995) (Fig. 6C). In *S. meliloti*, the *ccrM* transcript also peaks at the late pre-divisional (G2) cell stage (De Nisco *et al.*, 2014) and in *Agrobacterium tumefaciens* re-methylation commences at or near completion of DNA replication (Kahng and Shapiro 2001), corresponding to the times when CcrM is expressed and active in *C. crescentus* and *S. meliloti*. Nevertheless, the mere presence of CcrM in the cell does not guarantee that a given GANTC site is indeed methylated. Recent state-of-the-art methylome analyses at single bp resolution captured the progressive hemi-methylation and subsequent re-methylation of 4515 GANTC sites at five time points during the *C. crescentus* cell cycle (Kozdon *et al.*, 2013). Interestingly, 27 sites remained unmethylated throughout the cell cycle. Three of these sites are located in intergenic regions that are not permissive for integration of the Tn5 transposon (Christen *et al.*, 2011). As these regions can be deleted (Kozdon *et al.*, 2013), they are clearly dispensable for viability, suggesting that they are not accessible to Tn5-mediated insertion for other reasons. As constitutive expression of CcrM throughout the cell cycle from a constitutive promoter resulted in methylation of these sites (Gonzalez *et al.*, 2014), transposition could be temporally regulated or the sites are protected from methylation only at specific times during the cell cycle. It is conceivable that chromatin topology in G2-phase masks this region or that a G2-specific DNA-binding protein occludes these unmethylated sites. Thus, specific mechanisms exist that may restrict epigenetic modification. Interestingly, several of these unmethylated sites also overlap with known binding sites for MucR in *C. crescentus* (Fumeaux *et al.*, 2014), raising the possibility that MucR protects from methylation by CcrM. It is currently unknown if GANTC sites in other alpha-Proteobacteria show similar methylation protection.

Control of G2-phase promoters by the CtrA/SciP module

With the activation of *ctrA* transcription in S-phase by the CcrM/GcrA circuit, the stage is set for next transcriptional

