Second Messenger-mediated flagellum assembly during
the *Caulobacter Crescentus* cell cycle

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1 Introduction

1.1 The second messenger c-di-GMP

Nucleotide-based second messengers can serve as message carriers inducing a variety of cellular cascades by binding specifically to their targets, thus transducing signals originating from changes in the surroundings or in intracellular conditions into cellular responses. The bacterial second messenger cyclic-di-guanosine monophosphate (c-di-GMP) was first identified as an allosteric activator of cellulose synthesis in *Gluconacetobacter xylinus*[1,2] and was later shown to modulate several of the main activities of the bacterial cell such as transcription[3–5], translation[6,7], enzymatic activities[1,8], and specific proteolysis[9,10]. This influences multiple cellar processes such as motility, virulence, biofilm formation, cell cycle progression and cell differentiation[11,12]. In summary, c-di-GMP is regarded as a bacterial life style modulator[13] inducing the transit from a motile, single state to adhesive multicellular state. Low cellular levels of c-di-GMP are generally correlated with a motile and solitary lifestyle and with acute stages of infection of a range of bacterial pathogens, whereas high c-di-GMP concentrations promote a sessile, biofilm associated lifestyle and a chronic state of infection (Fig. 1).
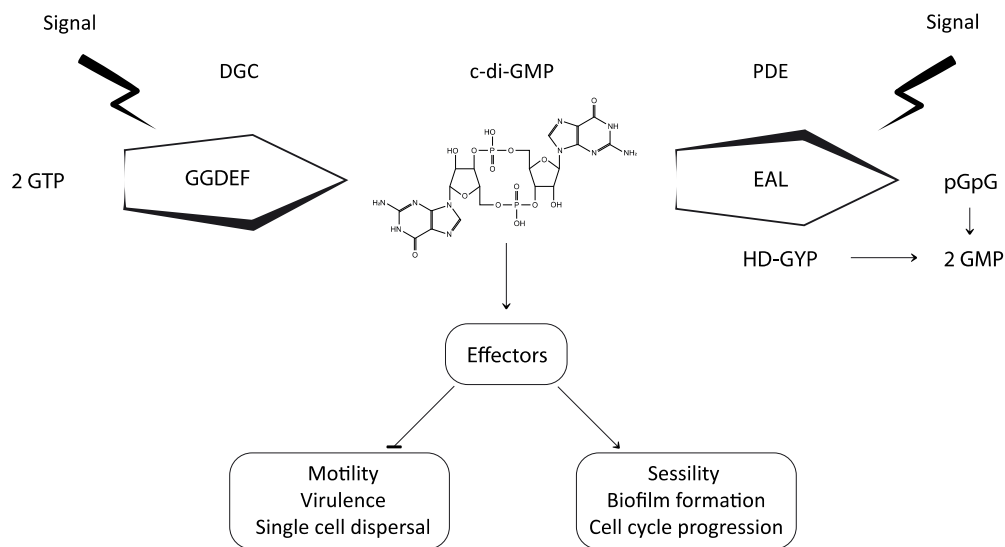
Figure 1: Overview of c-di-GMP signalling. DGC proteins harbouring the catalytic domain GGDEF and PDE proteins carrying the catalytic domain EAL regulate c-di-GMP cellular levels in response to environmental or intracellular signals. c-di-GMP binding proteins, so called effectors proteins translate c-di-GMP cellular levels into a cellular response and modulate the activity of target systems. Low levels of c-di-GMP promote motility and virulence, whereas high c-di-GMP cellular levels induce sessility and biofilm formation. Adapted from [14].

1.1.1 The making and breaking of c-di-GMP

C-di-GMP is the result of the condensation reaction of two GTP molecules. This process is catalysed by the activity of the widely distributed diguanylate cyclase enzymes (DGC)[15–18]. GTP binds to catalytic active GGDEF domains, which form a homodimer thereby allowing the anti parallel alignment of the GTP molecules and the formation of two intermolecular phosphodiester bonds[14]. C-di-GMP degradation is promoted by the activity of the specific phosphodiesterases (PDE) that harbor the active domain EAL or HD-GYP.

Active EAL domains[19] bind a single c-di-GMP molecule within their TIM-barrel-like fold[20,21] and catalyse the asymmetrical hydrolysis of an ester bond. This reaction utilizes a conserved glutamate as a general base that together with two Mg^{2+} cation(s)[22] coordinates and activates a water molecule for the nucleophilic attack. The attack of the hydroxide ion on the electrophilic phosphorus center leads to the breakage of the sugar phosphate bond producing the linear product dinucleotide 5'-

phosphoguanylyl-(3'-5')-guanosine (pGpG), which is then hydrolyzed to GMP by other unknown phosphoesterases[23]. Sequence alignment of EAL domains revealed the presence of 10 conserved charged or polar residues[24] that were identical in all active proteins. Four conserved residues residues Glu[523], Asn[584], Glu[616], Asp[646], (TBD1265) including the Glu residue of the EAL signature sequence bind the metal ion, whereas the conserved Glu[703] (TBD1265) functions as a general base, coordinating the catalytic water molecule (Fig. 2). The role of Asp[646] (TBD1265) is a matter of debate, nevertheless mutation of this residue caused significant changes in the catalytic parameters[21,22,24]. The functional role of conserved residues Arg[527], Glu[546], Lys[667], and Gln[723] (TBD1265) is presently unknown[24].
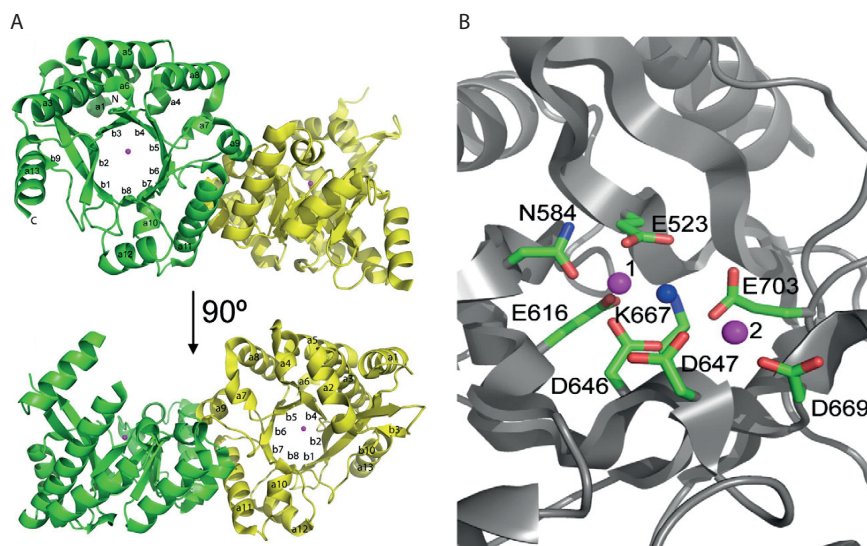
Figure 2 : Crystal structure of the TBD1265 EAL domain (PDB code 2r6o). Overall structure of the apoprotein dimmer (A). Close-up view of the active site with bound Mg2+ (denoted by magenta spheres). The potential catalytic water molecule is shown as a blue sphere, whereas the amino acid residues in contact with the metal ions and water are shown as sticks along a TBD1265 ribbon (B). Adopted from[24].

In addition to the catalytic center itself, a conserved motif known as loop 6[22,25] was shown to play an important role in the EAL domain activity[22]. It is presumed that this loop motif modulates the quaternary structure of the protein forming a dimer interface in the active conformation. Alteration of single amino acids in loop 6 or in Glu[268] (EAL$_{RocR}$), which interacts with the loop and stabilized its position changes the catalytic parameters of the protein dramatically[22] (Fig 3). This is an indication that

the loop 6 motif might be involved in signal transduction and activity regulation.

The metal-dependent phosphohydrolase HD-GYP domain is less common and able to degrade c-di-GMP directly into GMP[26]. The structure of HD-GYP domains reveal differences in the overall fold and an active site with a binuclear metal center[27].
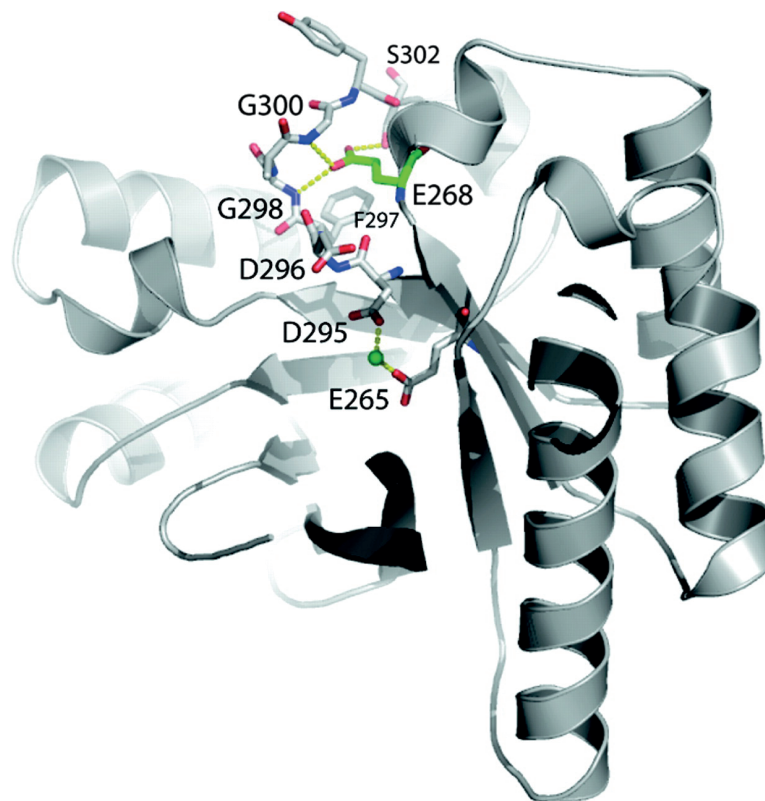
Figure 3: Structural model of EAL_RocR. The residues of loop 6 and Glu[268] are highlighted. The hydrogen bonds formed between Glu[268] and the loop residues Gly[300] and Ser[302] are represented by the broken lines. The Mg^{2+} ion is shown as the ball. Adopted from[21,22].

1.1.2 Regulation of c-di-GMP cellular levels

The cellular levels of c-di-GMP are tightly regulated with the enzymatic activities of DGCs and PDEs being subject to multiple regulation cascades. DGCs and PDEs are generally composed of the catalytic active domain fused to diverse sensory and regulatory domains such as PAS, HAMP, GAF and REC[28–30], which govern the activity of the GGDEF and EAL domains. This enables the integration of diverse signals into the c-di-GMP signaling system and modulates the cellular levels of c-di-GMP.

Moreover, proteins involved in the c-di-GMP turnover are subject to temporal and spatial regulation. In *Escherichia coli* for example, several GGDEF and EAL encoding

genes are under the control of the σ^s (RpoS)[31,32], a general stress response master regulator, which activates these genes only upon entry into stationary phase. In *C. crescentus,* spatial regulation and localization of c-di-GMP signaling elements in addition to specific proteolysis represent a further layer of regulation controlling c-di-GMP cellular levels. E.g. PdeA is a *C. crescentus* PDE[33] that co-localizes with its antagonist the DGC DgcB to the flagellated pole of the swarmer cell. During G1 to S phase transition, PdeA is recruited to the polar protease ClpXP where it is degraded setting DgcB activity free and leading to a c-di-GMP accumulation[10].

DGCs are subject feedback regulation by their own product. This regulatory mechanism is well-documented for PleD, a *C. crescentus* response regulator with two receiver domains arranged in tandem (REC1-REC2) and a GGDEF catalytic domain[8,34]. PleD is activated by phosphorylation of its first REC domain (REC1), which induces conformational changes of the REC1-REC2 stem that promote the formation of PleD homodimers. The resulting homodimer organization allows free rotation of the GGDEF domains that are connected to the REC1-REC2 stem over a flexible linker peptide. Once bound to GTP molecules, the GGDEF domains are reorganizing in a form that enables substrate proximity. The available crystal structures of PleD provided insight into the mechanism of non-competitive product inhibition by domain immobilization[17,34] (Fig. 4). Dimeric c-di-GMP is able to crosslink GGDEF domains in a nonproductive conformation either to a neighboring REC or GGDEF domain. C-di-GMP interacts with the GGDEF domain over the primary inhibitory site (I_p) characterized by an RxxD motif[8] and with a secondary inhibitory site (I_s) located on the surface of a neighboring GGDEF or REC domain. In both of the resulting domain organizations, the GGDEF active sites are immobilized resulting in PleD inactivation once the product levels reach the inhibition constant ($1\mu M$)[14,17].
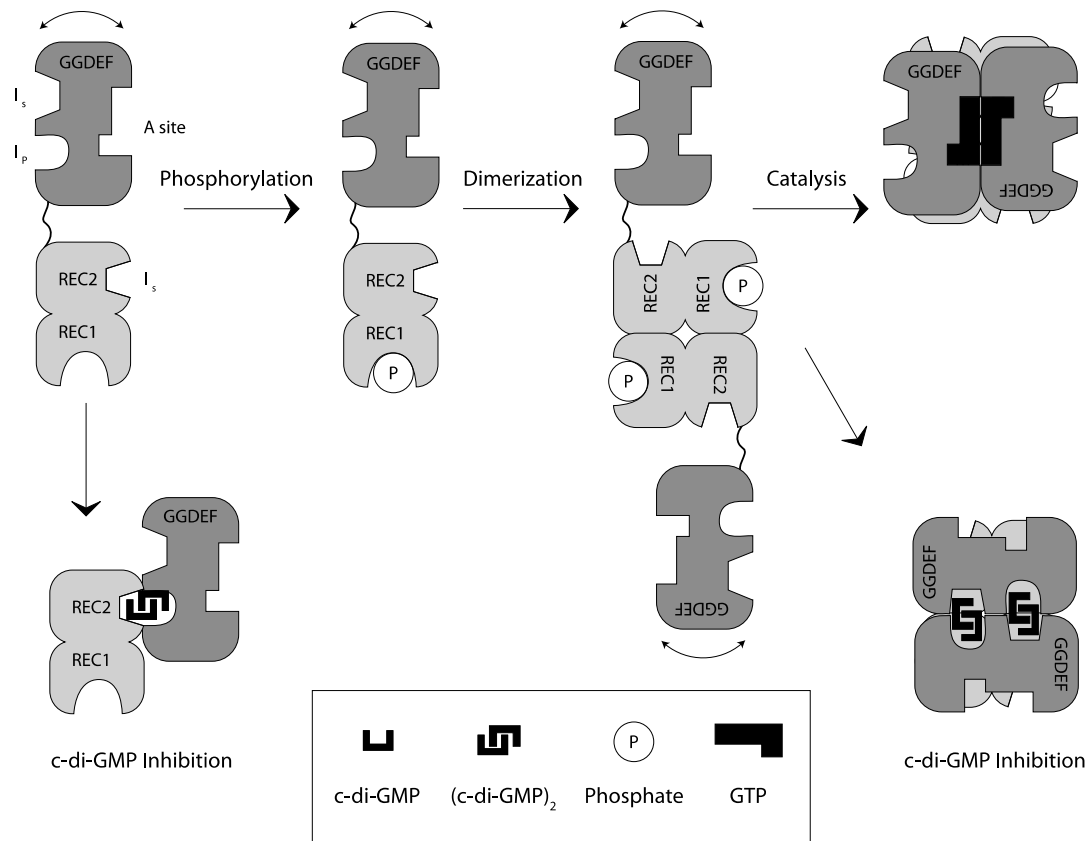
Figure 4: Model of PleD regulation. The catalytic GGDEF domain is connected to the receiver domains REC1-REC2 over a flexible linker peptide. Phosphorylation of the receiver domain REC1 results in dimerization and enzymatic activity. The two forms of domain organization resulting in allosteric product inhibition by domain immobilization are shown (lower panel). Inhibition occurs by binding of (c-di-GMP)$_2$ to the I_s at the REC2 and to the I_P at the GGDEF domain or by crosslinking two GGDEF domains over I_S and I_P. Adapted from[35].

GGDEF and EAL containing proteins display an additional composition as about one third of GGDEF containing protein and two thirds of the EAL containing proteins contain both domains (composite proteins)[29] It was shown for several composite proteins that one of these domains lost its catalytic activity and is involved in signal transduction mechanism[19,26,31,36] (see c-di-GMP effectors). This is for instance the case in *C. crescentus* PdeA, whose phosphodiesterase activity is strongly stimulated by GTP binding to the degenerate and catalytically inactive GGDEF domain[33]. However, several composite proteins retain both catalytic activities[37]. There is precedence for bifunctional enzymes involved in signal transduction, include protein

His-kinases/phosphatases of the two-component regulatory systems or the SpoT proteins catalyzing both synthesis and degradation of the bacterial nucleotide second messenger, (p)ppGpp[38]. This arrangement of two catalytic domains with opposite activities within one polypeptide might serve as an additional regulatory mechanism controlling the cellular levels of c-di-GMP.

1.1.3 c-di-GMP effectors

The paradigm of second messenger signal transduction requires an additional component. An adaptor molecule (effector) that is able to translate the cellular levels of c-di-GMP into a specific cellular response. So far, several c-di-GMP effectors were reported interfering with numerous cellular activities on several levels, including transcription, translation, allosteric regulation, and protein stability[39] During the evolutionary process, c-di-GMP effectors evolved from different initial molecules generating a high degree of variability of c-di-GMP signal integration (Table 1).

Degenerated GGDEF and EAL domains These proteins apparently have evolved from c-di-GMP enzymes that retained their ability to bind c-di-GMP in the allosteric (DGC) or substrate-binding site (PDE) though lost their enzymatic capacity. The *Pseudomonas aeruginosa* protein FimX[40–42] harbouring degenerate GGDEF and EAL domains is one example for this effectors class. Upon binding to c-di-GMP FimX localizes to one cell pole where it mediates type IV pili assembly[42]. Binding of c-di-GMP to the C-terminal EAL domain triggers a long-range (~ca. 70 Å) conformational change in the N-terminal REC domain and the adjacent linker promoting FimX localization[41,42]. Another example is PelD[43]. The Pel exopolysaccharide is involved in *P. aeruginosa* biofilm formation. Pel biogenesis is stimulated by c-di-GMP both on the transcriptional[3,44] and post-translational level[43,45]. Activation of the Pel biosynthesis machinery by c-di-GMP involves the c-di-GMP receptor protein PelD. Despite of limited primary sequence homology, secondary structure analysis of PelD revealed a near identical fold to the catalytic domain of the PleD diguanylate cyclase[45]. PelD binds c-di-GMP at a conserved RXXD motif that normally serves as

allosteric inhibition site for active diguanylate cyclases like PleD. Mutations in the RxxD motif of PelD abolished c-di-GMP binding and Pel stimulation[43].

AAA σ54 Regulatory Domain In *P. aeruginosa,* c-di-GMP modulates the activity of the transcriptional regulator FleQ[3], which antagonistically controls the expression of flagellar and PEL exopolysaccharide genes. FleQ is an enhancer binding protein belonging to the NtrC family of sigma54-specific transcription activators. It harbors an N-terminal input domain, an AAA$^+$/ATPase sigma54-interaction domain and a DNA binding domain. It is suggested that FleQ controls gene expression by a mechanism that involves binding to two sites in the promoter of the operon it controls. FleN and FleQ forming a protein complex, that in the absent of c-di-GMP induces a distortion of pel DNA and repress the *pelA* promoter activity. Once bound with c-di-GMP, FleQ undergoes a conformational change that induces a cascade of conformational changes in the FleQ/FleN/DNA complex and enables *pelA* promoter activity[44].

REC Domain The *Vibrio cholerae* transcription regulator VpsT[5] consists of an N-terminal receiver (REC) domain and a C-terminal helix-turn-helix (HTH) domain, with the latter mediating DNA binding. Unlike other REC domains, the VpsT REC domain is extended by an additional helix at its C terminus enabling c-di-GMP binding. This inversely controls extracellular matrix production and motility[5]. Upon c-di-GMP binding VpsT forms oligomers on DNA binding sites thus regulate promotors activity[46].

cNMP binding domain in *Xanthomonas campestris* the global regulator Clp[47,48] a member of the large CRP/FNR transcription family, induces a number of virulence genes (among others the cellulase gene *engXCA*, the protease gene *ptr1*, and the flagellin gene *fliC*[49]) upon a drop in the cellular level of c-di-GMP. Clp shows high sequence similarity to the *E. coli* catabolite activation protein CAP a cAMP receptor protein and contains a conserved cyclic nucleotide monophosphate (cNMP) binding

domain that binds c-di-GMP. The binding of c-di-GMP induces a conformational rearrangement affecting the affinity of Clp to its target promoters, thus repressing virulence genes transcription.

PilZ Domain The PilZ domain was identified in a bioinformatics analysis as c-di-GMP effector[50]. Additionally this domain is found at the C-terminus of cellulose synthetase BcsA of the *Gluconacetobacter xylinus*, the first enzyme shown to be regulated by c-di-GMP[2]. PilZ domains have been found to bind to c-di-GMP with variable affinities, ranging from sub-µM to µM[33,51–53] and to induce a variety of cellular cascades, including virulence, motility and exopolysacchatiedes synthesis. The PilZ domain has a β-barrel topology with a conserved short unstructured loop at the N-terminus, the so-called c-di-GMP switch. Conserved RxxxR and D/NxSxxG sequence motifs were demonstrated to mediate c-di-GMP binding[52]. The interaction of c-di-GMP to arginine residues in the switch portion induces a conformational change in the overall fold of the protein that facilitates the signal transduction as shown for YcgR. YcgR, an *E.coli* PilZ domain containing protein was demonstrated to bind to the stator complex of the flagellar motor and to regulate flagellar rotation[54,55] in response to c-di-GMP. Although it seems that protein-protein interaction is the preferred mode of action of the reported PilZ receptors, additional mechanism are reported. In *Klebsiella pneumoniae*, the PilZ domain protein MrkH binds DNA in a cyclic di-GMP-dependent manner to activate transcription of the type III fimbrae[4]. Due to the vast domain architectures of PilZ-containing proteins[50,52] additional signal transduction mechanism seems to be plausible.

Riboswitch c-di-GMP–controlled riboswitches regulate splicing[56] or translational initiation of mRNA. Two classes of riboswitches were identified: the first class is GEMM[57], an RNA domain localized in the 5' untranslated region of target mRNA that specifically binds c-di-GMP with high affinity and, in response, controls translation of downstream genes. Both ON- and OFF-switches of GEMM have been reported. The second class consists of a RNA c-di-GMP binding structure that upon binding induces

folding changes at atypical splice site junctions to modulate alternative RNA processing[58].

PNPase The polynucleotide phosphorylase (PNPase) is part of the RNA degradasome complex[59], a multiprotein complex involved in mRNA degradation. C-di-GMP was shown to directly interact with *E. coli* PNPase and, in response, increase its 3' oligonucleotide polymerase activity[60]. Moreover, a set of dedicated c-di-GMP catalytic enzymes, DosC (DGC) and DosP (EAL), seem to adjust c-di-GMP levels available to the PNPase within the degradasome complex in response to oxygen availability[60].

Protein	Organism	Domain	Biological output	Reference
GGDEF and/or EAL domain-containing proteins				
FimX	*P. aeruginosa*	REC/PAS/GGDEF/EAL	Twitching motility	[40,41]
PelD	*P. aeruginosa*	TM/TM/TM/TM/GAF/GGDEF	Exopolysaccharide (EPS) production	[43,45]
LapD	*P. aeruginosa*	HAMP/GGDEF/EAL	Biofilm escape	[61]
PopA	*C. crescentus*	REC/REC/GGDEF	Cell cycle progression	[9]
Transcription factors				
FleQ	*P. aeruginosa*	FLEQ/AAA/HTH	Motility, EPS	[3]
Bcam1349	*B. cenocepacia*	cNMP/HTH	Biofilm formation and virulence	[62]
VpsT	*V. cholerae*	LuxR-HTH	EPS production	[63]
Clp	*X. campestris*	cNMP/HTH	Virulence	[47,48,64]
PilZ domain-containing proteins				
YcgR	*E. Coli*	YcgR-N/PilZ	Motility	[54,55,65]

Alg44 (PA4608)	*P. aeruginosa*	PilZ	Alginate production	[66]
XC1028	*X. campestris*	PilZ	Motility, virulence and biofilm formation	[67]
PilZ (XCC6012)	*X. campestris*	PilZ	Virulence	[68]
PP4397	*P. putida*	YcgR-N/PilZ	N/A	[69]
PlzD (VCA0042)	*V. cholerae*	YcgR-N/PilZ	Virulence	[52]
PilZ (XAC1133)	*X. axonopodis*	YcgR-N/PilZ	Virulence	[70]
MrkH	*K. pneumoniae*	PilZ	Virulence and biofilm formation	[4]
Riboswitches				
Class I	*V. cholerae*	NA	Gene expression	[57]
Class II	*C. difficile*	NA	Translational control	[58]
Other RNA regulating proteins				
PNPase	*E. coli*	NA	RNA degradation	[60]

Table 1: Specific c-di-GMP effector molecules. adapted from[71].