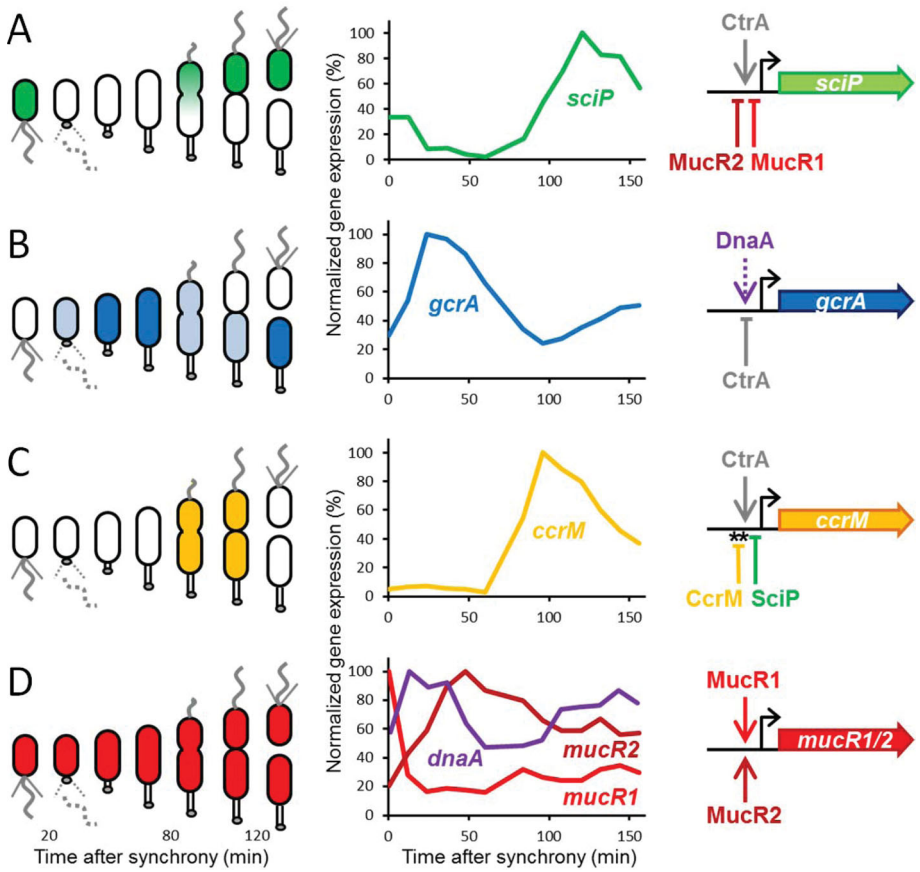

Figure 6. Abundance of GcrA, CcrM, SciP and MucR and their mRNAs during the *C. crescentus* cell cycle. **A–D.** The schematic on the left shows the spatio-temporal changes of each regulator during the cell cycle. The graphs in the middle show the fluctuation of the transcript during the cell cycle, computed from the global transcriptional profiling data (McGrath *et al.*, 2007) normalized to maximal expression for each gene. The schematic on the right summarizes all known interactions of the transcriptional regulators at the promoters of the respective regulator genes. **A.** SciP accumulation during the G1-phase. SciP is present in G1-phase and protoelytically removed during the G1→S transition. Re-synthesis of CtrA (in late S- and early G2-phases) precedes that of SciP (in G1-phase). **B.** GcrA accumulation during S-phase. DnaA control at the promoter of the *gcrA* gene is depicted as dotted arrow because the occupancy of the *gcrA* promoter by DnaA interaction has only demonstrated *in vitro*, but not yet been observed *in vivo*. **C.** Fluctuation of CcrM and regulation of the *ccrM* promoter. The asterisk (*) represents the methylation site (5′-GANTC-3′) in the *ccrM* promoter. **D.** MucR1, MucR2 and DnaA are grouped together as these three regulators are expressed at different time during the cell cycle but their steady-state levels do not fluctuate significantly.

module to act: the CtrA/SciP module that acts in G2-phase. Upon its synthesis, CtrA is phosphorylated (CtrA ~ P, Fig. 2 and 5A-B, but henceforth just referred to as CtrA) and activates expression of flagellar structural proteins, chemotaxis proteins and other developmental factors in the G2-phase of *C. crescentus* (Domian, Quon and Shapiro 1997; Laub, Shapiro and McAdams 2007) (Fig. 3), while repressing other genes such as *ftsZ*, *podJ* (Laub *et al.*, 2000; Fiebig *et al.*, 2014) and *gcrA* (Holtzendorff *et al.*, 2004). CtrA activates the G2-specific genes until SciP represses them upon entry into G1-phase (Chen *et al.*, 2006; Fumeaux *et al.*, 2014) (Fig. 6A). This is because the *sciP* gene, encoding the repressor/negative regulator of G2-phase promoters, is itself part of the G1-phase genes that CtrA activates during cytokinesis (Fumeaux *et al.*, 2014). A conserved 93-residue protein with a winged helix-turn-helix DNA-binding domain, SciP, is proteolyzed during the G1→S transition by the Lon protease (Gora *et al.*, 2010, 2013; Tan *et al.*, 2010) (Fig. 6A). *In vivo* SciP has a strong preference for promoters that CtrA activates in G2-phase versus the promoters of G1-phase genes (Fumeaux *et al.*, 2014). *In vitro* SciP has only very weak or barely detectable general DNA-binding ability on CtrA target promoters that are not among its preferred *in vivo* targets, but CtrA directly bound to these promoters can recruit SciP (Gora *et al.*, 2010, 2013). Moreover, *in vitro* competition assays revealed that CtrA recruits RNAP to these promoters (Gora *et al.*, 2010). Such recruitment is perturbed in the presence of SciP, presumably because SciP and RNAP compete for the same binding surface in CtrA. Interestingly, these findings point to a mechanism by which SciP silences its target promoters *in vivo* without displacing CtrA from the promoter (Gora *et al.*, 2010). As the DNA·CtrA·SciP ternary complex was shown to protect CtrA and SciP from the proteases ClpXP and Lon, respectively, stabilization of both proteins can affect cell cycle progression (Gora *et al.*, 2013). Evidently, the interaction between SciP and CtrA is not only an important determinant of CtrA proteolysis, but is critical for SciP function, as indicated by the finding that when SciP's ability to bind CtrA is compromised, it can no longer efficiently inhibit transcription of CtrA-activated genes (Gora *et al.*, 2010; Tan *et al.*, 2010).