1.2   *Caulobacter crescentus* cell cycle and development

The aquatic α-proteobacterium *Caulobacter crescentus* undergoes a complex cell cycle, which is characterized by an asymmetric cell division. Each division gives rise to genetically identical but morphologically distinct cells (Fig. 5). The motile daughter cell (swarmer cell) is equipped with polar adhesive pili and a rotary flagellum and needs to first differentiate into a non-motile stalked cell in order to initiate chromosome replication and cell division. This differentiation process is irreversible and involves multiple morphological changes; flagellum and pili are shed, and replaced by a long cellular extension, the stalk, that carries an exopolysaccharide adhesion, the holdfast, at its tip[72]. During the obligate swarmer-to-stalked cell

differentiation the chromosome replication block is suspended with cells transiting from G1-phase (gap) to S-phase (DNA synthesis). As cells progress through division a new flagellum and adhesive pili are synthesized at the pole opposite the stalk. In contrast to the newborn swarmer cell, the stalked cell progeny initiates chromosome replication and cell division immediately[73] thereby continuously producing new swarmer progeny[74]. Chromosome replication initiates only once per cell cycle[75] and is intimately linked to the cell developmental stage. Precise timing of assembly and loss of polar organelles is regulated by cell cycle and relay on signal transduction via the second messenger molecule c-di-GMP[10,76,77]. This provides an excellent platform to explore the c-di-GMP signal transduction in relation to polar organelles development.
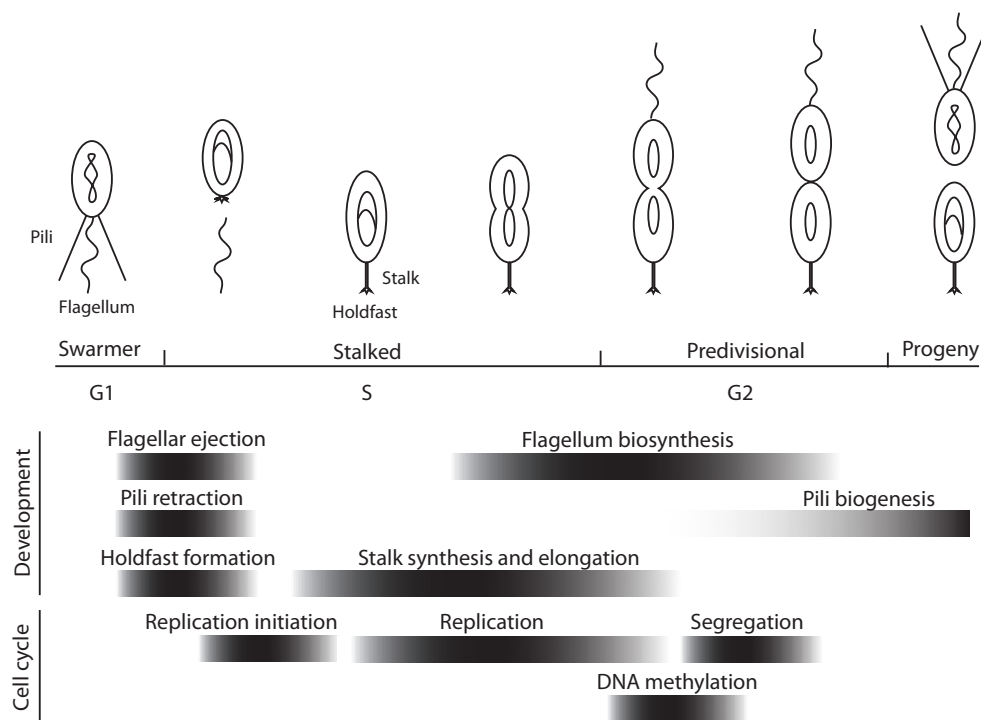
Figure 5: *C. crescentus* cell cycle and development. A schematic of the developmental stages of *C. crescentus* cell division and differentiation from the flagellated, piliated swarmer cell into a sessile surface attached stalked cell. Coincident with pole differentiation, the incipient stalked cell initiates chromosome replication. The grey bars represent morphogenetic and cell cycle events, bar position and length represent time windows. Adapted from[78].

1.3   The *C. crescentus* cell cycle regulation

The coordination of multiple processes that cooperatively assemble the *C. crescentus* cell cycle process requires numerous regulatory layers that orchestrate the single events spatially and temporally into one linear event. This is achieved by the oscillation of regulatory factors that create a signalling network. The main output of this regulatory network is the oscillation of CtrA, a response regulator that is composed of a receiver domain with a conserved histidine phosphorylation pocket and a C-terminal DNA binding domain[79,80]. CtrA recognizes a specific DNA motif[81], the CtrA box, that is located in the promoter region of its target genes. Once bound, CtrA and can act as repressor or activator. At least 95 genes are directly regulated by CtrA[82], among these are genes involved in chromosome replication, cell division and developmental processes[82], such as *fla*[79]*, che*[83]*, divK*[84] and *ftsZ*[85]. CtrA is regulated on multiple levels including phosphorylation, transcription, localization and proteolysis[84,86,87] that dictate CtrA levels and activity. In the swarmer cell, high levels of active CtrA-P result in the inhibition of chromosome replication[88] as CtrA binds to sites within the origin of replication (Cori). This neutralizes the activity of DnaA, a key bacterial replication initiation factor that also binds to the Cori[89,90]. During the swarmer-to-stalked cell differentiation CtrA is recruited to the incipient stalked pole where it is degraded by the polar ClpXP protease[91]. The removal of CtrA relieves the replication block and allows cells to enter S-phase. Removal of CtrA also leads to the transcription of *gcrA*[92]*,* which encodes an additional cell cycle regulator required for chromosome replication, cell elongation and polar development[93] (Fig. 6). The accumulation of GcrA during S-phase then leads to restart of *ctrA* transcription[94]. Buildup of CtrA levels in the predivisional cell subsequently inactivate the *gcrA* promoter and direct the degradation of DnaA by the ClpP protease[90]. In the late predivisional cell CtrA activity is sequestered asymmetrically by phosphorylation at the new swarmer pole and dephosphorylation and degradation at the stalked pole[80]. This asymmetric distribution differentially sequesters CtrA into the progeny cells during division, thereby contributing to the different cell cycle and cell differentiation fates of the two daughter cells.

1.3.1 *ctrA* transcription

The transcription of *ctrA* is regulated during the cell cycle via a hierarchical transcription cascade, in which every element controls the regulation of the next, creating a closed regulatory circuit composed of DnaA, GcrA, CtrA, and CcrM[82,94,95]. The transcription of *ctrA* is initiated from two promoters, P1 and P2[96]. While the weaker P1 promoter is negatively autoregulated, the stronger P2 promoter is part of a positive feedback loop. P1 is first activated in S-phase through the action of the other global transcription regulator, GcrA[94]. GcrA activation requires that the replication fork has moved through this region, leaving the P1 promoter in a hemimethylated state[75,97]. As CtrA levels build up, the P2 promoter is activated and at the same time CtrA stimulates the transcription of *ccrM*, encoding a DNA methyltransferase with high specificity for GANTC sites. CcrM methylation of GANTC sites represses the activity of the P1 promoter and activates transcription of *dnaA*[96], a key factor for replication initiation in the next round of cell division. The activity of P2 promoter leads to further accumulation of CtrA in the predivisional cell, which is then phosphorylated and increases its affinity to the target genes promoters[98]. Recently SciP[99,100], a small regulatory protein whose promoter is CtrA regulated, was shown to bind next to the CtrA binding box and to modulate the activity of CtrA controlled genes. Elevated cellular levels of SciP in the swarmer cell lead to reduction of CtrA levels since SciP inactivates the *ctrA* P2 promoter. Thus SciP serves as the negative feedback loop[99,100].
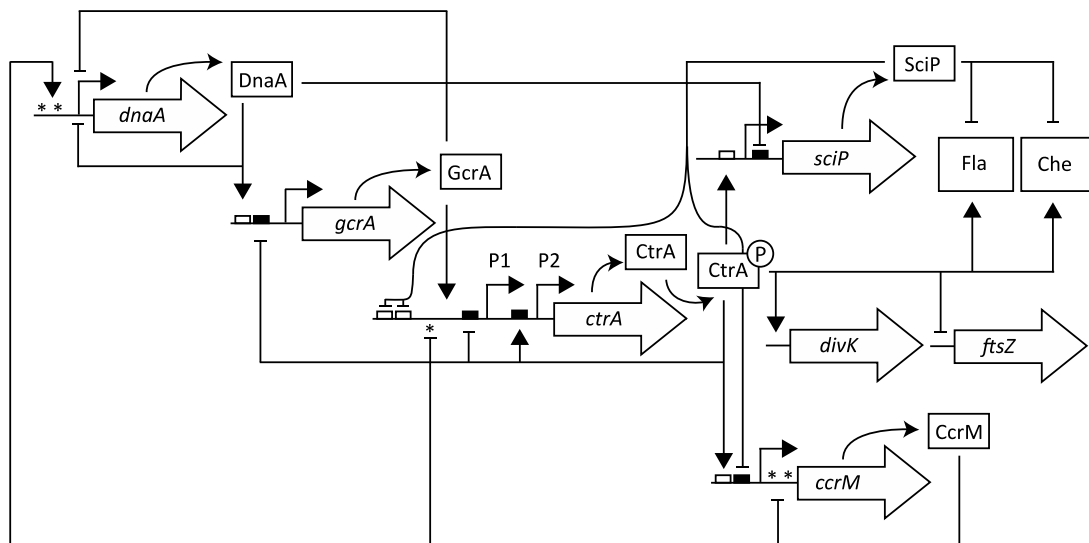
Figure 6: Topology of *ctrA* transcription and regulation. The regulatory circuit composed of *dnaA, gcrA, ccrM, sciP,* and *ctrA* is shown. Each regulator activates the next in the cascade and the inhibitory loop regulating the temporal activity of the promotors creating a closed cycle that regulates the oscillation of the cell cycle regulators. The main outputs of CtrA regulation and SciP inhibition are indicated. Adapted from[84,100].

1.3.2   CtrA phosphorylation

The oscillation of CtrA between a phosphorylated and dephosphorylated state and the asymmetric distribution of the different states in the predivisional cell is the result of an elaborate spatial distribution of the responsible phosphorelay system. The phosphorylation state of CtrA is determined by the bifunctional hybrid histidine kinase CckA[96,101] and the histidine phosphotransfer (HPT) protein ChpT[102]. Proteins regulating the CckA-ChpT phosphorelay are positioned asymmetrically at the poles of the predivisional cell, thereby creating a gradient of activated CtrA and eventually, after cytokinesis has taken place, establishing two different cell fates[103,104]. The histidine kinase DivJ[105] is localized to the stalked pole where it acts as a kinase for the single-domain response regulator DivK[106]. The flagellated pole in contrast, accommodates a second histidine kinase, PleC[103,104], that acts as DivK phosphatase. DivK shuttles between the poles with changing phosphorylation states, until cytokinesis is completed and the cell is compartmentalized and each cell type inherits different levels of DivK-P, which dictates a different cell programme[84,102]. In

the stalked cell, DivK-P binds to DivL[107], an unorthodox tyrosine kinase and prevents its interaction with CckA. In this situation CckA acts as phosphatase leading to the dephosphorylation of CtrA. In contrast, DivK-P levels are low at the flagellated pole allowing the interaction of DivL with CckA[108,109] and the stimulation of CckA autophosphorylation leading to CtrA phosphorylation (Fig. 7).
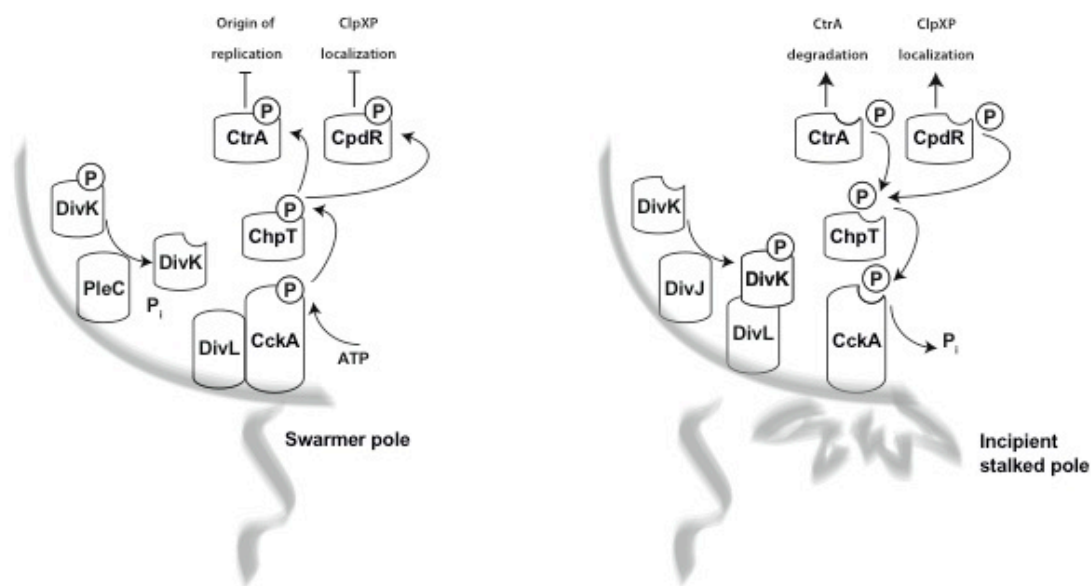
Figure 7: Model of the phosphorelay regulating spatially CtrA phosphorylation. The DivK phosphorylation state is determined by the phosphatase and kinase activity of PleC and DivJ. DivK~P interacts with DivL and inhibits DivL CckA interaction resulting in phosphatase activity of CckA and dephosphorylation of CtrA and CpdR. In contrast, dephosphorylation of DivK by PleC promotes DivL CckA interaction reversing CckA activity ultimately driving CtrA and CpdR phosphorylation. Adapted from[108].

Upon entry into S-phase, CtrA is specifically removed through proteolysis by ClpXP, an essential AAA$^+$ protease whose transcription is CtrA regulated[82,110,111]. The ClpX ATPase energizes the substrate unfolding and the translocation into the degradation cavity composed of tetradecamer complex of ClpP peptidase[112,113]. A degradation tag, a short N-or C-terminal amino acid sequence that is located directly on the substrate or on the adapter molecule, mediates substrate recognition[114]. During swarmer to stalked cell transition, ClpXP is localized to the incipient stalked cell pole[115]. This process coincides with CtrA localization to the same pole where the

degradation of CtrA takes place[110]. The proteolysis of CtrA is the integration point of two control mechanisms. The first originates from the PleC-DivJ-DivK phosphorylation switch that, through the modulation of CckA activity (see above), activates CpdR, a single-domain response regulator that is required for the localization of the ClpXP protease to the cell pole[116]. Phosphorylation of CpdR is controlled by the CckA-ChpT phosphorelay that also mediates CtrA phosphorylation and activity[117]. Thus, through the coincident modulation of CtrA and CpdR phosphorylation states, CckA coordinates cell type specific activity and stability of CtrA[117]. The second signal required for CtrA degradation is c-di-GMP. Elevated levels of c-di-GMP activate the effector protein PopA. PopA is an unorthodox response regulator harboring two receiver domains fused to a degenerated GGDEF domain that retained its ability to bind c-di-GMP via a conserved I-site (see above). Once bound, PopA recruits CtrA to the degradation machinery via its direct interaction with the RcdA helper protein[91] (Fig. 8).
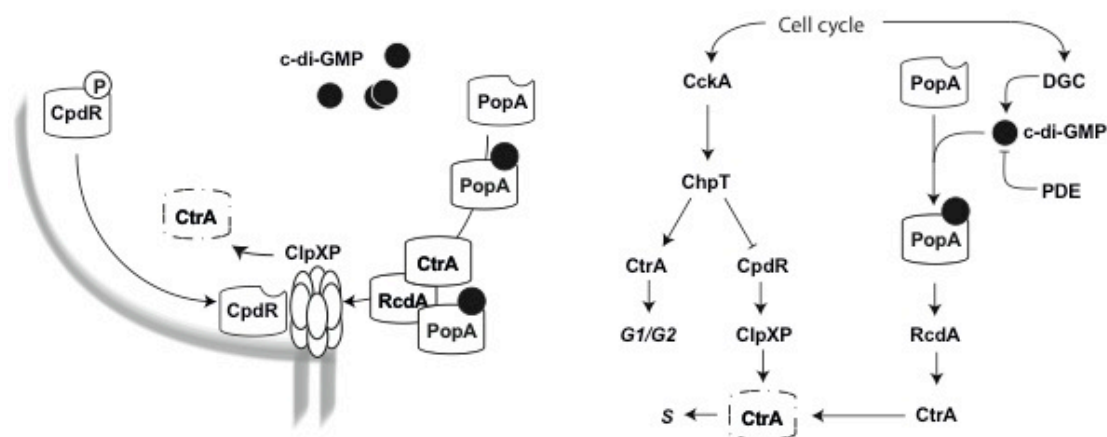
Figure 8: Model for CtrA degradation. The CckA-ChpT phosphorelay inversely regulates CtrA and CpdR activity through phosphorylation signal. Dephosphorylated CpdR recruits the ClpXP protease to the pole, whereas additional c-di-GMP mediate signaling activate the recruitment of CtrA over PopA to the same pole where ClpXP degrades CtrA. Adapted from[9].

1.4   c-di-GMP and *C. crescentus* development

The fluctuation of CtrA activity during the cell cycle is accompanied by fluctuations of c-di-GMP levels that control many aspects of polar organelle development such as flagella, holdfast, and stalk biogenesis[10,16,76,77]. While c-di-GMP levels are low in

swarmer cells, the concentration quickly increases during the transition to stalked cells. Likewise, in dividing cells a characteristic spatial gradient is observed with low c-di-GMP levels in the swarmer and high levels in the stalked compartment[118]. This characteristic distribution is governed through strict temporal and spatial control of several enzymes involved in c-di-GMP turnover. The swarmer cell maintains low c-di-GMP levels through the activity of the PDE PdeA[10]. At the same time the DGC PleD is kept in its inactive state in swarmer cells by the PleC phosphatase. The PleD response regulator harbors a catalytic GGDEF domain and is directly controlled by the PleC-DivJ-DivK phosphorylation switch[16,102]. While PleC keeps PleD in its inactive state in the swarmer cell, PleD is activated by DivJ mediated phosphorylation during the stalked cell transition[76]. At the same time, PdeA is degraded by the ClpXP protease, freeing the activity of its antagonist DGC, DgcB[10]. Together this leads to an accumulation of c-di-GMP in sessile stalked and predivisional cells. Asymmetric distribution of PleD and PdeA in the predivisional cell sets up a spatial gradient of c-di-GMP with low cellular levels in the swarmer compartment and high cellular levels in the stalked compartment[118]. Mutants that lack PleD and DgcB show characteristic pole differentiation defects[119]. This includes the failure to eject the flagellaum and to assemble a polar stalk and adhesive holdfast[76,120] (Fig. 9). These observations strongly indicate a prominent role for c-di-GMP in *C. crescentus* cell fate determination[10,34,76,91].
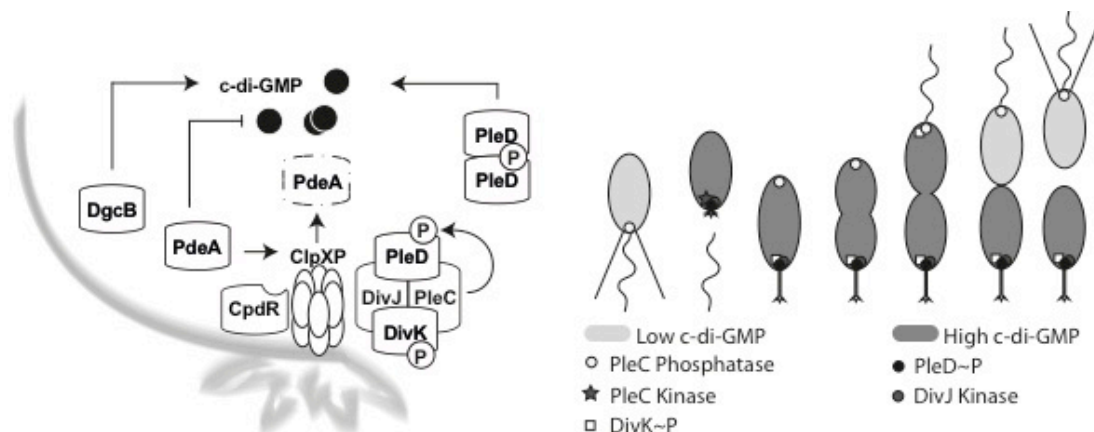
Figure 9: Model for c-di-GMP accumulation and distribution in the cell. During the swarmer to stalked pole transition, ClpXP mediates degradation of PdeA unleashing DgcB activity and PleD is activated by DivJ-DivK-PleC leading to PleD localization and rapid c-di-GMP accumulation. The distributions of c-di-GMP, PleD, PleC, DivK and DivJ during the cell cycle are indicated (left panel). Adapted from[10,16,118].

1.5 The flagellum

Locomotion is an advantage for the bacterial cell as the ability to move into favorable surroundings is a crucial for growth and survival in nutrient poor environments. One means of locomotion is the flagellum, an elaborate nanomachine, which enables the cell to harness the proton or sodium ion motive force to generate a mechanical force by the rotation of a long filament. The rotation of the flagellum is controlled by an array of chemoreceptors that can sense a wide range of environmental signals (chemotaxis). Changes in the surroundings induce a rotational response, clockwise or contra clockwise that facilitates the forward locomotion allowing bacteria to reach a beneficial environment.

1.5.1 The *C. crescentus* flagellar architecture

The flagellar motor consists of a rotor and a stator part. The stator is assembled in the inner membrane of bacterial cells from two integral membrane proteins, MotA and MotB, that form a complex of four MotA and two MotB protomers[121]. It has been estimated that at least 11 copies of the MotA-MotB complex, anchored to the peptidoglycan over MotB[122], assemble around the rotary part of the motor. The rotor is composed of several proteins that assemble into ring structures in and around the inner membrane where they form the MS ring switch complex. The MS ring is made of 26 copies of FliF subunits[123,124] that serve as assembly platform for the switch complex composed of FliG, FliM, and FliN. The interaction between FliG and MotA is responsible for the torque generation[125], whereas FliM and FliN modulate the direction of motor rotation in response to signals from the chemoreceptor array[126]. The C ring accommodates the type three secretion system (T3SS)[127] that facilitates the secretion of the flagellar distal elements, the rod, the

hook and the filament. The rod is composed of three proteins FlgB, FlgC and FlgF forming a cylindric channel that spans the periplasmatic space and is embedded in the peptidoglycan layer and the outer membrane via the P-ring (FlgI) and the L-ring (FlgH) strucutures. These are the only flagellar elements that are secreted via the Sec pathway[128,129] and not via the T3SS. The rod structure has a double function as a structural element that connects the motor to the hook protein (FlgE) and as a part of the T3SS as the proteins that assemble the hook and filament travel over the periplasmatic space within the rod. Once the hook proteins are secreted, they are self assembled into a short tubular structure, which is thought to function as a universal joint to smoothly transmit the torque produced by the motor[130] to the filament and enables the secretion of the filament subunits. The filament itself, is composed of FljJ, FljK, FljL, FljM, FljN, and FljO[131] subunits, grows to a tremendous length up to 15 µM[132] and serves as a screw propeller to convert rotary motion of the motor into a mechanical drive (Fig. 10).
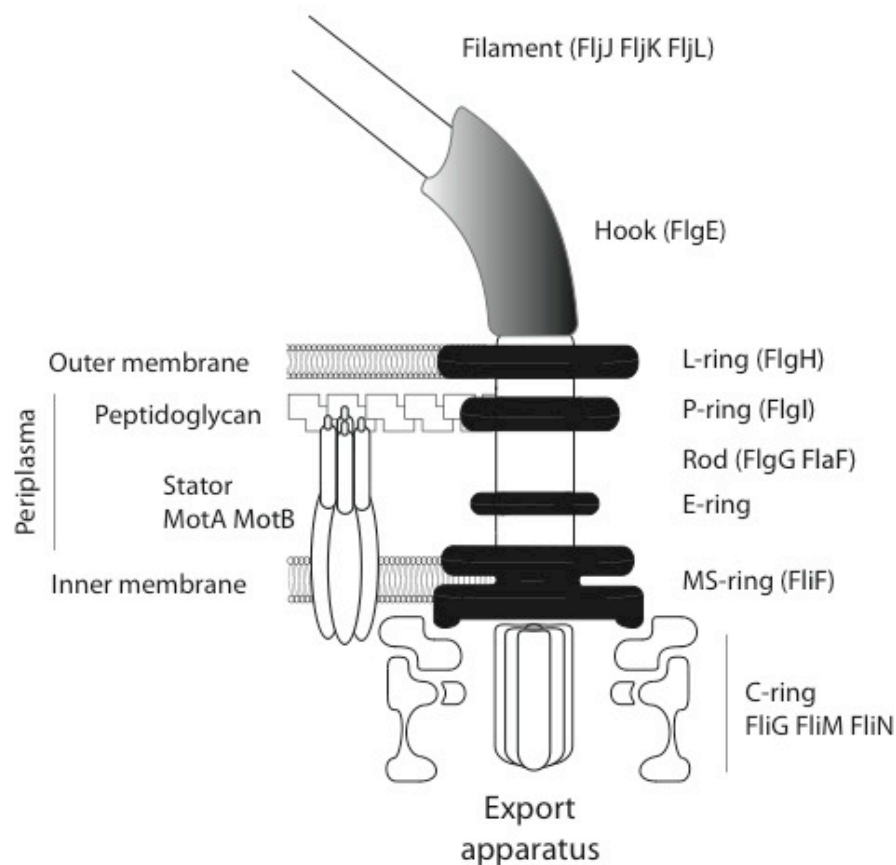
Figure 10: Schematic representation of the *C. crescentus* flagellum. The main structures are indicated as well as the proteins composing the structure. In addition, the position of the flagellum relative to the cell membrane is shown. The *C. crescentus* flagellum is presumed to have an additional ring structure (E-ring) which is unique and is missing in *E. coli* and *S. typhimurium*. Adapted from[133,134].

1.5.2   The regulation of flagellar biogenesis in *C. crescentus*

The *C. crescentus* flagellum biogenesis regulation is unique as flagellar gene expression is not only coordinated with the ongoing construction of the rotary device but is also tied to the cell cycle. The flagellum is assembled in the late predivisional cells and is active only for a short time during the cell cycle during which chromosome replication is inhibited (G1 phase). Upon swarmer cell differentiation into a stalked cell the flagellum is ejected. The flagellar genes cluster into operons that are regulated in a hierarchical manner forming transcriptional classes. These correspond to the flagellar structural checkpoints that are build sequentially and activate the next wave of gene expression. The activation of each class is dependent on the successful assembly of the previous class of proteins. Class I is reserved for the response regulator CtrA that acts as general activator of class II genes[79], the products of which assemble into the MS ring, the switch complex and the T3SS (basal body). In addition to the structural elements, class II includes regulatory elements that couple the transcription initiation of class III and class IV to the assembly of the flagellar basal body. FlbD is an NtrC-like transcriptional regulator that is required for the transcription initiation of sigma 54-depedent class III and IV genes[135,136]. The activity of FlbD is dependent on an additional factor, FliX[137]. FliX is 15 kD membrane associated protein required for class III and IV expression. It has been postulated that FliX, by some unknown mechanism, senses the assembly state of the class II encoded part of the basal body and in response regulates FlbD activity through a direct interaction[138]. Furthermore, it was shown that the stability of the two proteins is co-dependent and stabilized FliX deregulates FlbD activity and leads to the expression of class III protein in a class II mutant background. Once activated, the class III gene products are secreted via the T3SS and assembled into rod and hook structure. The class IV mRNA translation is inhibited, as long as the hook

structure is not assembled, by the activity of the class III gene product FlbT[139] that binds the flagellin mRNA 5' untranslated region[140]. The mechanism by which hook assembly is sensed and transmitted to FlbT is unknown (Fig 11).
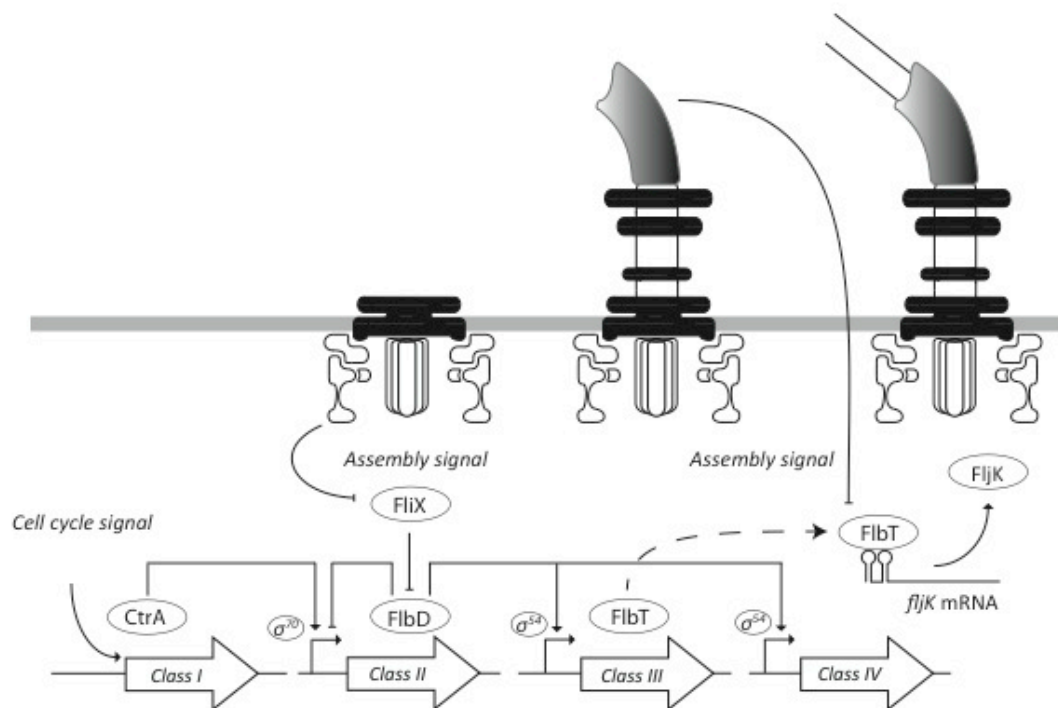
Figure 11: Regulation of flagellar gene expression and assembly during *C. crescentus* flagellar biogenesis: The regulatory cascade is indicated in the lower panel, the corresponding progression of flagellar assembly is indicated in the upper panel. Class II transcription is activated by CtrA. The class II gene product FliX inhibits FlbD by direct interaction, as long as the MS switch complex is not assembled. The nature of signal inactivating FliX is unknown. Class III and IV flagellar genes are activated by FlbD, whereas the class III gene product FlbT binds the flagellin 5' UTR mRNA (class IV) until the basal body hook, is fully assembeld. Adapted from[134,138].

1.5.3   The flagellum and c-di-GMP signalling

C-di-GMP induces the switch between a motile and a sessile life style of bacterial cells. In recent years several c-di-GMP mediated mechanisms affecting flagellar function were described. One example is the *E. coli* PliZ domain effector protein YcgR, which upon binding to c-di-GMP modulates flagellar rotation through a direct

interaction with the motor protein MotA. This interaction presumably results in strethened electrostatic interactions between MotA and the rotor component FliG generating a force that apposes motor rotation. A stepwise decrease of torque production and thus increased motor curbing was observed with increasing numbers of stator subunits interacting with YcgR[55]. Alternative models propose that activated YcgR binds to the switch complex and biases flagellum rotation towards a counter-clockwise orientation and thus smooth swimming[65].

In *C. crescentus,* the cellular levels of c-di-GMP are affecting the flagellum in several ways. A mutant lacking the DGC PleD fails to eject the flagellum during the swarmer to stalked cell transition, resulting in hypermotile cells and the stabilization of the FliF MS ring protein[76,110,120,141]. In contrast, a strain expressing a constitutive active allele of *pleD* (*pleD**) produces high cellular levels of c-di-GMP and, as a consequence, assembles an intact but paralyzed flagellum. The paralysis of the flagella at high c-di-GMP levels was associated with the activity of the c-di-GMP effector proteins DgrA and DgrB[33]. It was shown that *dgrA* and *dgrB* deletion mutants are able to retain motility even under high cellular levels of c-di-GMP. All of the above shows that the flagellar rotation and ejection in *C. crescentus* is c-di-GMP regulated.

In addition, c-di-GMP regulates flagellar assembly in *C. crescentus.* TipF[142] and TipN[142,143] were identified in a screen for mutants with reduced motility. Deletion mutants of *tipN* show delocalized flagella[142,143], whereas *tipF* mutants fail to assemble any external flagellar structures[142]. TipN was demonstrated to localize to the bacterial septum and to mark the position of the new pole. The observation that ectopic expression of *tipN* resulted in ectopic stalks argued that TipN is sufficient for the initiation of pole development. In addition, TipN is essential for normal pole development as *tipN* deletions misorient axis polarity, fail to localize the PleC phosphatase and polar organelles[143]. TipF localizes to the new pole in a TipN dependent manner, although no direct interaction was found. While *tipF* mutants are unable to assemble the flagellum, flagellar genes of class II, III, and IV are transcribed in this mutant background[144] and the respective proteins are presente in the cell but are not secreted. This indicates that an early checkpoint of flagellar

assembly is defect in the absence of TipF. This is supported by the observation that FliG failed to localize to the pole opposite the stalk in the absence of TipF[142]. The *tipF* gene encodes an EAL domain protein and putative PDE. Primary structure analysis of TipF EAL domain indicates that all of the catalytic residues are conserved (Table 2) with the exception of Lys^{332} (Gln^{647} TBD1265). In addition, TipF variants with a deletion in the EAL domain show the same phenotypes as *tipF* deletion mutants[142]. This creates a direct link between c-di-GMP signalling and flagellar assembly and/or positioning. However, the exact mechanism of this process is yet to be determined.

Proteins[A]	Conserved residues										Activity [B]
	1	2	3	4	5	6	7	8	9	10	
1. CB15 TipF	E211	R215	E233	N269	E311	D331	K332	K352	E396	Q415	?
2. TBD1265	E523	R527	E546	N584	E616	D646	D647	K667	E703	Q723	Yes
3. TDB1269	E	R	E	N	E	D	D	K	E	Q	Yes
4. TDB1660	E	R	E	N	E	D	D	K	E	Q	Yes
5. TDB1456	E	R	E	N	E	D	D	K	E	Q	Yes
6. EC YahA	E	R	E	N	E	D	D	K	E	Q	Yes
7. SFV 3559	E	R	E	N	E	D	D	K	E	Q	Yes
8. CV0542	E	R	E	N	E	D	D	K	E	Q	Yes
9. CV2505	E	R	E	N	E	D	D	K	E	Q	Yes
10. SO2039	E	R	E	N	E	D	D	K	E	Q	Yes
11 . NE0566	E	R	E	N	E	D	D	K	E	Q	Yes
12. EC YdiV	E	H	E	Q	L	G	N	M	G	Q	No
13. STM1344	E	T	V	A	A	A	N	M	A	Q	No
14. BS YkuI	E	R	I	D	E	D	N	K	E	Q	No
Residue role (interaction)	Me1	P2	G1	Me1, P1	Me1	Me1, Me2	Me2	Water-1, E616	Me2	E523, K667	

Table 2: Primary structure analysis of TipF EAL domain revealing a subset of conserved residues. Sequence alignment of the following proteins was used to identify conserved residues: T. denitrificans TBD1265 (Q3SJE6; aa 487–758), TBD1269 (Q3SJE2; aa 442–705), TBD1660 (Q3SIB5; aa 443–698), TBD1456 (Q3SIW6; aa 955–1212); E. coli YahA (P21514; aa 73–362); Shi. flexneri SFV_3559 (YhjK; Q83J53; aa 395–649); C. violaceum CV0542 (Q7P0M4; aa 362–612), CV2505 (Q7NV41; aa 146–401); She. Oneidensis SO2039 (Q8EFE2; aa 1–233); N. europaea NE0566 (Q82WU5; aa 1–415); E. coli YdiV (P76204; aa 1–237); S. typhimurium STM1344 (CdgR; Q8ZPS6; aa 1–237); B. subtilis YkuI (BSU14090; O35014; aa 1–407) (A). Hydrolysis of c-di-GMP (B). Adapted from[24]

2   Aim of this work

The second messenger cyclic-di-guanosine monophosphate (c-di-GMP) is known to control multiple aspects of bacterial growth and development. In *C. crescentus,* polar developmental such as the synthesis of polar organelles is c-di-GMP dependent. However, the adaptor molecules that sense c-di-GMP and mediate the signal to the appropriate targets are not identified yet.

The main goal of this work was to investigate whether and how putative downstream elements are involved in c-di-GMP signalling and to understand the respective signal transduction mechanisms. Previous results had linked the EAL domain containing protein TipF to flagellar assembly. The focus of this work was to investigate the c-di-GMP signal transduction mechanism involved in TipF activation and the identification of the processes of c-di-GMP mediated flagellar assembly. In an additional project, we identified the flagellar glycosylation protein FlmA as a putative c-di-GMP binding protein. Follow up studies should investigate c-di-GMP binding specificity of FlmA.

3 Results

3.1 Re-setting a flagellar polarization pathway during a bacterial cell cycle with a second messenger

Nicole Davis*, Yaniv Cohen*, Stefano Sanselicio, Coralie Fumeaux, Ricardo Guerrero-Ferreira, Elizabeth Wright, Urs Jenal, Patrick Viollier

Statement of my work

I contributed to this work by performing the experiments presented in Figures 2, 3C-D, 4A, 7A-E, S1, and S2A-B

ABSTRACT

Morphogenesis relies on the implementation and subsequent amplification of polarity cues, often imparted by nucleotide signals, at key times during the eukaryotic cell cycle. Analogous mechanisms are not known for the bacterial cell cycle. We uncovered a polarization mechanism in the bacterium *Caulobacter crescentus* that is triggered and amplified by cyclic-di-GMP signaling upon the G1→S transition to seed flagellum morphogenesis at the new pole via the TipF receptor protein. We show that c-di-GMP-binding induces the polarization of TipF and the recruitment of downstream effectors, including flagellar switch proteins and the PflI positioning factor, into a complex with the TipN landmark protein that preselects the polar site. Importantly, c-di-GMP not only induces TipF polarization and activation, but also its stability, thereby enforcing the removal of this factor from the maturating old pole during the G1→S transition. This novel c-di-GMP-governed reinforcement mechanism ensures a robust amplification and swift removal of TipF-dependent polarization during each cell cycle.

INTRODUCTION

Cell polarity is fundamental to intracellular organization and morphogenesis in all forms of life, yet little is known on how polarity-dependent cues are implemented, reinforced and removed during the bacterial cell cycle. The cell cycle regulator Cdc42, a member of the Rho-family of GTPases, is critical for polarization in baker's yeast *Saccharomyces cerevisiae* and many other eukaryotes. In response to cell cycle signals, Cdc42 polarizes and promotes the nucleation of actin cables that, in turn, enhance the polarization of Cdc42 in its active GTP-bound form (Cdc42•GTP). Polarized Cdc42•GTP recruits polarity effectors to the bud site and ultimately drives bud emergence. Underlying this polarization are "landmark" proteins that are positioned in the mother and inherited by newborn cells to dictate the future bud site(Slaughter, Smith, & Li, 2009).

Here we describe a conceptionally analogous polarized nucleotide-signaling pathway that triggers and reinforces morphogenesis during a bacterial cell cycle. We show that polar flagellation in the Gram-negative α-proteobacterium *Caulobacter crescentus* (henceforth *Caulobacter*) is cued by the activation, polarization and stabilization of a bis-(3'-5')-cyclic dimeric guanosine monophosphate (c-di-GMP) receptor protein (TipF) upon the G1→S transition to seed flagellar assembly at the pre-determined polar site. We show that TipF recruits the PflI placement factor into a flagellar positioning complex containing the TipN landmark protein while also binding and recruiting the switch component of flagellar base to the newborn pole. Importantly, the c-di-GMP-induced stabilization and localization amplifies the

polarization while synchronizing this specific morphogenetic process with other c-di-GMP induced developmental events that are triggered during the G1→S transition.

A myriad of polarized macromolecular assemblages were recently uncovered in bacteria and time-lapse imaging indicates that mechanisms must exist that coordinate protein polarization with cell cycle progression to ensure their appropriate inheritance in the progeny. The bacterial flagellum is such a polar assemblage, and while, polar flagellation is not uncommon, the mechanisms of polarization during the cell cycle are largely mysterious. Arguably the preeminent model system for bacterial cell cycle studies, *Caulobacter* has long been known to assemble polar flagellum in a cell cycle-controlled fashion. At the predivisional cell stage *Caulobacter* is overtly polarized, bearing a cylindrical extension of the cell envelope (the stalk) at the old pole and a newly assembled flagellum whose rotation is activated at cytokinesis at the opposite pole. Cytokinesis yields a motile swarmer cell that resides in a G1-like, non-replicative state and a dividing stalked cell that in S-phase. Two master transcriptional regulators of the cell cycle, CtrA and GcrA, reinforce the transcriptional program at sequential stages of the cell cycle. CtrA is present in G1-phase, protoelytically removed during the G1→S transition and reappears later in S-phase. GcrA accumulates during the G1→S transition, inducing the synthesis of CtrA along with polarity and cell cycle proteins.

The synthesis of early flagellar structural constituents, including the FliF MS-ring protein and two components of the switch complex (FliG and FliM), is induced by CtrA late in S-phase. The newly synthesized flagellar

parts are assembled from the inside of the cell outward, first with an organizational platform in the inner membrane (the MS-ring) from to which the switch complex on the cytoplasmic side of the membrane is tethered. The assembly of subsequent structures follows suit, ultimately ending with the elaboration of the external parts such as the hook and the flagellar filament (see. Fig. 3F).

Superimposed on these temporal patterns, are spatial cues that guide flagellar proteins to the appropriate site of assembly at the new cell pole opposite the stalk. The new cell pole is marked by TipN a polytopic coiled coil protein that is pre-positioned at this site when the new poles emerge from cytokinesis (Fig. 1)(Huitema, Pritchard, Matteson, Radhakrishnan, & Viollier, 2006; Lam, Schofield, & Jacobs-Wagner, 2006). At cytokinesis, TipN is recruited to the division plane form the poles, a redistribution that ensures that cell polarity cues are available for the flagellar polarization in the next cell cycle. Indeed, flagella are mispositioned in the absence of *tipN* mutants, indicating that TipN is required for the proper placement, but not the assembly, of the flagellum(Huitema et al., 2006; Lam et al., 2006). A flagellar misplacement defect has also been observed in mutants lacking the PflI positioning factor, a bitopic membrane proteins with a proline-rich domain at the C-terminus facing the cytoplasm that is localized to the future flagellated pole before the synthesis of early flagellar components. While the spatial relationship between TipN and PflI remains unexplored, TipN recruits TipF, a polytopic membrane protein that positively, regulates for flagellum biogenesis (Huitema et al., 2006). In the absence of TipN, TipF localizes to sites of misplaced flagella raising the possibility that mislocalized TipF is responsible

for the flagellar misplacement phenotype of a *tipN* mutant. This is supported by the observation that *tipF* deletion mutants lack external flagellar structures such as the hook and filament (Huitema et al., 2006).

TipF features C-terminal EAL domain facing the cytoplasm which in other related proteins confers c-di-GMP specific phosphodiesterase activity(Christen, Christen, Folcher, Schauerte, & Jenal, 2005). C-di-GMP is a ubiquitous bacterial second messenger that promotes an adhesive, surface-grown multi-cellular state called biofilm, while its absence favors the motile adventurous single cell state. C-di-GMP levels are regulated by the antagonistic enzymatic activities of diguanylate cyclases (DGC) and phosphodiesterases (PDE). DGCs harbor a catalytically active GGDEF domain that promotes the condensation of two GTP molecules into c-di-GMP. In contrast, EAL or HD-GYP domain catalyze the asymmetric hydrolysis of c-di-GMP into linear pGpG(Christen et al., 2005; Schirmer & Jenal, 2009). In *C. crescentus* multiple DGCs and PDEs modulates c-di-GMP levels during the cell cycle to promote development and cell cycle progression(Abel et al., 2011; R. Paul et al., 2008; R. Paul, Weiser, Amiot, & Chan, 2004). Whereas G1 swarmer cells contain low levels of c-di-GMP, levels quickly increase during G1$\rightarrow$S transition(R. Paul et al., 2008). Then, at cell division c-di-GMP levels diminish in the flagellated G1 (swarmer) chamber, while being maintained in the stalked compartment (Christen et al., 2010).

The observation that TipF localizes to the site of flagellar assembly at the new cell pole together with the finding that a *tipF* mutant fails to assemble a complete flagellar structure, argued that TipF may act as a phosphodiesterase locally at the newborn pole to promote flagellar

assembly(Huitema et al., 2006). A role for TipF as PDE at the site of flagellum assembly would be in line with the general consensus that high levels of c-di-GMP inhibit flagellar-based motility through different possible mechanisms(Boehm et al., 2010; Wolfe & Visick, 2008). Here we explore the spatiotemporal relationship of c-di-GMP and TipF in polar flagellum biogenesis during the *Caulobacter* cell cycle.

RESULTS

c-di-GMP binds to and activates TipF at the newborn pole

To dissect the mechanism and function of TipF at the newborn pole, we first considered the several unusual sequence features in TipF compared to c-di-GMP -specific phosphodiesterase (PDE) belonging to the same family. For example, TipF features a Lys at position 332 instead of an Asp in related proteins (Fig. 1D). Moreover, a Glu-Ser-Phe (ESF, residues 211-213) triplet replaces the defining Glu-Ala-Leu (EAL) motif normally found in related PDEs. To explore whether the sequence conservation reflects a functional requirement for the residues at these positions, we engineered alanine mutations at several conserved positions implicated in coordinating the cofactor (Mg^{2+} or Mn^{2+}), the substrate or the nucleophile (E211A, D331A and K352A, filled triangles in Fig. 1D). Neither of these mutant proteins could support TipF function (motility on 0.3% soft agar plates) when expressed in a $\Delta tipF$ background from P_{xyl} on a low copy plasmid, indicating that all of these residues play a key role in TipF function (Fig. 1E).

To test if TipF is an active PDE, we purified soluble, hexa-histidine tagged forms of TipF wild type and TipF(E211A) mutant. Both proteins were truncated variants lacking the first 110 amino acids, which include the two predicted trans-membrane domains. Circular dichroism (CD) spectra suggested that the secondary structures of purified TipF wild type and E211A mutant were unchanged (Fig. S1A). We next assayed PDE activity of both proteins by HPLC analysis using c-di-GMP as substrate (Fig. 2A). No cleavage of c-di-GMP to the linear pGpG was observed with either TipF wild type or the E211A mutant (Fig. 2A, right panel), even after extended incubation times of up to 24 h (Fig. S1B, 1C). By contrast, control PDE YahA from *E. coli* converted all of the c-di-GMP to pGpG in less than 5 min (Fig. 2A left panel).