The finding that SciP does not efficiently associate with the promoters of G1-phase genes *in vivo* suggests that this CtrA-dependent interaction is weak or very short-lived (Fumeaux *et al.*, 2014). By contrast, SciP efficiently associates with promoters that are activated by CtrA in G2-phase. Using these top SciP *in vivo* target sites, a consensus motif for SciP target DNA has been proposed [5′-(G/A)TTAACCAT(A/G)-3′] (Fumeaux *et al.*, 2014) that is remarkably similar to the 'extended' CtrA target motif

[5′-TTAACCAT-3′] (Spencer *et al.*, 2009). This extended motif is a variation of the 'standard' or classical CtrA recognition motif [5′-TTAA-N7-TTAA-3′] and is bound *in vitro* under conditions that artificially favor the dimerization of *C. crescentus* CtrA (Spencer *et al.*, 2009). Might therefore SciP promote dimerization of CtrA or depend on it? The fact finding that regions that SciP binds *in vivo* are slightly juxtaposed but not completely overlapping with the CtrA peaks suggests that SciP could associate peripherally CtrA on target promoters at such 'extended' CtrA motifs (Fumeaux *et al.*, 2014). A different consensus motif with the core GC-rich sequence 5′-GTCGC-3′ was advanced by Tan *et al.* (2010). While the former motif was proposed based exclusively on the 50 preferred *in vivo* target sites of (untagged) SciP as determined by ChIP-Seq experiments (Fumeaux *et al.*, 2014), without further experimental verification such as mutagenesis, the latter motif was derived on weak and concentration-independent protection in footprinting studies using His_6-SciP (SciP harboring an N-terminal a hexahistidine tag) and the *ctrA* promoter, a relatively poor *in vivo* substrate of SciP (Fumeaux *et al.*, 2014), and reinforced with lower-resolution ChIP data of FLAG-SciP (SciP harboring an N-terminal FLAG tag) and indirect transcript profiling data. The reason for discrepancy is not clear, but mutagenesis experiments showed that mutation of the proposed 5′-GTCGC-3′ motif does not alter *pilA* promoter activity (Gora *et al.*, 2013). This conclusion is consistent with the finding that SciP does not bind the *pilA* promoter *in vivo* but very efficiently targets the promoters of motility genes *in vivo* (Fumeaux *et al.*, 2014). With the identification of motility promoters as top SciP *in vivo* targets, the stage is now set to revisit the binding properties and target sequence *in vitro* by footprint analysis using these promoters as probes.

Though SciP has also been implicated in regulation of motility in *Rhodobacter capsulatus* (Mercer *et al.*, 2012), *C. crescentus* cells lacking SciP are not only non-motile, but also grow slowly and are filamentous (Gora *et al.*, 2010). Why the absence of negative regulator of transcription has such a detrimental effect on cell physiology is not intuitive, since many cell cycle proteins are regulated at the level of abundance and it is unknown which genes' premature expression could have such dramatic consequences. Clearly, the expression of at least one SciP target gene must be properly negatively regulated to avoid adverse effects on growth and/or division. As SciP does not associate with targets that CtrA negatively regulates, such as *Cori* and the *podJ* and *ftsZ* promoters, *in vivo* (Fumeaux *et al.*, 2014) (Fig. 4C), it seems that excessive firing of CtrA-activated promoters must account for the phenotypic problems of *sciP* mutants. In support of this, CtrA target sites in *Cori* are dispensable for viability (Bastedo and Marczynski 2009) and CtrA does also not appear to bind at or within the origin of replication of *S. fredii* (Fumeaux *et al.*, 2014).