In the absence of detectable PDE activity, we asked if TipF wild type or the E211A mutant can bind c-di-GMP using an iso-thermal calorimetry (ITC)-based binding assay (Fig. 2B). Successive injections of 10 µL of a 118 µM solution of c-di-GMP into the ITC reaction chamber containing 32.5 µM of WT TipF was accompanied with the characteristic heat release indicating specific and high affinity binding of c-di-GMP (Fig. 2B, left panel). The resulting integrated titration peaks were fitted to a sigmoidal enthalpy curve and a dissociation constant (K_D) of 0.4 (+/- 0.2 µM) between WT TipF and c-di-GMP was derived. By contrast, only background (non-specific) heat release was observed when an equimolar amount of TipF(E211A) was in the chamber (Fig. 2B, middle panel, note the different scales in the panels). Together, these results demonstrate that TipF is not a PDE and that while TipF wild type binds c-di-GMP with high affinity, the E211A mutant is unable to bind the

ligand. On the basis of these results we hypothesize that c-di-GMP binding is necessary for TipF to signal motility and that TipF(E211A) is non-functional because it fails to bind c-di-GMP and, in response, in be activated. In support of this view, we show below that depleting *Caulobacter* cells of c-di-GMP by overexpression of PA5295, a potent heterologous PDE from *Pseudomonas aeruginosa*, phenocopies the localization and flagellar assembly defect caused by the absence TipF or the "c-di-GMP-blind" E211A mutant (see Fig. 5E, 5F).

If TipF is activated without hydrolysing c-di-GMP, then analogous mutations that are known to specifically cripple catalytic activity of PDEs (without interfering with c-di-GMP binding) should still retain function. To test this prediction, we engineered mutants Q197A, R215L and E396A (Fig. 1D, open triangles) and found the mutant proteins R215L and E396A to confer motility in Δ*tipF* cells indistinguishable from that of WT TipF, while Q197A could also supported motility albeit with reduced efficiency (data not shown).

On the basis of these genetic and biochemical experiments, we conclude that TipF is a c-di-GMP binding protein and signals motility function(s) upon binding of the second messenger.

TipF cues the polar assembly of the flagellar base.

How TipF cues motility is not well understood. Transmission electron micrographs previously suggested that TipF signals the construction of the polar flagellum, or at least the external structures, since the flagellar hook and the filament (Fig. 3F) are absent form Δ*tipF* cells(Huitema et al., 2006). Moreover, live-cell fluorescence imaging in wild type (WT) and Δ*tipF* mutant

cells expressing a derivative of the FliG switch (C-ring) protein (Fig. 3F) harboring a carboxy (C-) terminal fusion to GFP (FliG-GFP) in addition to the endogenous FliG, showed that localization of FliG-GFP to the new pole depends on TipF (Fig. 3C, S2A). To demonstrate that TipF promotes the formation of the switch structure at the cell pole, we conducted live cell imaging of the FliM switch protein (Fig. 3A, 3F) harboring a C-terminal moiety to GFP (FliM-GFP) that was expressed from P_{van} on a plasmid. By analogy to other flagellar systems, FliG is predicted to interact with FliM and to tether the switch to the MS-ring base plate in the inner membrane. Our finding that FliM-GFP is diffuse in the cytoplasm of Δ*fliG* cells, but sequestered to the flagellated pole in WT cells, is consistent with this notion (Fig. 3A). Next, we imaged FliM-GFP in *tipF$^+$* and Δ*tipF* cells that carry the *fliM*::Tn*5* mutation (to avoid interference from untagged, endogenous FliM) and observed diffuse fluorescence in Δ*tipF* cells, akin to that of Δ*fliG* cells. By contrast, in *tipF$^+$* cells FliM-GFP was properly localized at the newborn pole opposite the stalk. Thus, consistent with the dependence of polar localization of FliM on FliG, when FliG localization is compromised in Δ*tipF* cells, FliM is also unable to localize in the absence of TipF*.

Since two switch proteins (FliG and FliM) are no longer polarized in the absence of TipF, we investigated if this is also the case for the MS-ring protein FliF. The MS-ring is the earliest known flagellar structure to assemble and tethers the switch complex to the cytoplasmic membrane. Similar to the situation for FliG-GFP, only few polar foci were seen when FliF-GFP was expressed in Δ*tipF* cells, arguing that neither the MS-ring, nor the switch complex assemble in Δ*tipF* cells (Fig. 3C, 3D, S2A and S2B). By contrast,

FliF-GFP and FliG-GFP show normal polar localization when expressed in Δ*fliF* and Δ*fliG* cells, respectively (Fig. S2A and S2B). Together this argued that TipF promoted polar clustering of FliG independent of FliF. To bolster this finding using an independent imaging approach, we conducted high-resolution analysis of WT and Δ*tipF* poles by electron cryo-tomography (ECT) (Fig. 3E, S2C and S2D) in which intracellular structures that are preserved in their native (cryogenically vitrified) state are visualized *in situ* by virtue of their natural contrast (electron density). Whole-cell imaging of WT and Δ*tipF* cells followed by structural refinement using segmentation algorithms of 2D projections from 3D tomograms revealed densities of the MS-ring and switch at the cytoplasmic membrane of WT poles, but no such structures at Δ*tipF* poles. As expected, flagellar (sub)structures traversing the OM and S-layer reflecting regions of the rod, P- and L-rings and the hook emanated from the poles of WT cells. These structures were also absent from Δ*tipF* cells. However, chemoreceptor arrays that normally assemble at the same pole and at the same time as the flagellum, but via a flagellum-independent pathway, were seen in the Δ*tipF* mutant pole thus confirming that the site was imaged where a flagellum should have been (Fig. 3E, right panel)*.*

The important role of TipF in the formation of the MS-ring and switch was further corroborated using strains in which FliG is tethered to FliF by way of FliF-FliG translational fusion (i.e. within single polypeptide) encoded within the endogenous *fliF* locus. This fully functional FliG-FliF fusion protein(Jenal & Shapiro, 1996) could not direct FliM-GFP into polar clusters in the absence of TipF, indicating that TipF function is beyond merely facilitating the recruitment of FliG to the membrane via FliF and that TipF stimulates polar clustering of

FliG (data not shown). In support of this conclusion, polar clusters of FliG, although dependent on TipF, still formed in Δ*fliF* cells (Fig. S2A). Moreover, below we show that TipF interacts directly with FliG in a yeast-two-hybrid assay.

Taken together, these experiments support the conclusion that the formation of the polar MS- and C-rings is cued by TipF and that TipF possibly acts at the earliest known (nucleation) step in flagellum biogenesis, switch and MS-ring formation, respectively.

The PflI flagellar positioning factor is recruited into a polar complex by TipF

In addition to the structural components of the flagellum, a polarly localized ancillary protein, PflI, was recently identified that is required for proper positioning of the flagellum. PflI, a bitopic membrane protein with a proline-rich C-terminal domain facing the cytoplasm, is expressed and recruited to the future flagellum assembly site before most flagellar proteins(Obuchowski, 2008). How PflI is localized to the newborn pole is unclear, but it does not require the MS-ring protein FliF. We uncovered three lines of evidence that prompted us to explore whether PflI localization was dependent on TipF. First, we observed that TipF synthesis precedes that of FliF and other flagellar proteins whose expression is activated by the CtrA master transcriptional regulator (see Fig. 7F). Second, we noted that, similar to PflI, FliF is also dispensable for the polar sequestration of TipF (data not shown). Finally, we, found that mCherry tagged TipF (TipF-mCherry) expressed from the endogenous *tipF* locus in lieu of untagged TipF co-

localizes with PfII-GFP expressed from P$_{van}$ on a plasmid (Fig. 1C). Moreover, since Δ*tipF cells* (unlike Δ*pfII* cells) are aflagellate, we wondered if TipF recruits PfII to the pole. To test this possibility, we expressed PfII variants harboring a C-terminal fusion to YFP or GFP either from its own promoter at the native chromosomal locus or from a plasmid in Δ*pfII* cells and imaged in the wild type or Δ*tipF* context. While the expected polar foci of PfII-YFP (and PfII-GFP) were readily seen in *tipF*$^{+}$ cells, only dispersed fluorescence from PfII-YFP or PfII-GFP was seen in the envelope of Δ*tipF* cells (Fig. 3B and S3A). Conversely, PfII does not noticeably affect TipF localization, as indicated by the apparent normal localization of TipF-GFP (expressed in lieu of endogenous TipF from the *tipF* locus) in Δ*pfII* compared to WT cells (Fig. S3B).

To identify which region of PfII is required for TipF-dependent localization, we imaged mutant PfII-GFP derivatives lacking either the trans-membrane domain [PfII(Δ4-28)], the coiled-coil region [PfII(Δ28-92)] or one of two adjacent stretches in the proline-rich region [PfII(Δ93-142) or PfII(Δ142-194)] expressed from P$_{van}$ on a plasmid in Δ*pfII* cells (Fig. S3C). While the deletion of the coiled-coil domain had no dramatic affect of PfII localization, deletion of the trans-membrane domain or the proline-rich region strongly interfered with polar sequestration. PfII lacking the proline-rich region is distributed in the membrane, as is the case for full-length PfII-GFP expressed in the absence of TipF, suggesting that TipF recruits PfII to the newborn pole, directly or indirectly, via the proline-rich domain.

Since TipF is recruited to this site by the TipN landmark protein (Fig. S3B), we predicted that PfII localization should also be dependent on TipN.

Indeed, PfII-YFP or PfII-GFP foci are mispositioned near or within the stalk in Δ*tipN* cells, a pattern resembling the misplacement of TipF-GFP in the absence of TipN (Fig. 3B and S3A). The formation of these (mislocalized) PfII foci in Δ*tipN* cells is still TipF-dependent as indicated by the fact that upon introducing a Δ*tipF* mutation into Δ*tipN cells,* PfII-GFP adopts a disperse disposition in the envelope (Fig. S3A). Conversely the Δ*pfII* mutation does not seem to change the mislocalization of TipF-GFP in Δ*tipN* cells (Fig. S3B). Thus, flagellar assembly and recruitment events at the newborn pole proceed in the order: TipN > TipF > [(PfII)(FliF/G/M)].

To test if this localization dependency is reflected in physical interactions among the proteins, we conducted pull-down experiments from extracts derived of cells harboring an empty vector control or a plasmid expressing either a TipF or PfII derivative with a C-terminal TAP (Tandem Affinity Purification) tag from the P*van* promoter. Owing to the instability of TipF (described below), we conducted the pull-down experiments in mutant backgrounds (Δ*tipN* and *flbD*::Tn*5,* when appropriate) in which TipF abundance is increased (Fig. 4A). This likely results from a post-transcriptional mechanism as *tipF* transcription is unchanged in a *tipN* and *flbD* mutant (Fig. S4A). Immunoblotting of TipF-TAP and PfII-TAP pull-down samples with antibodies to PfII (Fig. 4B) or TipF (Fig. 4C) provided evidence that TipF and PfII interact (directly or indirectly), albeit the interaction seems relatively weak. By contrast, co-immunoprecipitation of PfII with TipF-GFP using monoclonal antibodies to GFP was more efficient (Fig. S4B). We conclude that PfII resides in a complex with TipF. To investigate if TipF and PfII also associate with TipN, we probed blots containing the TipF-TAP (Fig.

4D) and PflI-TAP (Fig. 4E) pull-down samples with polyclonal antibodies to TipN and detected TipN in these samples as well, but not in the control samples. Thus, TipF and PflI also interact with TipN.

Lastly, in an attempt to identify TipF client proteins in an unbiased fashion, we conducted a yeast-two-hybrid screen with soluble TipF (lacking trans-membrane segments, residues 1-90 aa) as bait to probe a genomic prey library of *C. crescentus*. The screen was conducted in a yeast strain that ectopically expressed the diguanylate cyclase YdeH from *E. coli*, thereby mimicking a c-di-GMP containing environment. When selecting for interaction of bait and pray by scoring for growth in the absence of adenine, we identified two positive clones encoding Gal4-AD fusions to two C-terminal fragments of FliG (FliG$_C$), residues 192-340 and residues 236-340 (Fig. 4F).

In sum, we conclude that TipF interacts directly with FliG$_C$ and recruits PflI into a flagellar positioning complex with TipN at the newborn pole.

c-di-GMP signals TipF localization and activation

Having established that polar localization of PflI and flagellar assembly are regulated by the c-di-GMP-receptor TipF, we investigated if c-di-GMP is required for TipF function. To this end, we first asked if TipF(E211A) that is unable to bind c-di-GMP and cannot support motility, can promote the polar localization of PflI. As shown in Fig. 6A, neither the E211A derivative, nor the D331A or K352A mutants were able to tag PflI to the cell pole. Furthermore, the E211A mutation phenocopied flagellar assembly defect of the Δ*tipF* deletion as reflected in a failure to express the FlgE hook protein (Fig. 3F, 5F).

If TipF signaling is triggered by binding of c-di-GMP, then cells depleted for c-di-GMP should also be unable to localize PflI and to express the FlgE hook protein similar to Δ*tipF or tipF(E211A)* mutant cells. To test this prediction, we heterologously expressed the potent *P. aeruginosa* PDE PA5295(Duerig, Nicollier, Schwede, & Amiot, 2009) in strains carrying the *pflI-yfp* reporter and imaged the resulting cells. PA5295, but not the catalytically inactive mutant PA5295-AAL [carrying an analogous mutation to TipF(E211A) at position E328], was previously shown to reduce c-di-GMP levels beyond detection(Duerig et al., 2009) We found that induction of PA5295 caused the dispersion of PflI-YFP from the pole into the envelope and prevented FlgE expression, akin to Δ*tipF* or *tipF(E211A)* mutant cells (Fig. 5A). Interestingly, however, c-di-GMP depletion has a much stronger effect on transcription of the *flgE* gene than the Δ*tipF* or *tipF(E211A)* mutation, indicating that c-di-GMP modulates another step of flagellum biogenesis independently of TipF (see below).

Next, we explored if c-di-GMP depletion also affects TipF-GFP or TipN-GFP localization. As shown in Fig. 5B, 5C TipF was completely delocalized from the poles upon c-di-GMP-depletion, while TipN localization was only weakly affected. Thus, c-di-GMP promotes TipF localization and activity. If so, then a mutation in TipF that prevents c-di-GMP binding and activity, such as TipF(E211A), should be unable to localize when c-di-GMP is present. Live-cell fluorescence imaging confirmed that TipF(E211A)-GFP is delocalized when expressed from P_{xyl} promoter on a plasmid in Δ*tipF* cells (Fig. 6B). Next, we also determined the subcellular localization of the other TipF mutants and found TipF(D331A)-GFP to be dispersed and TipF(K352A)-GFP to cluster at

the stalked pole but not at the newborn pole (Fig. 6B). Thus, these mutations interfere with c-di-GMP binding or structural changes that are induced by c-di-GMP to prevent TipF and PflI from localizing to the newborn pole and inducing flagellar assembly at this site.

Bypassing the requirement of c-di-GMP for TipF signaling

To illuminate how c-di-GMP activates TipF and induces its polar localization, we isolated intragenic suppressor mutations that restore motility to *tipF(E211A)* or *tipF(D331A)* cells. These suppressor mutations are either missense mutations in two proximal residues (F280S or F284L) within the c-di-GMP-binding (EAL) domain or a duplications of a triplet amino acid (either ADA or DTV, residues 121-124 or 128-130, respectively) within the coiled-coil motif in front of the EAL-domain (Fig. 1B, 1E). Importantly, the two missense mutations were isolated irrespective of the genetic background, i.e. *tipF(E211A)* or *tipF(D331A)*. Since F280S and F284L are not allele-specific suppressors, we suspected that they would both also be able to overcome the localization defect of D331A and E211A by driving the proteins from dispersion into an active and polarized state. C-terminal fusions of GFP to the four TipF double mutants (E211A/F280S, E211A/F284L, D331A/F280S and D331A/F284L) revealed that these proteins indeed regained the capacity to cluster at the pole and to recruit PflI-YFP to polar sites (Fig. 6C, D).

Next, we tested if these TipF double mutants were able to resist delocalization induced by depletion of c-di-GMP. In the presence of c-di-GMP, WT TipF-YFP and the two E211A double mutant derivatives (E211A/F280S and E211A/F284L) were all polar (Fig. S5A). By contrast, depletion of c-di-

GMP delocalizes WT TipF-YFP, while polar foci of the E211A/F280S and E211A/F284L derivatives persisted (Fig. 5D). In support of the conclusion that the E211A/F280S and E211A/F284L mutants are active and localized in the absence of the signal induced by c-di-GMP, we found that the E211A/F284L mutant protein, like the E211A single mutant, is unable to bind or hydrolyze c-di-GMP *in vitro* (Fig. 2A, B), although its secondary structure is conserved (Fig. S1).

To test if these c-di-GMP-bypass mutants confer motility in the absence of c-di-GMP, we determined the FlgE steady-state levels (cell-associated and the shed hook accumulating in the supernatant) in WT and *tipF* single and double mutant cells by immunoblotting (Fig. 5F). These experiments showed that FlgE levels are reduced in c-di-GMP-depleted cells, even in the presence of the E211A/F280S and E211A/F284L TipF versions. This effect seems to be mediated by a second, c-di-GMP-dependent event that acts on *flgE* transcription, as indicated by the reduction of β-galactosidase activity from the P*flgE*-*lacZ* transcriptional reporter to 30±1% (E211A/F280S) and 30±1% (E211A/F284L) compared to WT, much below that observed in the Δ*tipF* single mutant (57±1%) (Fig. 5E). Consistent with the conclusion that at least one additional c-di-GMP-dependent event exists that controls the assembly and possibly the placement of the flagellum, expression of the hook gene as monitored using P*flgE*-*lacZ* activity is further reduced to 21% when c-di-GMP is depleted in Δ*tipF* cells. A similar c-di-GMP-dependent effect was seen on transcription of the *fljL* flagellin gene, using the P*fljL*-*lacZ* transcriptional reporter (Fig. S5B). In this case, however, c-di-GMP-depletion reduced *fljL*

transcription in $\Delta tipF$ cells from near full WT levels (*fljL* transcription is not affected by $\Delta tipF$ mutation) to 32±2%.

We conclude that TipF localization is at least partially uncoupled from c-di-GMP in two suppressor mutants and that c-di-GMP controls, in addition to the TipF branch, at least one more pathway in flagellum assembly.

TipF cell cycle abundance is tightly regulated and dependent on c-di-GMP

Immunoblotting experiments using polyclonal antibodies to TipF raised the possibility that the TipF protein is stabilized upon binding to c-di-GMP. The abundance of wild type TipF expressed from P_{xyl} (i.e. using the transcriptional and translational regulatory signals of the *xylX* gene) in $\Delta tipF$ cells is significantly higher that those of the TipF(E211A) mutant variant under the same conditions (Fig. 5F), suggesting that TipF is regulated at the level of stability by c-di-GMP. Indeed stability measurements of wild type TipF and TipF(E211A) under normal (Fig. 7B, D) or c-di-GMP depleted (Fig. 7A, C) conditions (using PA5295 as described above) approximated the half-life of WT TipF at a level of more than 2 hours in the presence of c-di-GMP and below 20 min in its absence. By contrast, for TipF(E211A) we determined half-life values of about 10-20 min irrespective of the presence or absence of c-di-GMP, demonstrating that TipF(E211A) is not significantly destabilized upon c-di-GMP depletion (Fig. 7A, C). Remarkably, the half-life of E211A/F284L (~40 min) was higher than that of TipF(E211A) but lower than that of WT TipF. Again, the stability of this mutant was largely insensitive to the presence of absence of c-di-GMP, consistent with the robust polarization of

TipF(E211A/F284L) even in the absence of c-di-GMP (see above). Thus, in the absence c-di-GMP TipF is unable to bind c-di-GMP and adopt an active conformation, it is delocalized from the cell pole and, at the same time is de-stabilized. These events can be uncoupled from c-di-GMP with a mutation of F284L, which not only activates the TipF(E211A) mutant form constitutively, but at the same time protects it from proteolysis *in vivo.*

In search for a candidate protease that destabilizes TipF *in vivo*, we found that ClpX, the ATP-dependent chaperone component of the ClpXP protease, controls the cell cycle-dependent accumulation of endogenous (untagged) TipF expressed from its native chromosomal location. Poisoning ClpXP activity by expression of a dominant negative (catalytically inactive) ClpX variant (ClpX*)(Osterås, Stotz, Schmid Nuoffer, & Jenal, 1999) from $P_{xyl,}$ results in TipF accumulation. Immunoblotting revealed TipF to be absent from G1-phase (swarmer) cells, induced during the G1-S transition (40 min) and again destabilized at the time of division (Fig. 7F). Blocking ClpX function using the *clpX** allele, resulted in TipF accumulation in G1 cells, presumably owing to a failure to degrade TipF in the swarmer progeny (Fig. 7E). Together with the observation that TipF stability critically depends on the presence of c-di-GMP, this strongly argued that TipF is rapidly degraded in the swarmer progeny as a result of a drop in c-di-GMP levels in this cell type(Christen et al., 2010).

As a result of the ClpXP-mediated elimination of TipF in G1 swarmer cells, TipF accumulation is dictated primarily by the time of its synthesis. Since the cell cycle pattern of TipF resembles that of GcrA, a master transcriptional regulator that activates transcription of a myriad of cell cycle-

regulated genes at the G1-S transition and is itself induced at this time, we hypothesized that GcrA induces the expression of TipF at the G1-S transition. In support of this idea, previous mRNA profiling experiments using DNA microarrays showed that *tipF* mRNA levels diminish when GcrA is depleted(Holtzendorff et al., 2004). Probing a P*tipF*-*lacZ* transcriptional reporter in a GcrA-depletion strain (Fig. 7G), we found that *tipF* promoter activity depends on GcrA. Moreover, quantitative chromatin-immunoprecipitation (qChIP) analysis using polyclonal antibodies to GcrA showed that GcrA binds to the *tipF* promoter (Fig. 7H). The induction of TipF expression at the transcriptional level by GcrA and the concordant surge in c-di-GMP levels at the G1-S transition ensure that TipF is active and ready to prime flagellum biosynthesis at the newborn pole, while a drop in c-di-GM in the incipient G1 daughter cells results in the removal of TipF, thus resetting flagellar polarity cascade.

DISCUSSION

With the coordinated induction of TipF and c-di-GMP at the G1→S transition, a robust polarized pathway for flagellum biogenesis is launched and simultaneously tuned to other c-di-GMP dependent differentiation events, including stalk biogenesis and the removal of the replication inhibitor/transcriptional activator CtrA. The implementation, the robust propagation and then the sudden re-setting of polarity during the cell cycle is a hallmark of bacterial, fungal and metazoan model systems, but the

underlying signals and mechanisms are not well understood, let alone in bacterial systems.

The c-di-GMP mechanisms unearthed here illustrate how during a primitive bacterial cell cycle, flagellar polarity is first signaled and implemented, and then ultimately re-set. At least three sequential mechanisms are involved and mediated by distinct effectors: TipN, c-di-GMP and ClpXP. The first mechanism, active during the late stages of constriction at the end of the preceding cell cycle, involves the deposition of the TipN landmark signal at the newborn pole to allow the correct interpretation of the polarity axis in the progeny. This ensures that the subsequent events, triggered by c-di-GMP at the G1$\rightarrow$S, are executed at the correct (newborn) pole (Fig. 1A).

The second mechanism involves a double selection by two parallel "AND" gates (the "GcrA-gate" and "c-di-GMP-gate") that must be met for TipF-dependent implementation of flagellar polarity to proceed. The GcrA-gate (Fig. 1A) licenses TipF expression with the induction of the transcriptional regulator GcrA that activates transcription of genes encoding polarity (PodJ, controls pilus assembly), development (PleC, regulates CtrA), division (MipZ, inhibits the FtsZ tubulin) and cell cycle (HU, nucleoid organizing protein) determinants, along with *tipF*. While the accumulation of GcrA seems to be coupled to the initiation of chromosome replication via the DnaA replication initiator protein(Collier, McAdams, & Shapiro, 2007) and is thus under a cell cycle cue, other unknown mechanism may help enforce this gate to promote the fluctuation of GcrA. With the synthesis of TipF, the surge of c-di-GMP at the G1$\rightarrow$S(Christen et al., 2010; R. Paul et al., 2008) directly induces the

activation and subsequent recruitment of TipF and its client proteins to the newborn (TipN-marked) pole to promote flagellum assembly (the "c-di-GMP-gate").

The third mechanism is discernable and involves the onset of a diminution in TipF levels during cytokinesis, when flagellar biogenesis is completed and the flagellum is energized. While it is unclear if the ClpXP protease regulates TipF stability as function of the cell cycle as for many of its substrates, ClpX activity is required to prevent the ectopic accumulation of TipF in the G1 progeny cell. Under conditions that preclude binding of c-di-GMP by TipF, either by mutation of the c-di-GMP binding site in TipF or by depletion of c-di-GMP from cells, polar localization is prevented and TipF is quickly turned over. The levels of c-di-GMP are rapidly depleted during compartmentalization in the G1 (swarmer) progeny, while they are maintained in the S (stalked) progeny. Therefore, a critical role can be attributed to this c-di-GMP reduction in the ClpX-dependent removal of TipF in the G1 progeny and, thus, in re-setting flagellar polarity for the G1 cell.