Control of G1-phase genes by the CtrA/MucR module

The second transcriptional module that CtrA specifies *in C. crescentus* is the G1-phase module in which CtrA activates promoters of G1-phase genes, while MucR1/2 repress them prior to entry into G1 (Fumeaux *et al.*, 2014). While the mechanism(s) by which MucR1/2 repression is lost or can be overcome is currently unknown, MucR1/2 clearly adopt a critical role in instating G1-phase gene expression and turning off G2-phase promoters via SciP (Fumeaux *et al.*, 2014). Premature accumulation of SciP in G2-phase has adverse effects on the cell cycle due to a premature shutdown of CtrA-activated promoters which are normally only silenced when SciP accumulates in G1-phase (Gora *et al.*, 2010; Tan *et al.*, 2010). The G1-specific accumulation of SciP seems to be largely due to control of protein synthesis, as evidenced by driving its expression from the xylose-inducible promoter for constitutive induction during the cell cycle (Gora *et al.*, 2013). Regulation of SciP synthesis normally restricts its accumulation to G1-phase (Fig. 6A). In *C. crescentus*, the paralogous repressors MucR control transcription of G1-specific genes that are activated by CtrA such as *sciP* (Fumeaux *et al.*, 2014) (Fig. 2B and 3). As MucR do not target CtrA-activated promoters that fire in G2-phase, a simple model was advanced in which de-repression of G1-phase genes directs the accumulation of SciP, which will lead to an immediate shutdown of G2-phase CtrA-activated promoters (Fumeaux *et al.*, 2014). The G1-specific transcript levels could rise until a threshold of SciP is reached that would dislodge RNAP from all CtrA-activated promoters (Gora *et al.*, 2010) or until CtrA/SciP is degraded during the G1→S transition (Domian, Quon and Shapiro 1997; Gora *et al.*, 2013). In this model, MucR adopts a critical role in regulating G1-specific gene expression and, by extension, may act as the trigger for the switch from the G2-phase transcriptional program to the G1-phase program (Fumeaux *et al.*, 2014). As MucR steady-state levels do not fluctuate significantly during the cell cycle (Fumeaux *et al.*, 2014) (Fig. 6D), the mechanism of activation is probably occurring at the level of MucR activity.

MucR proteins feature an ancestral zinc finger-type DNA-binding domain, comprising a recognition helix (α) and a two-stranded beta-sheet (β) embedded within a central ca. 58-residue globular domain of $\beta\beta\beta\alpha\alpha$ architecture (Malgieri *et al.*, 2007; Baglivo *et al.*, 2009). How DNA binding of MucR and conserved alpha-proteobacterial orthologs might be regulated is unclear, but the N- and C-termini could certainly provide entry points for post-translational regulation. Biochemical studies have shown that DNA-binding of *S. meliloti* and *Brucella abortus* MucR depends strongly on the presence of divalent cations (Bertram-Drogatz *et al.*, 1998; Caswell *et al.*, 2013). In support of the proposed important role of MucR in switching transcriptional programs in *C. crescentus*, the *mucR* mutation is pleiotropic in several lineages where *mucR* has been genetically analyzed (Cooley and Kado 1991; Bittinger *et al.*, 1997; Martin *et al.*, 2000; Bahlawane *et al.*, 2008; Mueller and Gonzalez 2011; Mirabella *et al.*, 2013). Many MucR orthologs from different alpha-Proteobacteria are interchangeable and can support MucR function in *C. crescentus* or *S. meliloti* (Mirabella *et al.*, 2013). Moreover, pangenomic ChIP-seq analysis revealed that MucR from *S. fredii* targets several promoters of genes orthologous to those that MucR1/2 targets in *C. crescentus* (Fig. 5C-D) (Fumeaux *et al.*, 2014). It is therefore likely that the role of MucR in controlling cell cycle transcription (and by inference its regulation) is conserved in this large class of bacteria that occupy distinct ecological niches and that the basic architecture of the regulatory network has been maintained in these different lineages (Fig. 1B). While ecological adaptation probably resulted in the recruitment of niche-specific functions into the MucR regulon, there are compelling hints that MucR controls G1-specific functions in other systems, specifically those that are required for interactions with host cells. For example, the G1-phase was recently shown to harbor the functions required for host cell invasion by *B. abortus* (Deghelt *et al.*, 2014). Moreover, a *B. melitensis* or a *B. abortus mucR* mutant is attenuated in infection of macrophages *in vitro* or of mice *in vivo* (Arenas-Gamboa *et al.*, 2011; Caswell *et al.*, 2013; Mirabella *et al.*, 2013). MucR (also known as Ros) regulates virulence genes in the plant pathogen *A. tumefaciens* (Cooley, D'Souza and Kado 1991; Cooley and Kado 1991) and is known to promote symbiosis in *Rhizobium etli* (Bittinger *et al.*, 1997). Therefore, MucR seems to be required for

the transcriptional fine-tuning of cell cycle functions, explaining why it is dispensable for viability. However, MucR binds the *ctrA* promoter region and regulates *ctrA* expression in *C. crescentus* (Fumeaux *et al.*, 2014), indicating a remarkable degree of interconnectivity within this module as part of a systemically operating regulatory circuit (Fig. 3). Adding to this complexity, the cross-regulation by these module components promotes the correct consecutive accumulation of these regulators at the correct time in the cell cycle or serves as part of (auto-)regulatory mechanism that maintains a steady state as shown in Figs 2 and 3 (Fioravanti *et al.*, 2013; Murray *et al.*, 2013; Fiebig *et al.*, 2014; Fumeaux *et al.*, 2014).

Gradual transitions from modules

If three sequential transcriptional modules regulate cell cycle transcripts, why is a gradual fluctuation in transcripts observed (Laub *et al.*, 2000; McGrath *et al.*, 2007; Fang *et al.*, 2013), rather than three transcript peaks arising from sequential transcriptional pulses? While this question remains to be addressed experimentally, several explanations can be envisioned. One possibility is that post-transcriptional delay mechanisms promote graduation of select transcripts. In fact, it has been proposed that G1-phase transcripts could be subject to post-transcriptional regulation (Gora *et al.*, 2010). Such global post-transcriptional regulators acting on G1-phase transcripts remain a possibility.

Alternatively, graduation of transcripts could arise from staggered transcriptional control by negative regulators and/or different promoter affinities causing transcript peaks to merge into one another upon their induction through (indirect) transcriptional hierarchies within or even across modules. Indeed the GcrA/CcrM module induces the expression of CtrA (Reisenauer and Shapiro 2002; Holtzendorff *et al.*, 2004; Fioravanti *et al.*, 2013; Murray *et al.*, 2013), which then activates the expression of CcrM during G2. CtrA also acts in the ensuing module with MucR to restrict the expression of SciP and other proteins to G1-phase for the consequent down-regulation of G2-phase promoters by SciP (Gora *et al.*, 2010; Tan *et al.*, 2010; Fumeaux *et al.*, 2014).

Impact of (p)ppGpp on cell cycle transcription

It has recently emerged that cell cycle transcription is not only regulated systemically, but is also responsive to systemic cues. For example, Crosson and colleagues found that (p)ppGpp (guanosine 3′,5′-bispyrophosphate) (Boutte and Crosson 2013), an alarmone that is induced under during carbon and nitrogen exhaustion, can influence the cell cycle when cells are growing in rich medium, i.e. under conditions where carbon and nitrogen should not be limiting (Lesley and Shapiro 2008; Boutte and Crosson 2011; Boutte, Henry and Crosson 2012; Brimacombe *et al.*, 2013; Zan *et al.*, 2013; Gonzalez and Collier 2014; Wang *et al.*, 2014). At the macroscopic level, (p)ppGpp delays progression from G1→S-phase in exponentially growing *C. crescentus* (Lesley and Shapiro 2008; Boutte and Crosson 2011; Gonzalez and Collier 2014). Basal levels of the alarmone are also synthesized by SpoT when *C. crescentus* cells are starved for fatty acids (Stott *et al.*, submitted), while (p)ppGpp levels are strongly augmented in response to carbon starvation (Lesley and Shapiro 2008; Boutte and Crosson 2011).