The diminution of c-di-GMP in compartmentalized G1 cells is governed by the PleC histidine kinase/phosphatase that is sequestered to the flagellar pole and partitions exclusively with the G1 progeny to de-activate the PleD response regulator with its c-di-GMP-synthase (GGDEF) output domain by de-phosphorylation(R. Paul et al., 2008). The resulting trough in c-di-GMP should disperse TipF from the pole and induce proteolytic removal, thereby terminating flagellar assembly. This event is coordinated with the onset of flagellar rotation regulated by the DgrA/B PilZ-domain proteins, paralogous c-di-GMP receptor proteins that prevent precocious activation of flagellar

rotation in the in the presence of bound c-di-GMP(Christen et al., 2007). The PleC-dependent reduction in c-di-GMP levels in the motile G1 progeny, is thought to release the inhibition of flagellar rotation by DgrA/B. PleC not only de-phosphorylates the PleD c-di-GMP synthase/response regulator, but also the DivK single domain response regulator, a cell fate cell fate determinant that promotes the transcriptional activation of G1-phase genes by CtrA (perhaps reinforced by other factors)(Biondi et al., 2006) and the accumulation of the swarmer cell specific c-di-GMP phosphodiestrase PdeA(Abel et al., 2011). Thus, the removal of TipF that clears the flagellar polarization pathway coincides with the implementation of the G1 transcriptional landscape by CtrA and with a reduction of c-di-GMP levels that directly promotes motor function in this cell type. By contrast, the transcriptional re-programming by GcrA and the resulting induction of TipF at the onset of S-phase thus reinstates the c-di-GMP-dependent flagellar polarity with the coincident activation of the PleD-kinase DivJ.

An imbalance in c-di-GMP abundance in the two progeny cells also occurs in the compartmentalized *Pseudomonas aeruginosa* pre-divisional cell. Interestingly, the FimX EAL-GGDEF hybrid protein of *P. aeruginosa* that promotes the assembly of retracting Type IV (polar) pili (Tfp) for twitching motility shares biochemical and cytological properties with TipF. FimX is a polarized EAL domain protein that can influence the positioning of Tfp(Kazmierczak, Lebron, & Murray, 2006; Navarro, De, Bae, Wang, & Sondermann, 2009). It also binds c-di-GMP and this causes a (long-range) conformational change in the adjacent domain(Qi et al., 2011). How FimX scouts the pole, how it signals Tfp assembly and how its localization is

coordinated with cell cycle progression is unknown. Our *tipF* suppressor genetics points to a functional relationship between triplet duplications in the coiled-coil motif and missense mutations in the EAL domain. Helical wheel analysis predicts the alpha-helicity to be maintained by the insertion, while causing a shift of the hydrophobic region that could cause structural reorganization presumed to underlying the functional modifications of TipF and interactions with its flagellar and polarity targets.

Within the flagellar polarization cascades TipF bears perhaps the most functional resemblance to the FlhF GTPase studied in *vibrios*, *pseudomonads* and *Campylobacter jejuni*. FlhF is itself polarized and recruits the MS-ring protein FliF to the polar site of flagellation in *V. cholera*(Correa et al., 2005; Kusumoto et al., 2006; Pandza et al., 2000). However, despite the pervasive polar flagellation found across most bacterial lineages, the principal target of FlhF in polar flagellation and the underlying temporal relationships are unknown. By contrast, TipF associates with PflI, TipN and the flagellar switch protein FliG. The soluble portion of TipF (encompassing the predicted coiled–coil and EAL domains) interacts directly with the C-terminal portion of FliG. FliG is emerging as an important regulatory hub for diverse modes of motility control. EpsE of *Bacillus subtilis* and YcgR of *E. coli/Salmonella enterica* are thought to arrest flagellar rotation by disengaging or curbing the flagellar motor(Blair, Turner, Winkelman, Berg, & Kearns, 2008; Boehm et al., 2010; K. Paul, Nieto, Carlquist, Blair, & Harshey, 2010). Moreover, the nucleoid binding protein H-NS and the enzyme fumarate reductase (FRD) bind to FliG, perhaps to regulate motility in response to metabolic fluctuations or to maintain the homeostasis of motility with chromosome organization or

environmental conditions that H-NS responds to(K. Paul, Brunstetter, Titen, & Blair, 2011). With the important of role of FliG switch protein in regulating the direction of rotation of the flagellum it is perhaps not surprising that the aforementioned FliG-binding proteins regulate rotation. However, because of its central role early in flagellar erection, assembly regulators that act on FliG might also be expected. TipF is the first representative of this class with a function(s) that may go beyond regulating FliG. In fact, polar sequestration of FliG and FliF is independent of each other but strongly affected by TipF. Our evidence that another, TipF-independent, but c-di-GMP dependent flagellar assembly step exists (Fig. 5E-F) provides another example of the unexpected regulatory complexity in flagellar assembly. As established from seminal work in other systems, flagellation is fine-tuned through external signals. As shown here, this pathway also ingrates information from pre-existing spatial landmarks for polar c-di-GMP-mediated signals that are periodically removed and re-established, thus reinforcing small nucleotide polarization pulses that drive morphogenesis at key times in the bacterial cell cycle.

Material and Methods

Strains, plasmis, and media

The bacterial strains and plasmid used in this study are listed in Table S1. *Caulobacter crescentus* strains were grown in peptone yeast extract (PYE, Ely, 1991) or in minimal media supplemented with 0.2% glucose or 0.3% D-xylose (M2G or M2X, Ely, 1991) at 30°C with constant shaking (150 rpm). When selection was required antibiotics in the following concentrations were added: (solid/liquid media in µg/ml): gentamycin (5/0.5), kanamycin (20/5), nalidixic acid (20/not used) and oxytetracycline (5/2.5). For inducible gene expression the medium was supplemented with 1 mM vanillate, 0.3% xylose or 1 mM IPTG. For synchronization experiments newborn swarmer cells were isolated by Ludox gradient centrifugation (Jenal, 1996) and released into fresh minimal medium. For synchronization with inducible constructs, 1 mM vanillate was added to the growth medium 2 h prior synchrony.

E. coli strains were grown in Luria Broth (LB) at 37 °C. When necessary for selection the following antibiotic concentrations were used: (solid/liquid media in µg/ml) ampicillin (100/50), gentamycin (20/15), kanamycin (50/30) and oxytetracycline (12.5/12.5).

Microscopy

DIC and fluorescence microscopy were performed on a DeltaVision Core (Applied Precision, USA)/Olympus IX71 microscope equipped with an UPlanSApo 100x/1.40 Oil objective (Olympus, Japan) and a coolSNAP HQ-2 (Photometrics, USA) CCD camera. Cells were placed on a patch consisting of 1% agarose in water (Sigma, USA). Images were processed using ImageJ software (NIH, USA) or Photoshop CS3 (Adobe, USA) software. Bactria cell and protein localization signals were analyzed with MicrobeTracker (V0.931) and MATLAB software. Cryo-electron Tomography

was preformed as described in(Kudryashev, Cyrklaff, Wallich, Baumeister, & Frischknecht, 2010).

Protein purification

Rosetta BL21pLysS *E.coli* strain was transformed with respective plasmids and used for protein overexpression. Cultures with an optical density at 600nm of 0.4 were induced with 1mM isopropyl-b-D-thiogalactopyranoside (IPTG) and incubated for 4h at RT with constant shaking, cells were collected by centrifugation. The cell pellets were resuspended in buffer consisting of 20mM Tris-HCl pH7, 250mM NaCl, 5mM imidazole, 10mM $MgCl_2$, 1% glycerol and Complete mini cocktail of protease inhibitors, EDTA free at the concentrations specified by the manufacturer (Roche). The cells were lysed using French press, and the crude extracts were centrifuged at 15,000g for 40 min at 4^0C. The cleared lysates were incubated with preequillibrated Profinity IMAC Ni-resin (Bio-Rad) for 1h at 4^0C and washed subsequently with lysis buffer and washing buffer containing 20 mM imidazole. The proteins were eluted using 350mM imidazole.

Phosphodiesterase Assay

$TipF_{WT}$, $TipF_{E211A}$ and $TipF_{E211A/F284L}$, were tested for c-di-GMP phosphodiesterase activity. As a control YahA, a class A phosphodiesterase from *E. coli* was used. 10mM final concentration of each protein was incubated with 100mM c-di-GMP in 50mM Tris-HCl pH8, 50mM NaCl, 5mM $MgCl_2$, 1mM DTT at RT. Samples were taken at time points 0, 1h, 2h and overnight. The reactions were stopped by heating at 99^0C for 5 min, diluted with 5mM NH_4HCO_3 pH8 buffer, filtered (0.22mm), and analyzed on a Resource Q ion-exchange chromatography column (GE Healthcare).

Circular Dichroism Spectroscopy.

Circular dichroism (CD) measurements were performed with an AVIV 26A DS spectropolarimeter. Spectra were recorded at room temperature from 250 to 190 nm, with a resolution of 1.0 nm, response time of 3 s, bandwidth of 1 nm and 3 accumulations. CD samples were prepared by adjusting the protein concentration to 30 µM in 50 mM Tris buffer at pH 8. A control spectrum of pure buffer solution was subtracted from all samples.

Isothermal titration calorimetry

The interaction of TipF with cyclic-di-GMP was measured with a VP-ITC isothermal titration calorimeter from MicroCal (Northampton, MA). Four different experiments were performed with three different protein batches measured under the same conditions (50 mM Tris/HCl, 50 mM NaCl, pH 8.0 at 25°C). All integrated titration peaks produced sigmoidal enthalpy curves for the interaction between TipF and cyclic di-GMP. The average dissociation constant, K_D, was 0.4 (+/- 0.2 mM), the stoichiometry of binding, n, was 0.35 (+/- 0.1) and the enthalpy of reaction was -2.1 (+/- 0.3 kcal/mol). Two additional experiment were performed at 35°C, the average dissociation constant, K_D, was 0.3 (+/- 0.1 mM), the stoichiometry of binding, n, was 0.3 (+/- 0.02) and the enthalpy of binding, ΔH^0, was -4.75 (+/- 0.25) kcal/mol. All solutions were degassed and equilibrated at desired temperature before using. The delay between the injections was set to 5 min to ensure re-equilibration between injections. Data were evaluated using Origin software (OriginLab) provided by Microcal (Northampton, MA). $TipF_{E211A}$ and $TipF_{E211A/F284L}$ binding experiments were contacted under the above-specified conditions.

Motility Assays

The spontaneous, non-allele specific *tipF* suppressor mutants were obtained by plating 2.5µl of an overnight culture of PV1928 (P$_{xyl}$-*tipF(E211A)*); PV2152 (P$_{xyl}$-*tipF(D331A)*); and PV2151 (P$_{xyl}$-*tipF(K352A)*) to 0.3% PYE swarm agar supplemented with Tetracycline (1µg/ml). The plates were incubated for seven days after which spontaneous motile colonies developed. The plasmids were isolated, sequenced, and shown to contain the initial primary mutation (E211A, D331A, or K352A) and an additional second-site mutation within the tipF coiled-coil or EAL domains.

Yeast two-hybrid analysis

Yeast two hybrid screen was preformed using the system described in. The EAL domain of TipF fused to the Gal4-DBD (DNA binding domain) was expressed in *Saccharomyces cerevisiae* PJ69-4A from plasmid pGBD Kan^R. YdeH, an active DGC that produces c-di-GMP, was expressed in the same strain from plasmid p426-cm-YdeH or the empty plasmid p426. For screening, a *C. cresentus* library was transformed into the strain containing the bafore mentioned plasmids. The library contains *C. crescentus* genomic DNA fragments between 500 to 3000 bp, obtained after partial digest of genomic DNA using the enzymes HinPIL, MspI and Taq1, fused to the *gal4-AD* (activator domain) in three reading frames (J. Luciano). Transformants were screened on SD medium lacking leucine, tryptophan, uracil and either adenine or histidine. Prey-plasmids of positive clones were isolated and retransformed in PJ69-4A containing the Gal4-DBD-TipF-EAL or the Gal4-DBD. Interaction strength and self-activity were tested with these strains on

selective medium to check for the activation of reporter genes. Inserts of confirmed interactors were sequenced to ensure in-frame coding sequence with the Gal4-AD.

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Table S1 Strains and Plasmids

Strains

Name	Genotype / Description	Source or Reference
NA1000	CB15N Laboratory strain derived from CB15	Evinger and Agabian, 1977
UJ3638	NA1000 Δ*tipF* Markerless in frame deletion of *tipF*	Huitema et al., 2006
UJ3635	NA1000 Δ*tipN* Markerless in frame deletion of *tipN*	Huitema et al., 2006
PV2025	NA1000 Δ*tipF* P$_{xyl}$-*tipF*	This Study
PV1928	NA1000 Δ*tipF* P$_{xyl}$-*tipFE211A*	This Study
PV2152	NA1000 Δ*tipF* P$_{xyl}$-*tipFD331A*	This Study
PV2151	NA1000 Δ*tipF* P$_{xyl}$-*tipFK352A*	This Study
ND562	NA1000 Δ*tipF* P$_{xyl}$-*tipFE211A/F280S*	This Study
ND568	NA1000 Δ*tipF* P$_{xyl}$-*tipFE211A/F284L*	This Study

ND570	NA1000 *ΔtipF* P*xyl*-*tipFD331A/F280S*	This Study
ND564	NA1000 *ΔtipF* P*xyl*-*tipFD331A/F284L*	This Study
ND280	NA1000 *ΔtipF* P*xyl*-*tipFF280S*	This Study
ND283	NA1000 *ΔtipF* + P*xyl*-*tipFF284L*	This Study
ND284	NA1000 *ΔtipF* + P*xyl*-*tipFADA insert*[128-130]	This Study
ND282	NA1000 *ΔtipF* + P*xyl*-*tipFDTV insert*[121-124]	This Study
ND562	NA1000 P*van*-*tipFE211A/F280S-sEGFP*	This Study
ND564	NA1000 P*van*-*tipFD331A/F284L-sEGFP*	This Study
ND568	NA1000 P*van*-*tipFE211A/F284L-sEGFP*	This Study
ND570	NA1000 P*van*-*tipFD331A/F280S-sEGFP*	This Study
ND319	NA1000 *ΔpflI* P*van*-*pflI-GFP*	This Study
ND424	NA1000 *ΔtipFΔpflI* P*van*-*pflI-GFP*	This Study
ND342	NA1000 *ΔtipNΔpflI* P*van*-*pflI-GFP*	This Study
ND480	NA1000 *ΔtipNΔtipFΔpflI* P*van*-*pflI-GFP*	This Study
ND607	NA1000 *ΔtipF* P*van*-*fliM-sEGFP*	This Study
ND611	NA1000 *fliM::Tn5* P*van*-*fliM-sEGFP*	This Study
ND572	NA1000 *ΔpflI tipF-gfp*	This Study

ND573	NA1000 *ΔtipNΔpfliI tipF-gfp*	This Study
NR1760	NA1000 *ΔtipN tipF-gfp*	This Study
ND316	NA1000 *ΔpfII P$_{van}$-pfII$^{Δ4-28}$-GFP*	This Study
ND317	NA1000 *ΔpfII P$_{van}$-pfII$^{Δ28-92}$-GFP*	This Study
ND318	NA1000 *ΔpfII P$_{van}$-pfII$^{Δ93-142}$-GFP*	This Study
ND558	NA1000 *ΔpfII P$_{van}$-pfII$^{Δ142-194}$-GFP*	This Study
ND588	*NA1000::tipN-gfp* P$_{van}$-*PA5295*	This Study
ND589	*NA1000::tipN-gfp* P$_{van}$-*PA5295AAL*	This Study
ND590	*NA1000::pfII-venus* P$_{van}$-*PA5295*	This Study
ND591	*NA1000::pfII-venus* P$_{van}$-*PA5295AAL*	This Study
ND578	*NA1000::tipF-gfp* P$_{van}$-*PA5295*	This Study
ND579	*NA1000::tipF-gfp* P$_{van}$-*PA5295AAL*	This Study
ND762	*NA1000 pflgE-lacZ/290* P$_{xyl}$-*PA5295AAL*	This Study
ND787	*NA1000 pflgE-lacZ/290* P$_{xyl}$-*PA5295*	This Study
ND831	NA1000 *ΔtipF pflgE-lacZ/290* P$_{xyl}$-*PA5295AAL*	This Study
ND751	NA1000 *ΔtipF pflgE-lacZ/290* P$_{xyl}$-*PA5295*	This Study
ND839	NA1000 *ΔtipF vanA*::P$_{van}$-*tipF-venus pflgE-lacZ/290* P$_{xyl}$-*PA5295AAL*	This Study

ND837	NA1000 *ΔtipFvanA*::P$_{van}$-*tipF-venus pflgE-lacZ/290* P$_{xyl}$-*PA5295*	This Study
ND843	NA1000 *ΔtipF;vanA*::P$_{van}$-*tipFE211AF280S-venus pflgE-lacZ/290* P$_{xyl}$-*PA5295AAL*	This Study
ND841	NA1000 *ΔtipF;vanA*::P$_{van}$-*tipFE211AF280S-venus pflgE-lacZ/290* P$_{xyl}$-*PA5295*	This Study
ND835	NA1000 *ΔtipF;vanA*::P$_{van}$-*tipFE211AF284L-venus pflgE-lacZ/290* P$_{xyl}$-*PA5295AAL*	This Study
ND833	NA1000 *ΔtipF;vanA*::P$_{van}$-*tipFE211AF284L-venus pflgE-lacZ/290* P$_{xyl}$-*PA5295*	This Study
ND749	*NA1000 pfljL-lacZ/290* P$_{xyl}$-*PA5295AAL*	This Study
ND777	*NA1000 pfljL-lacZ/290* P$_{xyl}$-*PA5295*	This Study
ND796	NA1000 *ΔtipF pfljL-lacZ/290* P$_{xyl}$-*PA5295AAL*	This Study
ND811	NA1000 *ΔtipF pfljL-lacZ/290* P$_{xyl}$-*PA5295*	This Study
ND804	NA1000 *ΔtipF;vanA*::P$_{van}$-*tipF-venus pfljL-lacZ/290* P$_{xyl}$-*PA5295AAL*	This Study
ND802	NA1000 *ΔtipF;vanA*::P$_{van}$-*tipF-venus pfljL-lacZ/290* P$_{xyl}$-*PA5295*	This Study
ND808	NA1000 *ΔtipF;vanA*::P$_{van}$-*tipFE211AF284L-venus pfljL-lacZ/290* P$_{xyl}$-*PA5295AAL*	This Study
ND806	NA1000 *ΔtipF;vanA*::P$_{van}$-*tipFE211AF280S-venus pfljL-lacZ/290* P$_{xyl}$-*PA5295*)	This Study
ND800	NA1000 *ΔtipF;vanA*::P$_{van}$-*tipFE211AF284L-venus pfljL-lacZ/290* P$_{xyl}$-*PA5295AAL*)	This Study
ND798	NA1000 *ΔtipF;vanA*::P$_{van}$-*tipFE211AF284L-venus pfljL-lacZ/290* P$_{xyl}$-*PA5295*).	This Study
UJ6287	NA1000 *ΔtipF* P$_{van}$- *PA5295* P$_{xyl}$-*tipF*	This Study
UJ6288	NA1000 *ΔtipF* P$_{van}$- *PA5295* P$_{xyl}$-*tipFE211A*	This Study

UJ6289	NA1000 *ΔtipF* P*van*- *PA5295* P*xyl*-*tipFE211AF284L*	This Study
UJ6292	NA1000 *ΔtipF* P*van*- *PA5295AAL* P*xyl*-*tipF*	This Study
UJ6293	NA1000 *ΔtipF* P*van*- *PA5295AAL* P*xyl*-*tipFE211A*	This Study
UJ6294	NA1000 *ΔtipF* P*van*- *PA5295 AAL* P*xyl*-*tipFE211AF284L*	This Study
UJ 1793	NA1000 *ΔfliF* Markerless in frame deletion of *fliF*	Jenal et al., 1996
LS 2356	NA1000 *ΔfliG* Markerless in frame deletion of *fliG*	Jenal
ND306	NA1000 *ΔpflI* Markerless in frame deletion of *pflI*	This Study
ND359	NA1000 *ΔtipFΔpflI* Markerless in frame deletion of *pflI* and *tipF*	This Study
ND454	NA1000 *ΔtipNΔtipFΔpflI* Markerless in frame deletion of *pflI, tipN* and *tipF*	This Study
UJ413	NA1000 fliM::Tn5 *Tn5* insertion in *fliM*	P. Aldridge
UJ 284	NA1000 *ΔpleD*	P. Aldridge
UJ5292	pJ69-4A trp1-901 leu2-3,112 ura3-52 his3-200 GAL4 ΔGAL80 ΔLYS2::GAL1-HIS3 GAL2-ADE2 met2::GAL7-lacZ	James et al., 1996
UJ5649	pJ69-4A UJ5292 containing pGBD-tipF-kan, p426-TEF-ydeH-cm	Jutta Nesper
UJ5646	UJ5292 containing pGBD-tipF-kan, p426-TEF-cm	Jutta Nesper
UJ200	Disruption of the clpX gene by a Strep/SpecR cassette and integration of clpX at the xyl locus in NA1000	Jenal and Fuchs, 1998

Plasmids

Name	Description	Source
PCWR208	pLac290 P_{xyl}-*tipF*	Huitema et al., 2006
PCWR239	pLac290 P_{xyl}-*tipFE211A*	Huitema et al., 2006
PCWR261	pLac290 P_{xyl}-*tipFD331A*	This Study
PCWR262	pLac290 P_{xyl}-*tipFK352A*	This Study
pND100	pLac290 P_{xyl}-*tipFF280S*	This Study
pND101	pLac290 P_{xyl}-*tipFF284L*	This Study
pND102	pLac290 P_{xyl}-*tipFADA$^{28-130}$*	This Study
pND103	pLac290 P_{xyl}-*tipFDTV$^{121-124}$*	This Study
pND85	pLac290 P_{xyl}-*tipFE211A/F280S*	This Study
pND86	pLac290 P_{xyl}-*tipFE211A/F284L*	This Study
pND87	pLac290 P_{xyl}-*tipFD331A/F280S*	This Study
pND88	pLac290 P_{xyl}-*tipFD331A/F284L*	This Study
pND72	PCWR512 P_{van}-*pflI-CTAP*	This Study
pND71	PCWR512 P_{van}-*tipF-CTAP*	This Study
pND75	PCWR512 P_{van}-*tipFE211AF284L*-CTAP	This Study
pND56	pOK12 *ΔpflI$^{4-28}$*	This Study
pND57	pOK12 *ΔpflI$^{28-92}$*	This Study
pND58	pOK12 *ΔpflI$^{92-142}$*	This Study
pND59	pOK12 *ΔpflI$^{142-194}$*	This Study
pND77	pMT384 P_{van}-*pflI-sEGFP*	This Study
pND78	pMT384 P_{van}-*tipF-sEGFP*	This Study
UJ 6444	pET21 p_{lac}::His_6tipF$_{110-452}$	This Study
UJ 6450	pET21 p_{lac}::His_6tipF$_{110-452\ E211A}$	This Study
UJ 6445	pET21p_{lac}::His_6tipF$_{110-452\ E211A/F284L}$	This Study
PV1928	pCRW P_{xyl}-*tipFE211A*	This Study
PV2152	pCRW P_{xyl}-*tipFD331A*	This Study
PV2151	pCRW P_{xyl}-*tipFK352A*	This Study
SoA226	pXGFP4 P_{xyl}-*fliF-GFP*	Soren Abel
pCRW71	pCRW P_{xyl}-*fliG-GFP*	Huitema et al., 2006
UJ 6287	pRV P_{van}-*PA5295*	This Study
UJ 6292	pRV P_{van}-*PA5295$_{AAL}$*	This Study

pND95	P$_{van}$-*tipFE211A/F280S-sEGFP*	This Study
pND96	P$_{van}$-*tipFD331A/F284L-sEGFP*	This Study
pND97	P$_{van}$-*tipFE211A/F284L-sEGFP*	This Study
pND98	P$_{van}$-tipF*D331A/F280S-sEGFP*	This Study

Figure Legends:

Fig.1. Localization of TipN, TipF and PfII to the flagellated pole (A) Dynamic protein localization of TipN, TipF and PfII and its coordination with other cell cycle events in *C. crescentus*. TipN (green) localized at the non-flagellated pole recruits newly synthesized TipF (red), which in turn recruits the PfII flagellar positioning protein (yellow). (B) Domain organization of TipN, TipF and PfII. Shown are the predicted transmembrane domains (TM, gray), coiled coil domains (CC, black), and the phosphodiesterase domain (EAL, red) and the proline-rich domain (PR, yellow). (C) TipF and PfII co-localize. The localization of TipF-mCherry and PfII-GFP was analyzed using live-cell fluorescence microscopy in wild-type (WT) cells expressing TipF-mCherry in place of TipF from the *tipF* locus and PfII-GFP from the P$_{van}$ promoter on the medium copy number plasmid, pCWR510. (D) Alignment of the EAL domains from TipF and the c-di-GMP phospphodiesterases CC3396 of C. crescentus, VieA of *Vibrio cholerae*, RocR of *Pseudomonas aeruginosa* and YkuI of *Bacillus subtilis*. Key residues that are predicted to be required for phosphodiesterase activity (empty arrowheads) or required for c-di-GMP binding (filled black) are marked. In addition, the residues are indicated that bypass the requirement of E211 or D331 for motility (red triangles), the defining EAL motif and the K332 reside where Asp is normally present (underlined). (E) Motility assay of Δ*tipF* mutants harboring plasmids encoding WT, single or double mutant TipF. (DTV)$_2$ and (ADA)$_2$ denote the triple duplications of DTV or ADA residues at positions 121-124 or 128-130, respectively. Overnight cultures were spotted on PYE swarm (0.3% soft) agar

plates and incubated for 60 hr at 30ºC. Compact swarms indicate the motility defect caused by mutations in the c-di-GMP binding site whereas the suppressive mutations yield diffuse and enlarged swarms.

Fig. 2. TipF is enzymatically inactive but binds c-di-GMP. (A) No c-di-GMP hydrolytic activity is detected in recombinant (soluble) TipF, but in the control PDE YahA. One mM of purified proteins was incubated with c-di-GMP and incubated for 5min and then separated on a ResourceQ column to observe the cleavage product pGpG (left panel). No pGpG was detected after 1 h of incubation with WT or mutant (E211A or E211A/F284L) TipF with c-di-GMP. (B) Isothermal titration calorimetry experiments showing that c-di-GMP binds to the soluble portion of WT TipF, but not to the mutant derivatives E211A or E211A/F284L. The top panels show the raw ITC data curves collected at 25°C in binding buffer (50 mM Tris/HCl, 50 mM NaCl, pH 8.0). The bottom panels show the integrated titration peaks fitted to a one-site binding model (solid line). The average dissociation constant (K_D) of the EAL domain of TipF was estimated at 0.4 (+/- 0.2 μM), the stoichiometry of binding (n) to 0.35 (+/- 0.1) and the enthalpy of reaction to -2.1 (+/- 0.3 kcal/mol).

Fig. 3. TipF mediates the localization of flagellar proteins FliM, FliG, FliF and PflI to the cell pole. (A) The subcellular localization of FliM-GFP from the P_{van} promoter on a medium copy number plasmid in a *fliM* mutant (*fliM*::Tn5), in a *tipF* mutant (Δ*tipF; fliM*::Tn5) or in a *fliG* mutant (Δ*fliG; fliM*::Tn5) analyzed by live-cell fluorescence microscopy. (B) Localization of PflI-YFP expressed from P_{pflI} at the *pflI* locus in the presence (*pflI-yfp)* or the

absence of TipF (Δ*tipF; pfII-yfp*) or TipN (Δ*tipN; pfII-yfp*). (C, D) Localization of GFP-fusions to the flagellar proteins FliG (C, FliG-GFP) and FliF (D, FliF-GFP) expressed from P*xyl* at the *xylX* locus in a Δ*tipF* mutant. (E) Electron cryo-tomography imaging reveals densities corresponding to the intact flagellum including the MS-ring and the switch complex in or near the inner membrane (IM) of the WT, which are absent in the Δ*tipF* mutant. The color-coding of the segmented structures corresponds to the color scheme in (F). Flagellar structures are shown in red in the left panel. The white arrow in the right panel denotes the chemoreceptor arrays. (F) Schematic of the flagellum. The relative position of the MS-ring (FliF), the switch complex (including FliG and FliM), the hook (FlgE) and the filament (flagellins) are indicated, as are the envelope layers: inner (cytoplasmic) membrane (IM), peptidoglycan layer (PG), outer membrane (OM) and S-layer (SL).

Fig. 4. TipF directly interacts with FliG and forms a complex with TipN and PfII. (A) TipF steady-state levels as determined by immunoblotting using polyclonal antibodies to antibodies in lysates from wild-type, Δ*tipF*, Δ*tipN* and *flbD::Tn5*. (B-E) Tandem Affinity Purification (TAP) pull-down of TAP-tagged proteins expressed from P*van* on a medium copy plasmid, followed by immunoblotting using polyclonal to TipF, PfII, TipN and CtrA. The cell lysates derived from boiled cells shown in the lanes on the left of the panels provide negative and positive controls for the specificity for the antisera. CtrA immunoblots are shown as a control for loading. (B) TipF-TAP and TipF*-TAP (the E211A/F284L mutant is referred to as TipF*) were expressed from the P*van* promoter on a medium copy number plasmid in Δ*tipN* mutants. (C) TipF

is present in PfII-TAP purifications Δ*tipN* Δ*pfII* double mutant lysates. M, protein marker lane. (D, E) TipN is present in pull-downs of TipF-TAP (D) or PfIII-TAP (E) from *flbD::Tn5* lysates. (F) The C-terminus of FliG and the soluble part of TipF interact directly in a yeast two-hybrid (Y2H) assay. Yeast strains that harbor plasmids encoding a C-terminal fragment of FliG(192-340) fused to the GAL4 activation domain (AD) and TipF fused to the GAL4 DNA binding domain (DBD) or the DBD alone were tested for growth on selective media as indicated. The screen was conducted in a yeast strain that ectopically expressed the diguanylate cyclase YdeH from *E. coli*, thereby mimicking a c-di-GMP containing environment. Conditions that promote or inhibit growth are schematically represented (lower panel). The black (branched) arrow denotes promoter activation and thus growth, whereas the grey arrow indicates that the promoter does not fire efficiently and cannot support growth on selective media.

Fig. 5. Localization of TipF and PfII as well as expression of FlgE is c-di-GMP dependent

(A-D) Localization of PfII-YFP (A), TipF-GFP (B), TipN-GFP (C), and YFP tagged TipF-suppressor mutants (D) under normal (PA5295-AAL, +cdG) or c-di-GMP depleted conditions (PA5295-WT, -cdG). PA5295 variants were expressed the P_{xyl} promoter on a medium copy number plasmid in cells expressing the GFP fusions proteins from their respective promoters at the endogenous locus. In (D) TipF-YFP variants were expressed from P_{van} at the *vanA* locus in Δ*tipF* cells. (E) Effects of c-di-GMP concentration on transcription of the *flgE* hook gene by b-galactosidase assays of cells (WT,

Δ*tipF* or Δ*tipF* cells expressing mutant TipF from P*~van~* at the *vanA* locus) harboring a P*~flgE~*-*lacZ* transcriptional reporter fusion. (F) Effects of c-di-GMP depletion on FlgE steady-state levels in supernatants (Sup.) or cell lysates (Lys.) by immunoblotting using anti-FlgE antibody (α-FlgE). TipF mutants were expressed from the P*~xyl~* promoter on a low copy-number plasmid in Δ*tipF* cells.

Fig. 6. Suppressor mutations render TipF and PflI localization c-di-GMP independent.

(A-D) Localization of PflI-YFP (A, C) or TipF-GFP mutants (B, D) in Δ*tipF* cells harboring plasmids encoding TipF loss of function mutants expressed from the P*~xyl~* (A, C) or the P*~van~* (B, D) promoter a low copy-number plasmid.

Fig. 7. C-di-GMP levels affect TipF protein stability.

(A - D) Depletion of c-di-GMP by over-expression of a potent PDE reduces TipF steady-state levels. TipF, the c-di-GMP binding mutant E211A or the intragenic suppressor mutant E211A/F284L were expressed from P*~xyl~* on a low copy-number plasmid in Δ*tipF* cells grown in M2G containing xylose, following a shift to M2G containing vanillate to induce the expression of the PDE *PA5295* from *P. aeruginosa* or its active site mutant PA5295-AAL and repress P*~xyl~*. Samples were taken every 20 min and protein levels were quantified from the immunoblots and plotted as percentages of the highest value (A and B). The corresponding immunoblots, probed with an anti-TipF polyclonal antiserum, show the levels of the TipF variants during a period of 140 min after vanillate induction (C and D). (E) The dominant negative *clpX*~ATP~

allele was expressed from plasmid in M2G containing xylose or repressed by growing the cells in M2G. Swarmer cells were isolated and TipF was detected using an anti-TipF antibody and ClpX^{ATP*}::Flag using M2 antibodies. In the presence of WT ClpXP, TipF was not detectable while inactivation of ClpXP by the dominant negative allele encoding ClpX^{ATP*} led to stabilization of TipF. (F) The cell-cycle abundance of TipF resembles that of GcrA. Synchronized WT swarmer cells were followed throughout the cell cycle. CtrA, TipF and GcrA protein levels were determined by immunoblotting using specific polyclonal antisera. (G) GcrA activates *tipF* transcription. The activity of a P$_{tipF}$-*lacZ* transcriptional reporter was assayed in WT and in a strain (P$_{xyl}$-*gcrA*) in which GcrA expression is repressible by glucose in the absence of xylose. (H) GcrA binds to the *tipF* promoter as determined by quantitative chromatin-immunoprecipitation (qChIP) experiments using polyclonal antibodies to GcrA. The abundance of the *tipF* and *sciP* promoters was quantified in the immunoprecipitates.

Fig. S1. Secondary structure and activity of WT and mutant. TipF(A) Circular dichroism (CD) spectra of WT, E211A and E211A/F284L TipF. All spectra show the characteristic behavior associated with secondary structures, indicating that the purified proteins are folded. (B and C) FPLC chromatograms showing the lack of phosphodiesterase activity of WT, E211A and E211A/F284L TipF even after 24h.

Fig. S2. TipF promotes FliF and FliG localization. The localization pattern

of (A) FilF-GFP or (B) FliG-GFP expressed from P*xyl* at the *xylX* locus in WT, Δ*tipF,* Δ*fliG* and Δ*fliF* background. The relative positioning along the cell axis and number of foci were quantified. (C) Electron cryotomography image slices revealing the absence of flagellar structure in the Δ*tipF* mutant, whereas (D) flagella structures are clearly visible in WT cell poles.

Fig. S3. PflI localization determinants. Live-cell fluorescence microscopy images showing the localization of (A) PflI-GFP expressed from P*van* on a medium-copy plasmid in Δ*pflI*, Δ*pflI* Δ*tipF,* and Δ*pflI* Δ*tipF* Δ*tipN* background and (B) the localization TipF-GFP expressed from the endogenous promoter at the *tipF* locus in WT, Δ*pflI*, Δ*tipN*, and Δ*tipN* Δ*pflI* background. (C) Localization of PflI-GFP derivatives harboring a deletion in the transmembrane domain [PflI(Δ4-28)], the coiled-coil region [PflI(Δ28-92)] or one of two adjacent stretches in the proline-rich region [PflI(Δ93-142) or PflI(Δ142-194)] expressed from P*van* on a plasmid in Δ*pflI* cells.

Fig. S4. Transcription of *tipF* and co-immunoprecipitation PflI with TipF-GFP. (A) P*tipF*-*lacZ* activity assayed in WT, Δ*tipN*, *flbD::Tn5* strains. (B) Co-immunoprecipitation experiment with TipF-GFP expressed from the endogenous promoter at the *tipF* locus. TipF-GFP was precipitated with monoclonal anti-GFP antibodies, the precipitates were then probed for the presence of PflI by immunoblotting using polyclonal antibodies to PflI. The cell lysate lanes from boiled cells shown on the left of the panels provide negative and positive controls for the specificity for the antisera. CtrA

immunoblots are shown as a control for loading. Input designates the solubilized lysates that were used for the immunoprecipitation.

Fig. S5. Effect of TipF suppressor mutations on TipF localization and *fljL* transcription. (A) TipF-YFP variants were expressed from P_{van} at the *vanA* locus in Δ*tipF* cells and PA5295-AAL was expressed from the P_{xyl} promoter on a medium copy number plasmid. (B) Effects of c-di-GMP concentration on transcription of the flagellin gene *fljL*. The promoter of *fljL* was fused to *lacZ* and b-galactosidase activity was measured in WT, Δ*tipF* or Δ*tipF* cells expressing mutant TipF from P_{van} at the *vanA* locus under normal (PA5295-AAL, +cdG) or c-di-GMP depleted conditions (PA5295-WT, -cdG).
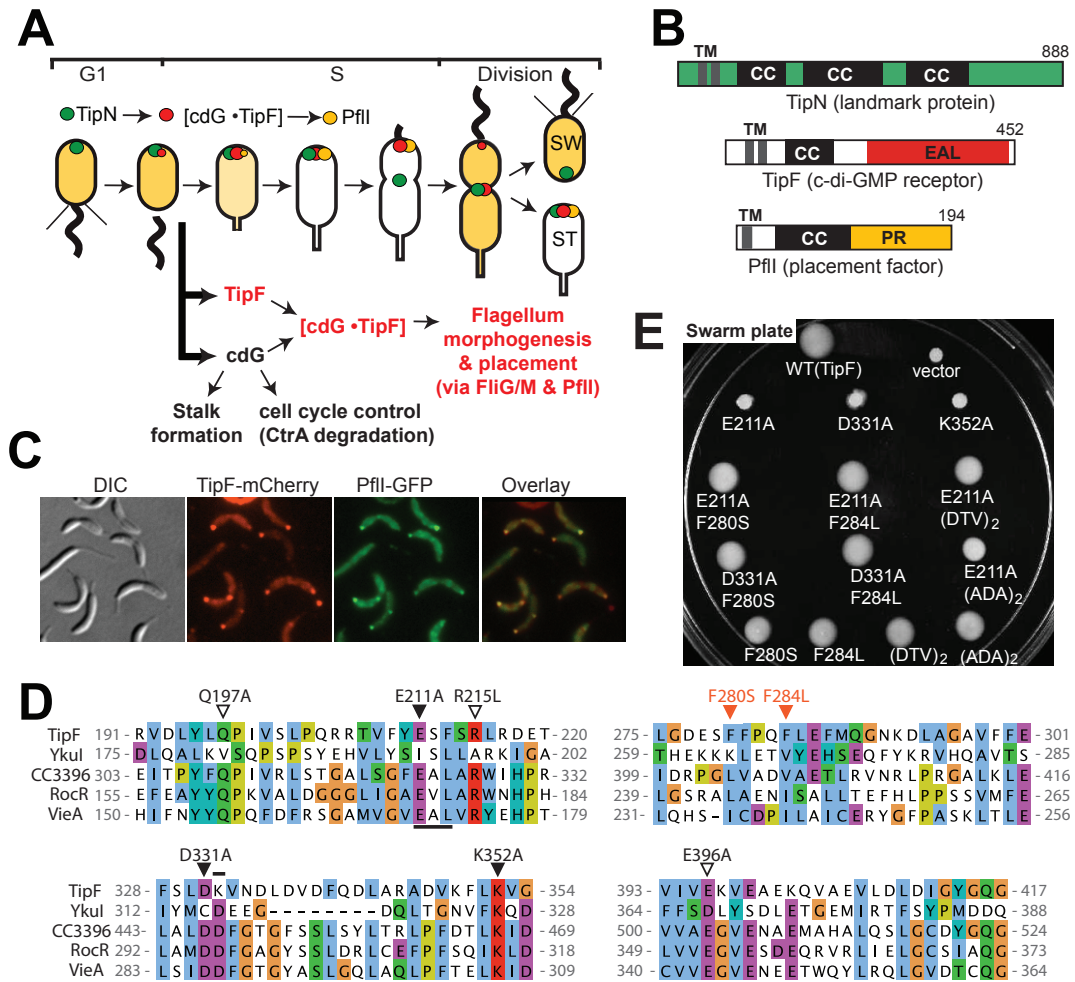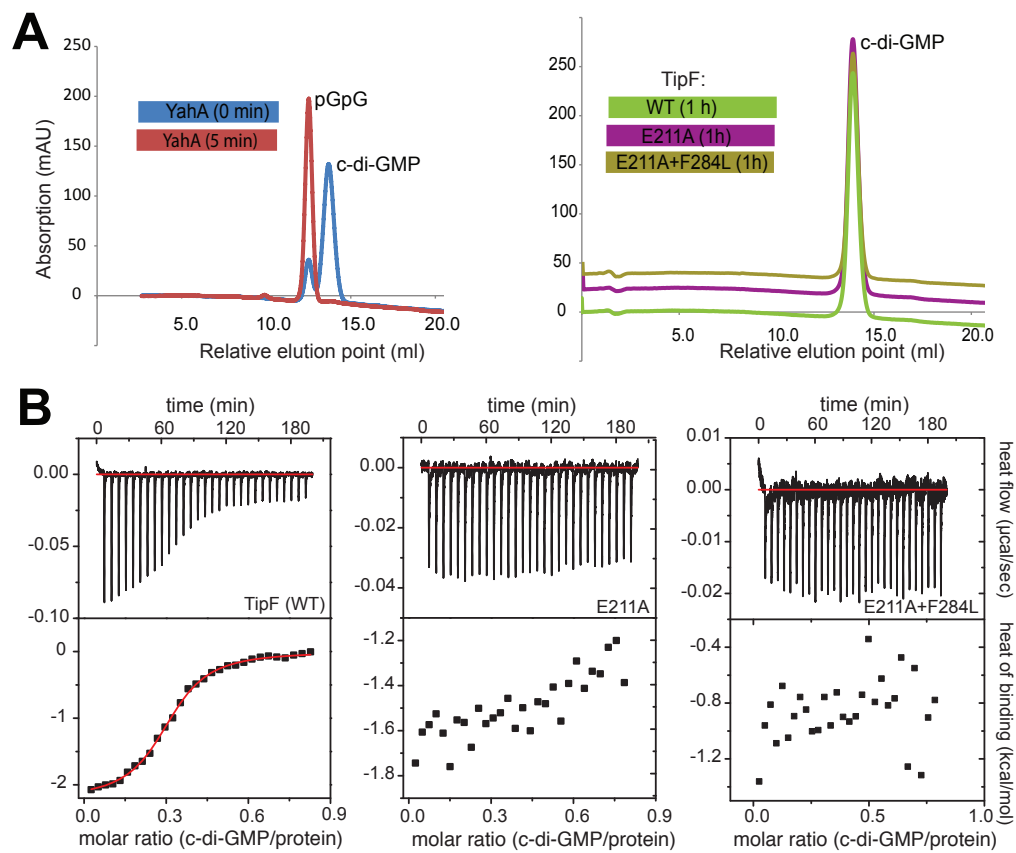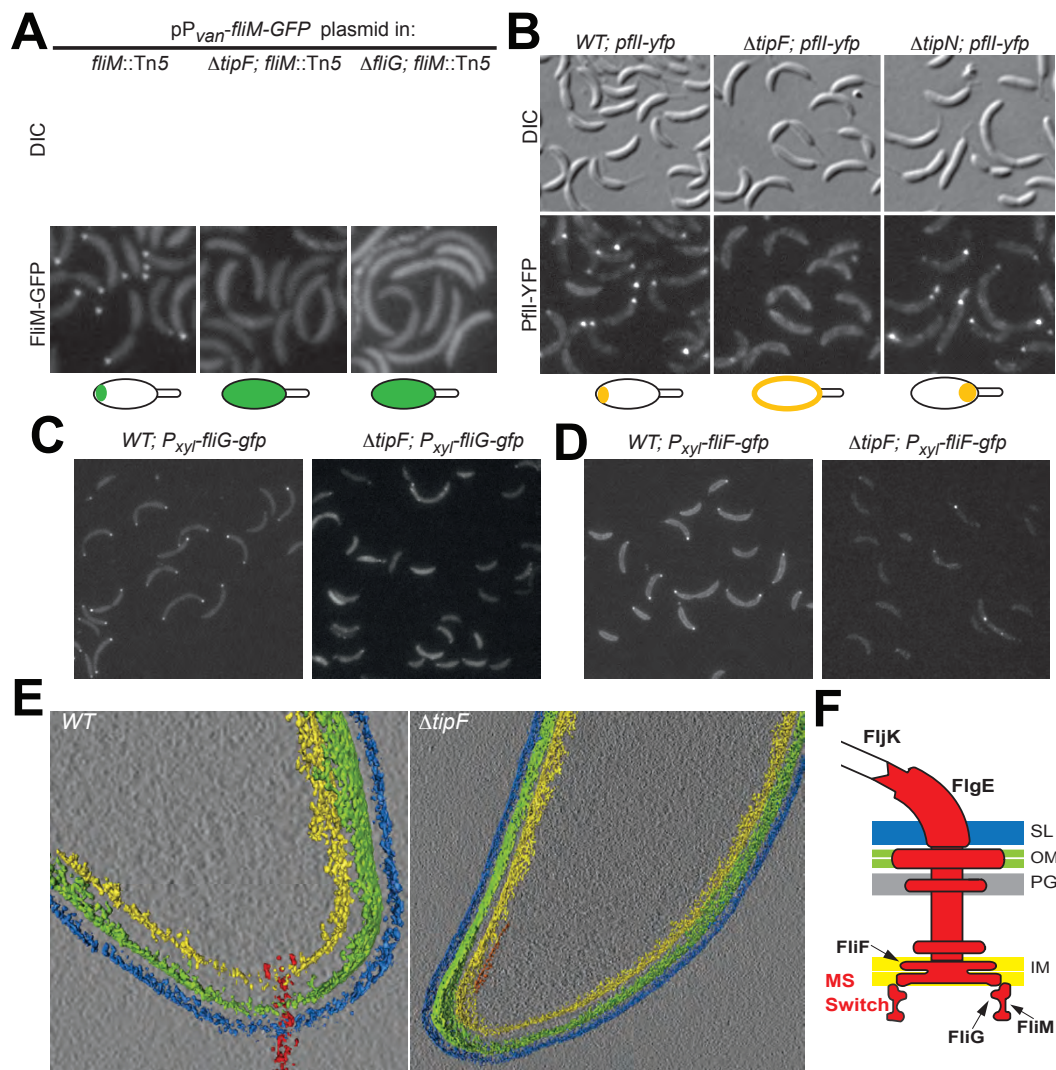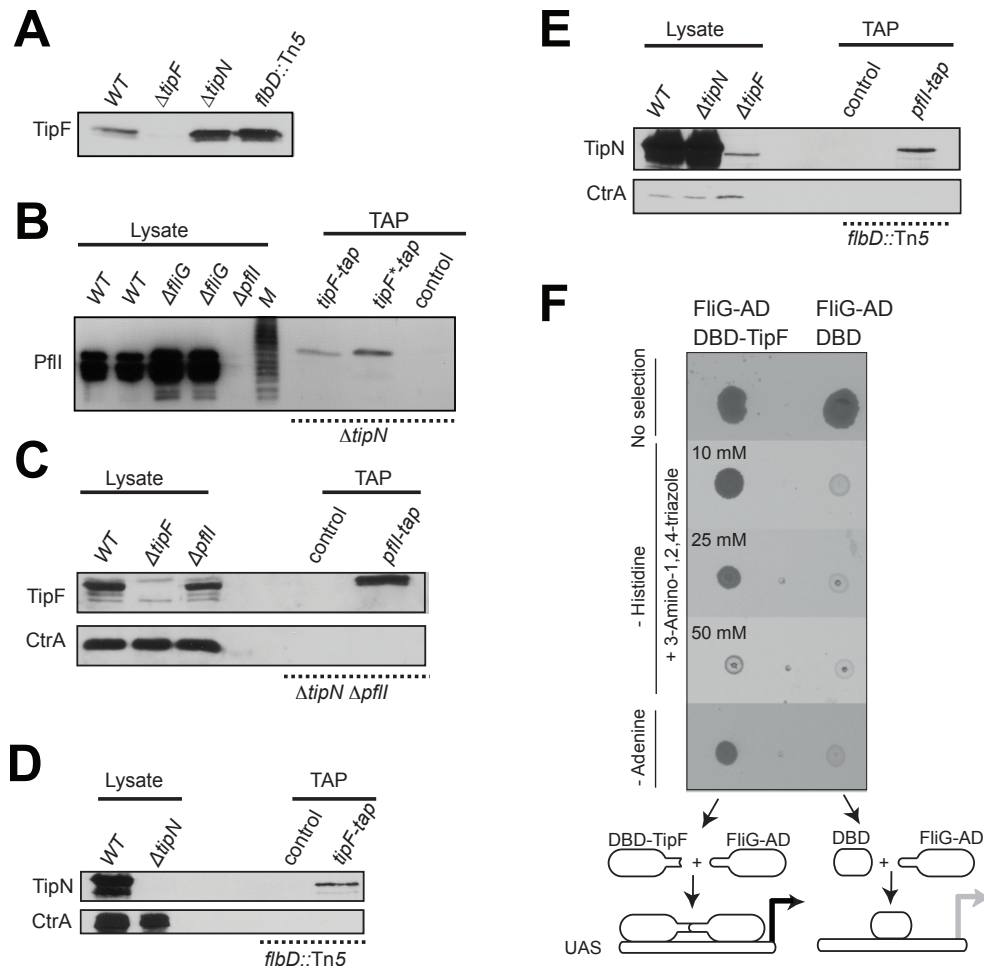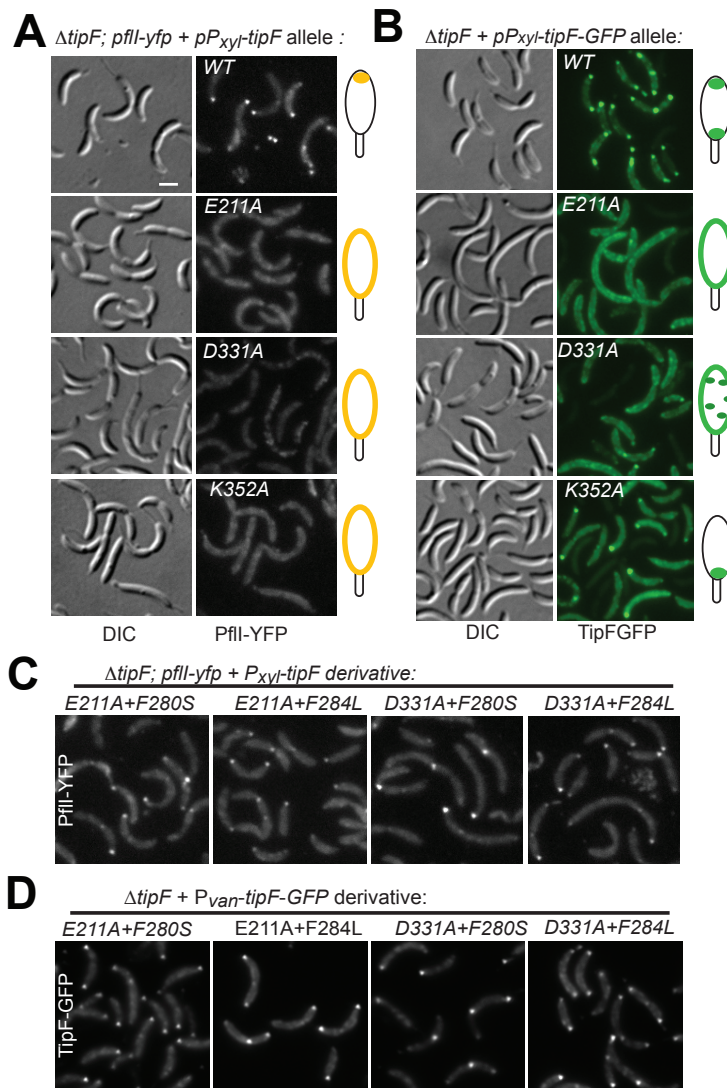
Fig. 1