At the molecular level, (p)ppGpp has been reported to influence the steady-state levels of DnaA and CtrA during carbon starvation (Lesley and Shapiro 2008; Boutte and Crosson 2011). Artificial induction of (p)ppGpp synthesis with a constitutively active variant of *E. coli* RelA synthase in *C. crescentus* results in stabilization of CtrA and reduced synthesis of DnaA in the absence of starvation (Gonzalez and Collier 2014). Notably, basal (p)ppGpp levels *in C. crescentus* maintain *ctrA* transcription and increase *dnaA* transcription in response to fatty acid starvation (Stott *et al.*, submitted), in contrast to the inhibition of *dnaA* transcription by (p)ppGpp in *E. coli* (Chiaramello and Zyskind 1990; Zyskind and Smith 1992).

The role of the (p)ppGpp alarmone on the cell cycle is also seen in other alpha-Proteobacteria. Induction of (p)ppGpp by carbon and nitrogen starvation also yields G1-arrested *S. meliloti* cells, a condition that was recently exploited for synchronization (De Nisco *et al.*, 2014). Moreover, the recent finding that G1-arrested *B. abortus* cells are the predominant invasive cell type that enter host cells (Deghelt *et al.*, 2014) and the fact that *B. melitensis*, *B. suis* and *B. abortus* mutants blocked in (p)ppGpp production are attenuated for virulence on mammalian cells (Kim *et al.*, 2005; Dozot *et al.*, 2006) further suggest that the mechanism of (p)ppGpp-mediated G1-arrest is crucial to promote productive interactions between bacterium and host.

The mechanism by which (p)ppGpp affects protein stability is currently unknown, but could be mediated by polyphosphate (polyP) (Rao, Gomez-Garcia and Kornberg 2009). These long chain polymers of inorganic phosphate are formed by sequential addition of inorganic phosphate by polyP kinase as it converts ATP to ADP. In *C. crescentus*, (p)ppGpp promotes polyP accumulation (Boutte and Crosson 2011). PolyP in turn has general chaperone activity, protecting proteins from unfolding and aggregation (Gray *et al.*, 2014), modulates the activity of the *E. coli* Lon protease (Kuroda *et al.*, 2001) and influences the expression of the sigma factor encoded by *rpoS* (Shiba *et al.*, 1997). It is thus conceivable that (p)ppGpp influences the abundance of CtrA or DnaA indirectly through polyP.

Re-wiring the CtrA module into quorum sensing control

Members of the *Rhodobacterales* also tune CtrA abundance to a cell-cell signaling pathway known as quorum sensing (QS) (Fuqua, Parsek and Greenberg 2001). In QS, a diffusible chemical signal released at by each bacterium at a constant rate serves as proxy of the number of cells within a population. As each cell produces the QS signal, a critical threshold in the level of the signal is attained with a pre-determined concentration of cells. QS is executed by a receptor that binds the signal and activates a signal transduction cascade inducing transcriptional changes. In *Dinoroseobacter shibae*, a symbiotic bacterium associated with marine algae, *ctrA* expression is also strongly down-regulated in a QS mutant strain (Patzelt *et al.*, 2013) and a comparable mutant of *Ruegeria* sp. KLH11 that does not express *ctrA* is non-motile (Zan *et al.*, 2013). This motility defect is corrected when CtrA is expressed from a constitutive promoter in the QS mutant, indicating that QS regulates motility through CtrA (Zan *et al.*, 2013). It is unclear how CtrA controls motility in *Ruegeria sp.* and in *R. capsulatus*, as no CtrA TTAA-N7-TTAA consensus motifs were discernible upstream of motility genes, suggesting that motility control is indirect. As (1) a SciP ortholog is encoded in the *Rhodobacterales* genomes (Fig. 1B), (2) SciP expression is activated by CtrA in *C. crescentus* and (3) the predicted SciP consensus motif overlaps with a CtrA half site in motility promoters, we predict that SciP is a regulator of motility in these genera as in *C. crescentus* (Fumeaux *et al.*, 2014). The situation seems to be slightly different for *D. shibae* where inactivation of *ctrA* also leads to down-regulation of flagellar gene expression, many of which harbor a consensus CtrA boxes in the putative promoters (Wang