Fig. 2

Fig. 3

A pP*van*-*fliM-GFP* plasmid in:

fliM::Tn5   Δ*tipF*; *fliM*::Tn5   Δ*fliG*; *fliM*::Tn5

DIC

FliM-GFP

B WT; *pfII-yfp*   Δ*tipF*; *pfII-yfp*   Δ*tipN*; *pfII-yfp*

DIC

PfII-YFP

C WT; *P*xyl-*fliG-gfp*   Δ*tipF*; *P*xyl-*fliG-gfp*

D WT; *P*xyl-*fliF-gfp*   Δ*tipF*; *P*xyl-*fliF-gfp*

E *WT*   Δ*tipF*

F FljK
FlgE
SL
OM
PG
FliF   IM
MS Switch
FliG   FliM

Fig. 4

Fig. 5

Fig. 6

A Δ*tipF; pfII-yfp + pP_{xyl}-tipF* allele :

DIC	PfII-YFP
WT	
E211A	
D331A	
K352A	

B Δ*tipF + pP_{xyl}-tipF-GFP* allele:

DIC	TipFGFP
WT	
E211A	
D331A	
K352A	

C Δ*tipF; pfII-yfp + P_{xyl}-tipF* derivative:

E211A+F280S   E211A+F284L   D331A+F280S   D331A+F284L

PfII-YFP

D Δ*tipF + P_{van}-tipF-GFP* derivative:

E211A+F280S   E211A+F284L   D331A+F280S   D331A+F284L

TipF-GFP

Fig. 7

A − cdG (P*van*-PA5295-WT)

Relative levels (%)

TipF (WT)
E211A/F284L
E211A

Time (min)

B + cdG (P*van*-PA5295-AAL)

Time (min)

C − cdG (P*van*-PA5295-WT)

(min) 0 20 40 60 80 100 120 140

D + cdG (P*van*-PA5295-AAL)

0 20 40 60 80 100 120 140

E P*xyl*::*clpX^{ATP*}-flag*

M2GX   M2G

α-TipF

α-Flag

F

G1   S   Division

(min) 0 20 40 60 80 100 120 140 160

CtrA

TipF

GcrA

G P*tipF-lacZ* activity in:

(%)   *WT*   P*xyl-gcrA*

xyl glu   xyl glu

H GcrA bound to:
(α-GcrA qChIP):

Relative abundance

P*tipF*

P*sciP*

Fig. S1

A

B

C

Fig. S2

A

| $\Delta pflI$ | $\Delta pfli\ \Delta tipF$ | $\Delta pflI\ \Delta tipN$ | $\Delta pflI\Delta tipF\Delta tipN$ |

DIC

PflI-GFP

B

| *WT* | $\Delta pflI$ | $\Delta tipN\ \Delta pflI$ | $\Delta tipN$ |

DIC

TipF-GFP

C

$\Delta pflI$ + P*van-pflI-GFP* derivative:

PflI-GFP

| $pflI^{\Delta 4\text{-}28}$ | $pflI^{\Delta 93\text{-}142}$ | $pflI^{\Delta 142\text{-}194}$ |

DIC

$pflI^{\Delta 28\text{-}92}$

Fig. S3

A

| ΔpflI | Δpfli ΔtipF | ΔpflI ΔtipN | ΔpflIΔtipFΔtipN |

DIC

PflI-GFP

B

| WT | ΔpflI | ΔtipN ΔpflI | ΔtipN |

DIC

TipF-GFP

C

ΔpflI + P$_{van}$-pflI-GFP derivative:

PflI-GFP

pflI $^{Δ4-28}$ pflI $^{Δ93-142}$ pflI $^{Δ142-194}$ pflI $^{Δ28-92}$ DIC

Fig. S4

A

P$_{tipF}$-lacZ activity in:

B

Fig. S5

A

+ cdG (No induction of P_{xyl}-PA5295-WT)

ΔtipF; vanA::Pvan-tipF-yfp derivative:

B

P_{fljL}-lacZ

3.2 Additional results TipF

3.2.1 TipF is cell cycle regulated but its degradation does not depend on known adaptor proteins of the ClpXP degradation pathway

Since TipF is required for flagellum assembly and flagellum biogenesis occurs at a specific time during the cell cycle, we have asked whether TipF is cell cycle controlled. Swarmer cells isolated from NA1000 were allowed to proceed through one cell cycle. Samples were taken every 20 min and subjected to immunoblot analysis using a TipF-specific antiserum. The experiment revealed a unique cell cycle dependent pattern of TipF levels (Fig. 1). TipF is first detectable after 40 min of the cell cycle right on the onset of c-di-GMP accumulation with a main peak at the time points were the flagellum is assembled.
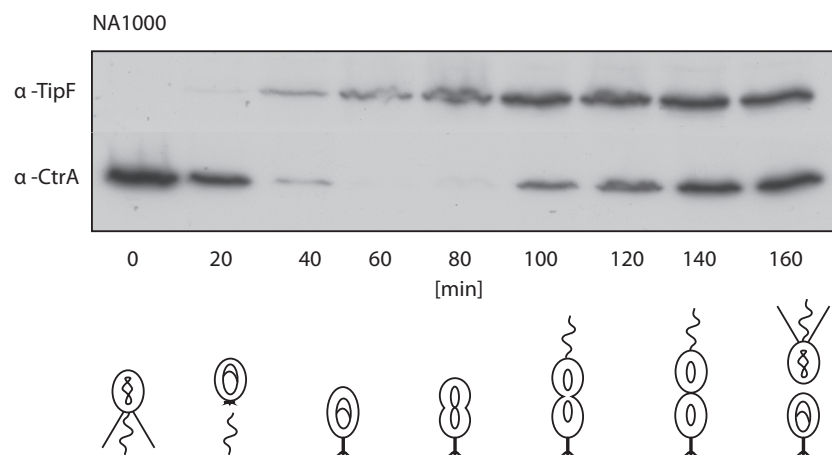
Figure 1: TipF levels are cell cycle regulated. A synchronized culture of *C. crescentus* NA1000 was subjected to immunoblot analysis using anti-TipF antibodies. TipF is appearing after 40 min within the cell cycle. Anti-CtrA immunoblot served as control for the synchronization quality.

The TipF cell cycle dependent pattern suggest that a rapid degradation of TipF occurred within the swarmer cells. This is in agreements with previous observations showing that swarmer cells have low c-di-GMP levels[118] and that TipF stability is c-di GMP mediated (chapter 1). Moreover TipF accumulation right on the swarmer to stalked cell transition is adjunct with c-di-GMP accumulation within the cells[10]. Since our data indicates that ClpXP degrades unbound TipF we have asked whether additional factors that are necessary for the ClpXP degradation pathway are required for TipF degradation as well. CpdR is reported to recruit ClpXP and the ClpXP

dependent substrate PdeA to the cell pole and in the absence of CpdR the levels of the ClxpXP substrates CtrA and PdeA are stable during the cell cycle[10,145]. RcdA is in addition needed to recruit the substrate CtrA to the degradation machinery in dependence of PopA and c-di-GMP[9] (Fig. 2B). In strains carrying either deletions in *cpdR*, *rcdA* or *popA* the levels of TipF show the same characteristic cell cycle accumulation (Fig. 2A) as in wild-type cells, showing that these proteins are not involved in the degradation of TipF.

Specific proteolysis is an important regulation mechanism in *C. crescentus*. ClpXP dependent proteolysis was demonstrated to temporally and spatially regulate key proteins as PdeA[10] and CtrA[9]. We report another key protein to be ClpXP dependent, but in contrast to the substrates described before, TipF degradation neither requires ClpXP localization nor the known adapter proteins. This raises the question how the degradation machinery recognizes TipF and how the machinery discriminates between the bound and the unbound state of TipF but this remains to be a subject of future experiments.
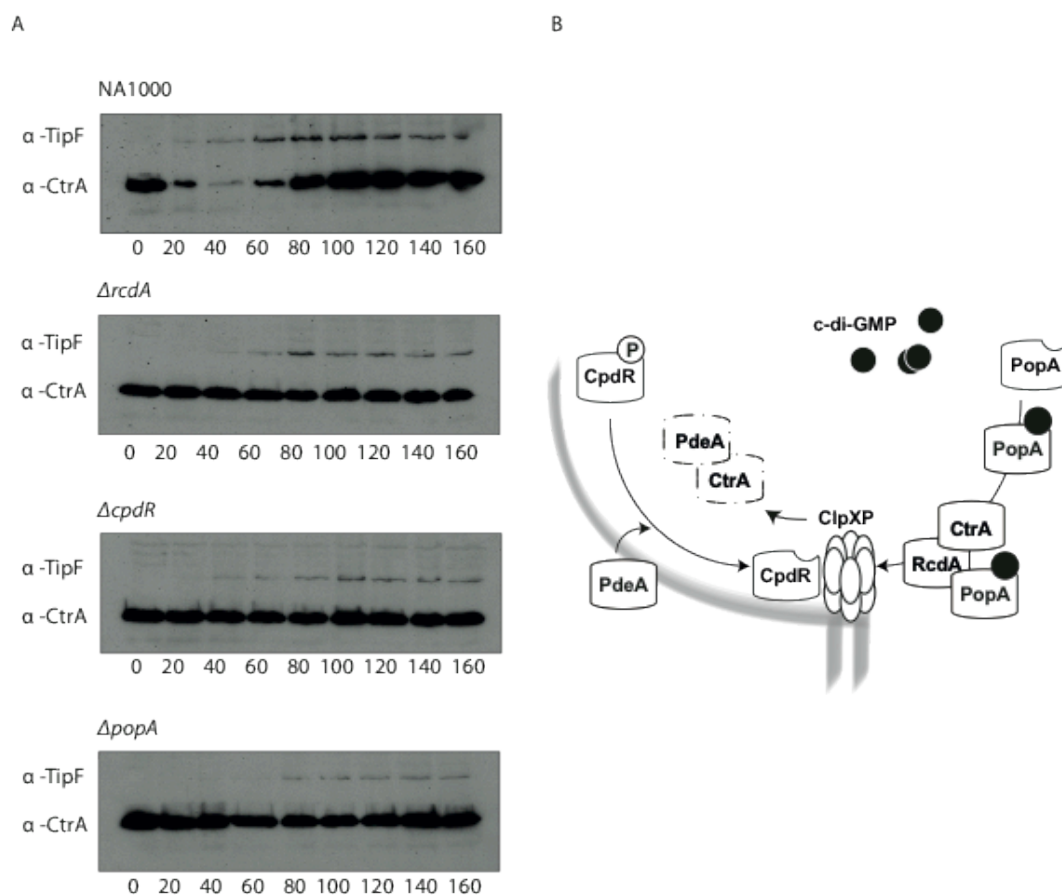
Figure 2: CpdR, RcdA, and PopA are not involved in ClpXP dependent degradation of TipF during the cell cycle. (A) Immunoblots of synchronized cultures of *C. crescentus* NA1000, *cpdR*, *rcdA,* and *popA* deletion strains probed with anti-TipF and anti-CtrA antibodies. (B) Schematic representation of the ClpXP degradation pathway. Shown are the degradation pathway of two known ClxP substrates, PdeA and CtrA, and the involvement of CpdR, PopA and RcdA in this degradation. CpdR recruits PdeA and the ClpXP protease to the cell pole where the degradation of PdeA and CtrA occurs. CtrA localization to the pole depends on RcdA and PopA. Adapted from[9,10].

3.2.2 c-di-GMP independent TipF mutants are stable in a strain lacking c-di-GMP and restore flagellum assembly

Abel and colleagues have constructed a *C. crescentus* strain that lacks all predicted diguanylate cyclases, consequentially this strain cannot synthesize c-di-GMP and all the c-di-GMP related signalling systems are inactivated. The cdG° strain is lacking all characteristic polar organelles of *C. crescentus* including the flagellum (Abel et al., unpublished). Since TipF is required for the biogenesis of the flagellum[142] and as we have shown that TipF is only stable when c-di-GMP is bound, we investigated the stability of TipF and the ability of intragenetic suppressor mutants to restore the assembly of the flagellum and motility in the cdG° background.

RNA sequencing revealed that the mRNA levels of *tipF* in the cdG° and wild-type are identical in the two strains (Abel et al., unpublished). In contrast no TipF protein was detectable in the cdG° strain (Fig. 3A), confirming that the protein is instable in the absence of c-di-GMP. In addition, over-expression of TipF from a xylose inducible promoter in a cdG° strain containing an additional deletion of *tipF* did not increase the protein levels in the absence of c-di-GMP, while expression of two different TipF intragenic suppressor mutants, active proteins that cannot bind c-di-GMP (described in Chapter 1.), are detectable in the cdG° strain (Fig. 3B).
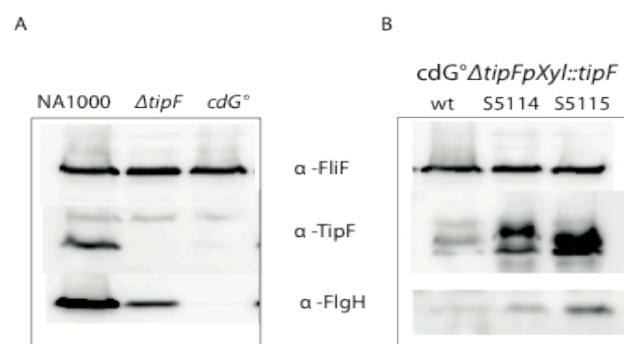

Figure 3: TipF and flagellar protein levels in the absence of c-di-GMP. (A) Cellular extracts of wild-type, cdG°, and Δ*tipF* strains were investigated in immunoblot analysis for the levels of TipF, FlgH, and FliF. (B) Cellular levels of TipF, FlgH, and FliF in a cdG°Δ*tipF* strain expressing either TipF wild-type protein or the TipF suppressors S5114 (TipF$_{E211A/F284L}$) and S5115 (TipF$_{D331A/F280S}$). All immunoblots were probed with anti -TipF, anti-FlgH and anti-FliF antibodies.

We also tested the stability of the selected TipF intragenic suppressors in the cdG°Δ*tipF* strain. TipF$_{wt}$, and TipF$_{D331A/F284L}$ were expressed in the cdG°Δ*tipF* strain by adding xylose to the cultures, and then shifted to a medium without the inducer and the stability of the proteins were investigated over time (Fig 4). In agreement with our previous findings, the intragenic suppressor was found to be stable for a longer period of time compared to the wild-type protein.
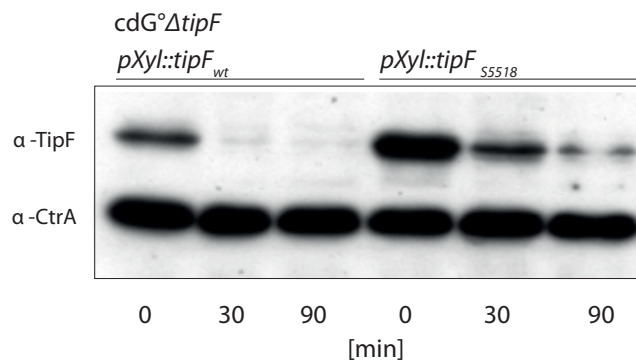

Figure 4: TipF suppressor is stable in the in the cdG° background. Cellular extracts of cdG°Δ*tipF* strains expressing the *tipF* alleles encoding for TipF$_{wt}$ and the suppressor S5518 (TipF$_{D331A/F284L}$) were subjected to immunoblot analysis. Proteins were induced in the presence of xylose for 3h, thereafter the cultures were shifted to a medium without the inducer and samples were taken every 30 min. The blot was probed with anti-TipF antibodies. Anti-CtrA immunoblots served as control.

The cdG°Δ*tipF* strain expressing the stabilized suppressor TipF$_{E211A/F284L}$ was also able to regain motility in liquid media (Movie 1). In contrast this strain did not swarm well on semisolid ager plates (Fig. 5A), suggesting additional defects. Therefore the flagellum structure of this strain was resolved by cryo-electron tomography (in cooperation with Misha Kudryashev). The basal body hook complex was intact (Fig. 5B) however the flagellum was misplaced and had only short filaments (Fig. 5C).

We also checked the levels of a selected flagellum protein, and observed that FlgH, the L-ring protein which is missing in the cdG° strain, shows increased but not wt levels in the cdG°Δ*tipF* strain expressing the stabilized suppressor TipF$_{E211A/F284L}$ (Fig. 3B). Interestingly, even more FlgH is present in a *tipF* deletion mutant, suggesting that c-di-GMP regulates FlgH in an additional TipF unrelated pathway.

The observation that the TipF suppressor mutant is stable in the absence of c-di-GMP and in turn is sufficient to restore flagellum assembly, indicates that TipF is

sufficient for flagellum assembly and that the interaction of the c-di-GMP signal within the flagellum assembly process is via TipF only. The TipF suppressor cannot restore motility on soft agar plates suggesting additional defects of the cdG° strain not connected to TipF. For instance the flagellum is not correct placed and that is most likely due to the delocalization of TipN, a localization factor that is required for the correct flagellum placement [142] and is delocalized in a cdG° strain (Abel, unpublished).

The fact that c-di-GMP is required for flagellum assembly demonstrates the complexity of c-di-GMP signaling in *C. crescentus* since c-di-GMP also regulates flagellum ejection [76] and inhibits flagellum rotation [33]. This seemingly contradicting activity of c-di-GMP can be explained by the temporal and spatial distribution pattern of c-di-GMP [118] and of the effector molecules, moreover the large variety of binding affinity displayed by c-di-GMP effectors [71] is a strong indication that different levels of c-di-GMP can induced different cellular response.
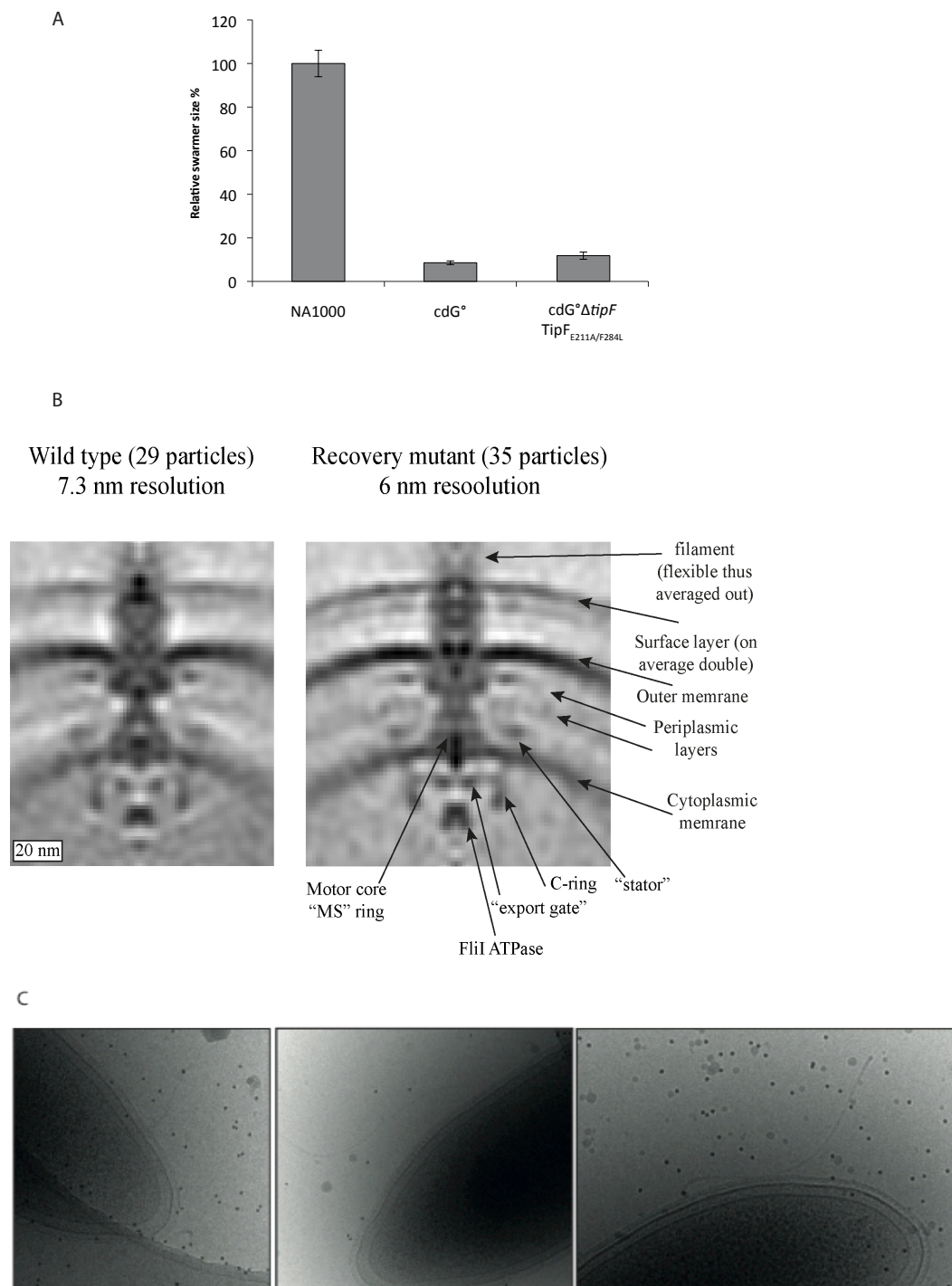
A

B

Wild type (29 particles)
7.3 nm resolution

Recovery mutant (35 particles)
6 nm resoolution

filament
(flexible thus
averaged out)

Surface layer (on
average double)

Outer memrane

Periplasmic
layers

Cytoplasmic
memrane

Motor core
"MS" ring

"export gate"

FliI ATPase

C-ring

"stator"

20 nm

C

Figure 5: TipF intragenic suppressor restores flagellum assembly in the cdG° strain. (A) The cdG° mutant displayed severely impaired motility, leading to a swarm size on semi-solid agar plates of only about 10 % compared to NA1000. Expression of TipF$_{E211A/F284L}$ in the cdG° strain containing a TipF deletion cannot restore this phenotype. (B) Flagella structures of *C. crescentus* NA1000 and cdG°Δ*tipF* expressing TipF$_{E211A/F284L}$ from a plasmid (Recovery mutant) observed by cryo-electron tomography. Flagellum elements are indicated. (C) cdG°Δ*tipF* expressing TipF$_{E211A/F284L}$ shows flagellum misplacement and filaments protruding from the cell body.