et al., 2014). Remarkably, it has recently been reported that CtrA regulates other QS components in *D. shibae* (Wang *et al.*, 2014) and the formation of virus-like particles (known as GTAs) in *R. capsulatus* (Mercer *et al.*, 2012). Remarkably, in *R. capsulatus* the motility functions also depend on the phosphorylation of CtrA, while other promoters require the unphosphorylated form of CtrA (Mercer *et al.*, 2010; Brimacombe *et al.*, 2013). Thus CtrA is used in different ways and, not surprisingly, as part of different modules.

CtrA can also regulate ancillary functions in lineages where it is dispensable for viability, for example the formation of metabolically dormant cyst cell types in *Rhodospirillum centenum* that was enhanced upon inactivation of CtrA (Bird and MacKrell 2011). Interestingly, evidence was recently provided that down-regulation of CtrA promotes the formation of nitrogen-fixing bacteroides during Rhizobium-legume symbiosis (Pini *et al.*, 2013). Thus, CtrA also negatively influences development in alpha-Proteobacteria.

CONCLUSIONS

The themes emerging collectively from these recent studies is that not only can these conserved module components be appropriated to control transcription of essential cell cycle genes, but also to regulate dispensable ancillary functions in several alpha-proteobacterial orders. Moreover, the module components function in pairs, acting either positively or negatively on transcription initiation, to restrict promoter firing, to specific cell cycle phases. As shown for the GcrA/CcrM pair, the components can act negatively in isolation, but act positively when together or they can function as classical activator/repressor pairs as in the case for CtrA/MucR (with the caveat that CtrA and MucR can act both as transcriptional repressors or activators) (Laub *et al.*, 2000; Fumeaux *et al.*, 2014).

It is also clear that systemic and environmental regulation of selected modules allows for gene expression to be exquisitely adjusted to the unique demands imposed by niches, from starvation stress adaptation in the environment to facilitating symbiosis and infection of (eukaryotic) host cells. MucR, for example, is required for virulence gene expression in *Brucella* species (Arenas-Gamboa *et al.*, 2011; Caswell *et al.*, 2013; Mirabella *et al.*, 2013) and CtrA has recently been implicated in the development of the infectious cell type in an obligate intracellular bacterium belonging to the order *Rickettsiales*, *Ehrlichia chaffeensis* (Cheng *et al.*, 2011). Moreover, CtrA controls motility and chemosensory functions that are needed to colonize host cells in the marine sponge symbiont *Ruegeria* sp. KLH11 (Zan *et al.*, 2013).

While the CtrA module appears to have been appropriated by the QS pathway in some bacteria, GcrA may have been adopted by bacteriophages (Gill *et al.*, 2012; Panis, Lambert and Viollier 2012; Murray *et al.*, 2013), perhaps to reprogram transcription in the host for viral progeny production with paralogous regulatory module. The fact that GcrA does not exhibit conspicuous similarity to known transcriptional regulators at the primary or secondary structure level indicates that other proteins not annotated as transcriptional regulators (e.g. conserved hypotheticals) may still hold some surprises to regulation of cell cycle transcription in alpha-Proteobacteria.

SUPPLEMENTARY DATA

Supplementary data is available at FEMSRE online.

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