3.2.3 A CtrA mutant is able to partially suppress a deletion of TipF

Intragenic TipF mutants can suppress the motility phenotype of a *tipF* deletion mutant. They were identified by screening for motility in the presence of an active site mutant in the EAL domain (chapter 3.1). In contrast no extragenetic motile suppressor mutants could be identified so far (PV personal communication), indicating that either TipF is the only factor required or that several factors have to be changed in parallel in order to allow swimming on swarm agar plates. Support for the later hypothesis came from the identification of motile suppressors of the cdG° strain, which were all found to be in *ctrA* (Abel et al., unpublished). CtrA is the master regulator in C. *crescentus* and regulates among other functions the transcription of the flagellar class II genes. One of the identified *ctrA* mutants in the cdG° strain is *sokA* (Abel et al., unpublished), this allele was described before to suppress *divK* [82]. We have shown that TipF is not detectable in the cdG° strain, since it is degraded in the absence of bound c-di-GMP (see above). Therefore we analyzed the levels of TipF in the cdG° *sokA* motile suppressor and indeed TipF was not detectable as in the cdG° strain (Fig. 6), indicating that assembly of the flagellum can be bypassed in the absence of TipF.
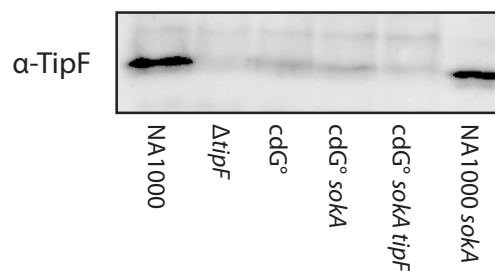

Figure 6: TipF cellular levels. Cellular extracts of wild-type (NA1000), Δ*tipF*, cdG°, cdG°*sokA,* cdG°*sokAtipF,* and *sokA* strains were investigated in immunoblot analysis for the levels of TipF. The blot was probed with anti-TipF antibodies.

To investigate whether *sokA* can also suppress the motility defect of a *tipF* deletion mutant in the wild-type C. *crescentus*, we analyzed a *sokAΔtipF* mutant. This strain was motile in liquid cultures and did slightly restore the motility on semi-solid ager plates (Fig. 7A). Since FliG directly interacts with TipF (chapter 3.1) and is

misslocalized in the absence of TipF[142] we analyzed the localization of FliG-GFP in the *sokAΔtipF* strain. Compared to the *tipF* mutant FliG is localized at higher degree to the pole but not as efficient as in the wild-type or in the *sokA* mutant (Fig 7B), which is in accordance with the results of the motility assay on swarm agar plates.
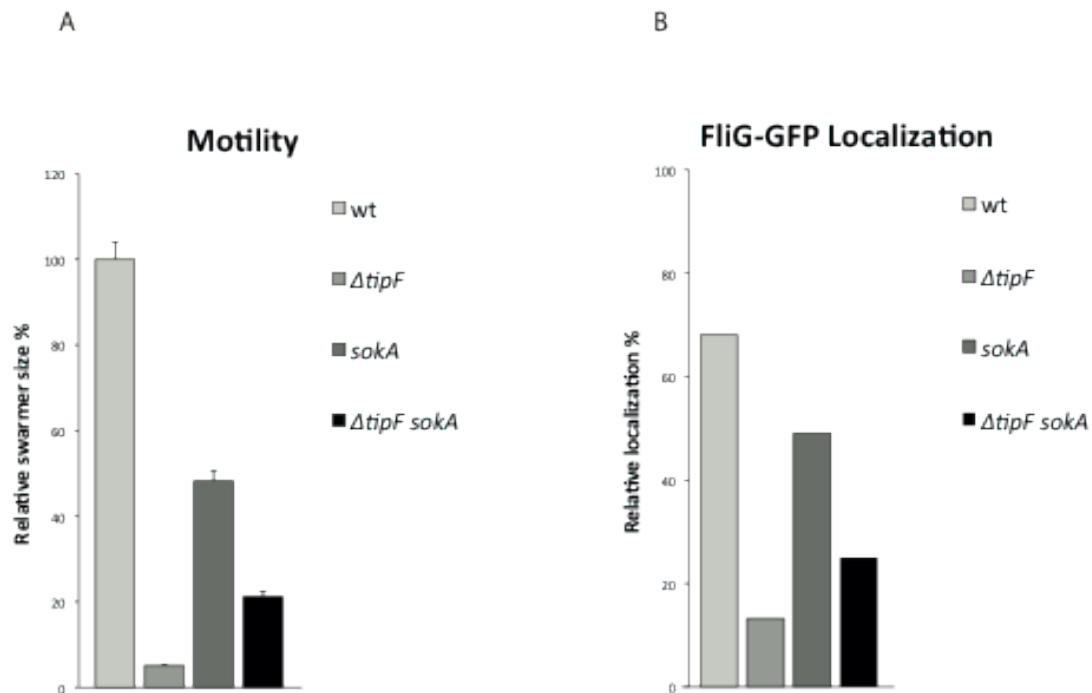

Figure 7: The CtrA mutant SokA partially bypasses necessity of TipF for flagellum assembly and function. (A) The indicated strains were inoculated on PYE swarm agar plates and incubated for 72 hrs at 30°. The swarm-sizes were measured and compared to the wt strain NA1000 (100%). (B) FliG-GFP was expressed in the indicated strains from a plasmid containing p*xyl*::*fliG-gfp* by adding xylose and the strains were analyzed microscopically. The number of FliG-GFP foci in the indicated strains were counted and compared.

Since a mutation in CtrA is able to partially restore Δ*tipF* motility we ask if the activity of CtrA is altered in Δ*tipF*. Synchronous cultures of wild-type and Δ*tipF* (Fig. 8A) were used to monitor the CtrA protein level along the cell cycle. Protein levels of CtrA in the Δ*tipF* mutant were found to be identical to the one of the wild-type. In addition we have monitored the protein levels of CcrM and FliF, whose expression is under control of CtrA (Fig. 8B and C). ccrM is a DNA methyltransferase, therefore flagella unrelated and FliF builds the flagellar MS ring. We have not noted any changes in appearance timing and level of CcrM and FliF, this suggests that there is
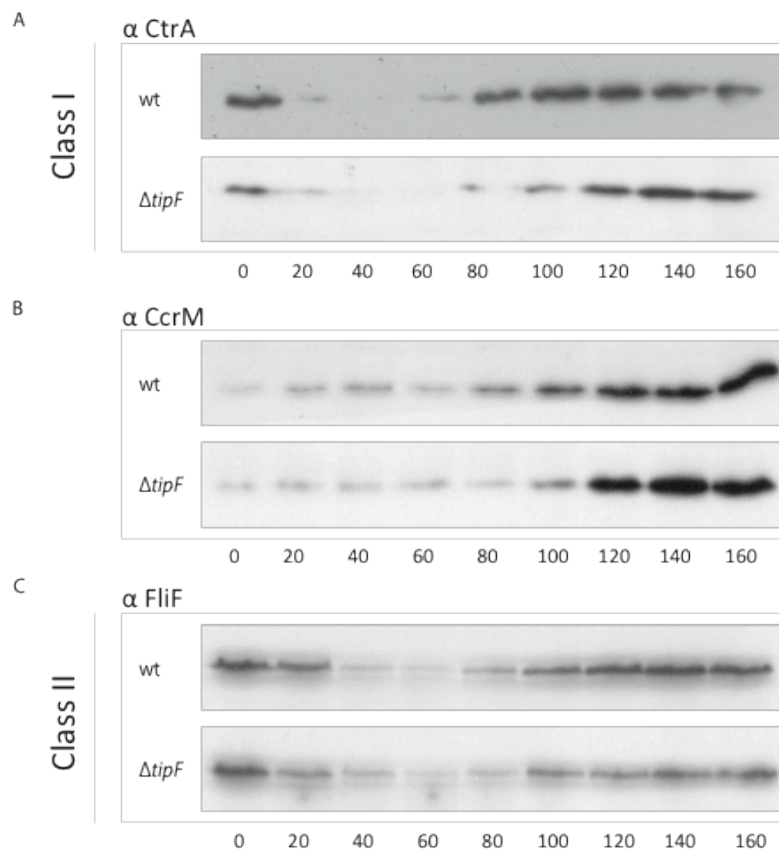
no alteration in the activity of CtrA in Δ*tipF* strain.

Figure 8: CtrA activity is not altered in a Δ*tipF* strain. (A) Synchonizied cultures of *C. crescentus* wild-type (NA1000) and Δ*tipF* were analyzed by immunoblots with anti-CtrA antibodies. CtrA levels and the cell cycle dependent pattern do not altered in Δ*tipF* strain. In addition the protein level and cell cycle dependent pattern of CcrM and FliF (flagellar gene class II) are shown in immonoblot probed with anti-CcrM antibodies (B) and immonoblot probed with anti-FliF antibodies (C), they are comparable to the wild-type strain.

Our results suggest that the assembly of the flagellum has an additional, TipF unrelated, pathway that can be activated by alteration of the CtrA activity through the *sokA* mutation[82,107]. This indicates that TipF is not the only factor involved in flagellum assembly and that this factor is CtrA regulated. One protein profiling those requirements is PflI. PflI was shown to regulate the positioning of the flagellum [146] in *C. Crescentus*. In addition the transcription of the *fliL* operon, in which *pflI* is located, is CtrA dependent [82].

We suggest that flagellar assembly and positioning is the integration point of multiple signalling cascades, as each suppressor mutant (SokA or TipF intragenic

mutation) is able to restore motility to some extent. Next we will test for the involvement of PflI in this process by overexpressing PflI in *tipF* deletion mutant.

3.2.4 The chemotaxis arrays in the *tipF* deletion mutant

Deletion of *tipF* results in several pleiotropic phenotypes, including the loss of flagellum, the reduced attachment ability, and the perturbation of cell division[142]. To analyze the morphology of the Δ*tipF* strain in more detail, we used cryo-electron tomography and in a number of samples we noted that the chemoreceptor arrays are absent (Fig. 9A). The chemoreceptors, or methyl-accepting chemotaxis proteins (MCPs), form the basis of the signaling apparatus modulating the direction of flagellum rotation[147]. To investigate whether TipF might be involved in the localization of MCPs we analyzed McpA, one of 18 MCPs (McpA-R) that are encoded by *C. crescentus*. Immunoblot experiments revealed that McpA is present in the Δ*tipF* strain (data not shown). McpA-GFP, expressed from a plasmid under control of the native promoter, was also localized in the *tipF* deletion mutant (Fig. 9B). Only slight differences were noticed, most likely due to the cell elongation of the Δ*tipF* mutant.

Our results suggest that TipF is not involved in the localization of McpA. However, we cannot rule out the possibility that McpA-GFP expression results in protein localization due to McpA stabilization and self-assembly properties[148]. In a next step, we will investigate the expression level and localization of additional chemotaxis array components in the *tipF* deletion mutant.
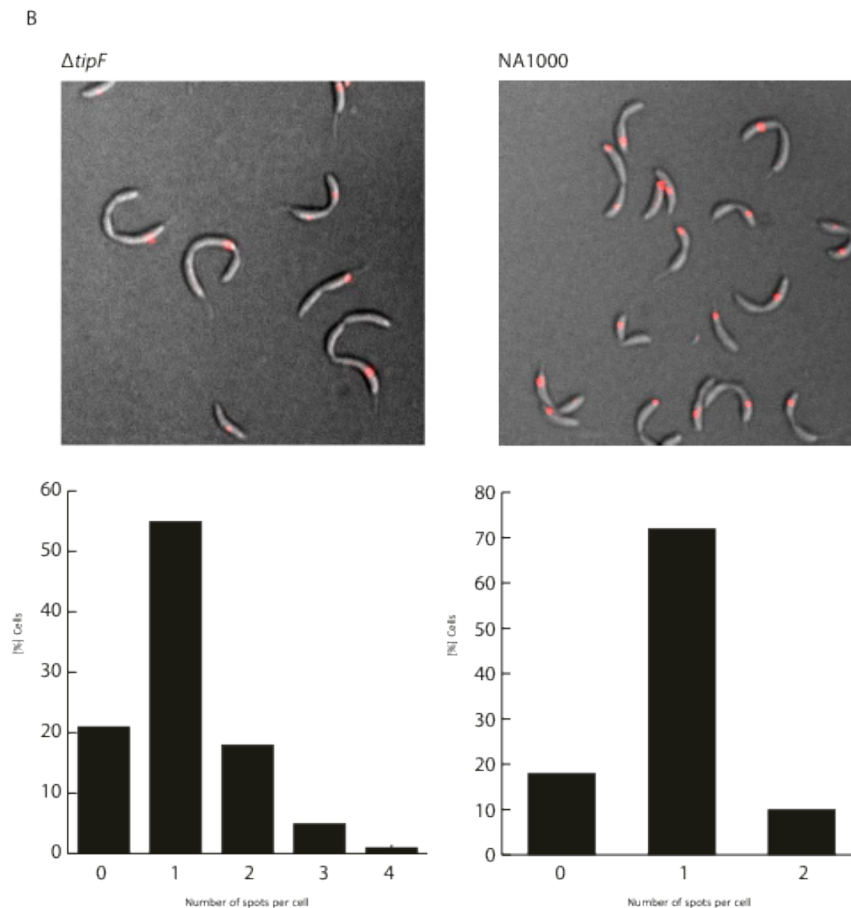
A
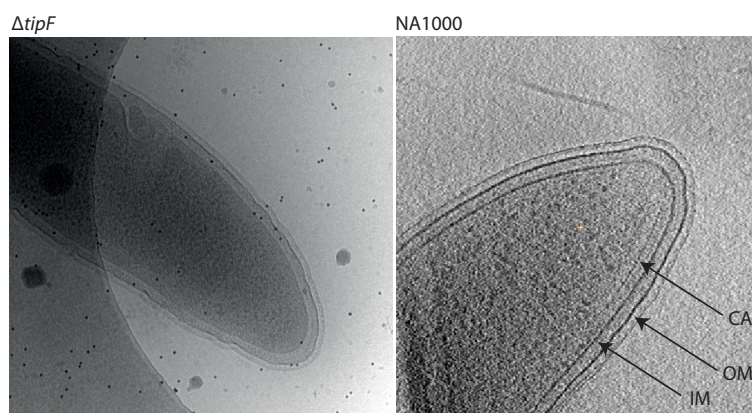
Figure 9: McpA localization is TipF independent. (A) Cryo-electron tomography images showing the chemoreceptor arrays in the NA1000 strain (indicated with an arrow CA-Chemoreceptor array, OM-Outer membrane and IM-Inner membrane) and the absence of those structures in the *tipF* deletion mutant. Indicated with an arrow are the Chemoreceptor array (CA), Outer membrane (OM) and Inner membrane (IM). (B) McpA localization in NA1000 and Δ*tipF* strain (top). McpA-GFP was expressed in both strains from a plasmid under control of the native promoter and analyzed by DIC and fluorescence microscopy. The analysis of the amount of spots per cell (bottom) revealed only a slight misplacement of McpA-GFP in the Δ*tipF* strain.

3.2.5   Yeast two-hybrid screen for proteins interacting with TipF

So far we have presented several evidences for the involvement of TipF in the assembly of the flagellum. Furthermore, a deletion mutant of *tipF* shows some other phenotypes, like a decrease in attachment (Fig. 10), reducedion of pili expression[142] and loss of the chemotaxis array. In order to map the molecular mechanism of those processes we setup a yeast two hybrid screen in an attempt to identify novel direct interacting proteins of TipF. Using the EAL domain of TipF as bait, we screened a genomic prey library of *C. cresentus*. To produce c-di-GMP during the screen we

expressed from the constitutive TEF yeast promoter the diguanylate cyclase YdeH from *E. coli*. In a first screen scoring for growth in the absence of adenine we identified a total of 4 clones. 3 were found to be Gal4-AD fusions to FliG (see also chapter 3.1), two corresponding to aa 192-340 and one to aa 236-340. In a second screen, we identified 5 clones activating HIS3. One had an insert that encodes a C-terminal part of MarR (CC_3112; aa 190-232) and one that codes for the C-termius of a CelD homolog (CC_2278; aa 304-401). The two prey clones did not interact with the Gal4-DBD itself but were able to activate the reporter gene HIS when the TipF EAL domain was present (Fig. 10).

Not much information is available about the two potential TipF interacting proteins. MarR is annotated as a transcription regulator containing an unorthodox dual wHTH DNA binding domain and no obvious input domain. The CelD homolog (CC_2278) is annotated as Gcn5-related N-acetyltransferase (GNAT). To investigate whether these proteins are involved in similar pathways as TipF, we generated clean deletion mutants in the *C. crescentus* strains CB15 and NA1000. The mutants were analyzed regarding their morphology, ability to swim and to attach to surfaces.
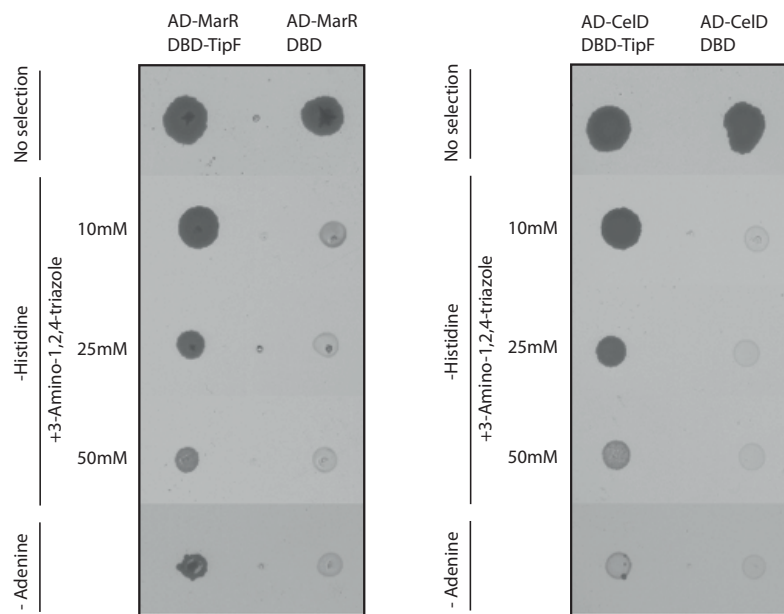
Figure 10: Interaction of TipF with MarR and CelD. The yeast reporter strain PJ69-4A expressing either Gal4-DBD-TipF EAL or Gal4-DBD and Gal4-AD-MarR aa 190-232 or Gal4-DBD-TipF EAL or Gal4-DBD and Gal4-AD-CelD aa 304-401 were assayed for histidine and adenine auxotrophy in the presence of an plasmid expressing YdeH (produces c-di-GMP).

The CC_2278 deletion mutant behaved like the wt strains but showed a subtle attachment defect (Fig. 11) when it was allowed to interact with a polystyrene surface for different periods of time (range from 0.5 hours to 19 hours). The mutant showed reduced attachment in early time points, nevertheless it was able to increase attachment levels when sufficient time was given (Fig. 11). This delayed attachment phenotype suggests that CC_2278 is involved in the temporal regulation of the holdfast. Next we will proof a direct interaction of CC_2278 and TipF by co-imunoprecipitation experiments and then investigate whether TipF is necessary to localize CC_2278. A potential interaction between TipF and CC_2278 is in particular interesting since it could serve as a signal transduction mechanism that regulates the switch between the two lifestyle forms motile and sessile. Several observation were made indicating that the flagellum serves as a mechanoreceptor that mediates an outside inside signal inducing a rapid attachment to the surface once flagellum rotation is inhibited [149]. Assuming that CC_2278 is involved in this flagellar mediated signal, its co-localization to the flagellar C-ring (FliG) via TipF could serve the signal
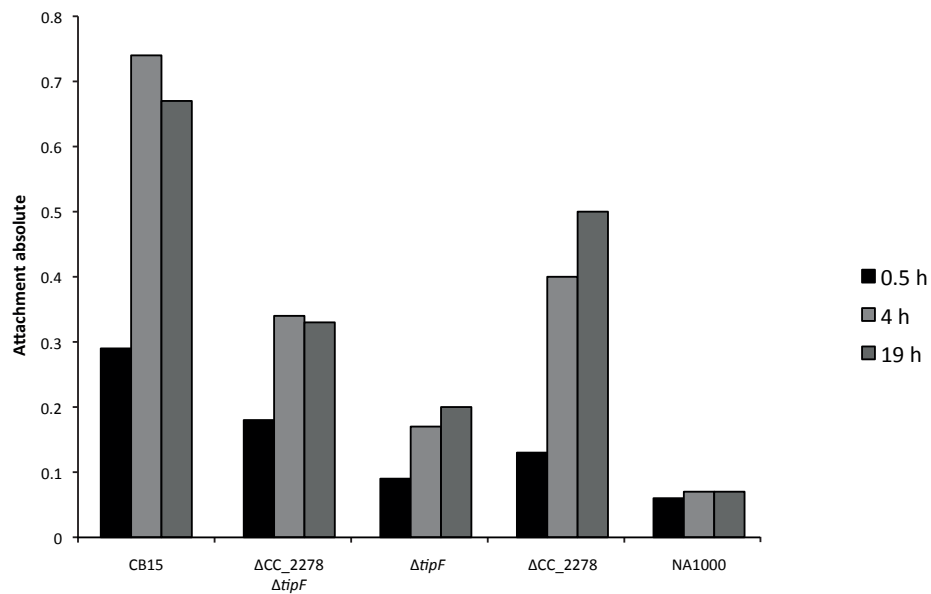
transduction mechanism.

Figure 11: Attachment defect of the CC_2278 deletion mutant. Cells were allowed to attach for 0.5, 4 or 19 hrs and stained with crystal violet. The stain was dissolved and the OD was measured (indicated at the Y axis). The strain NA1000 cannot attach and served as a control.

No obvious link between TipF and MarR could be found so far. In a motility assay on soft ager plates we observed no motility defects of the *marR* deletion strain as the swarm size was similar to the wt NA1000 (Fig. 12A). It did also attach as well as the CB15 wt strain, indicating that pili and the holdfast are present (Fig. 12B). The cellular level of TipF and time of expression were also determined in the *marR* deletion mutant. Immunoblots of samples of synchronized cells revealed that the TipF level and time of appearance remains unaltered in comparison to NA1000 (Fig 12C). The direct interaction between MarR and TipF has still to be proven by independent methods. If the two proteins do interact, further investigations are necessary to find out in which TipF dependent pathway MarR works.
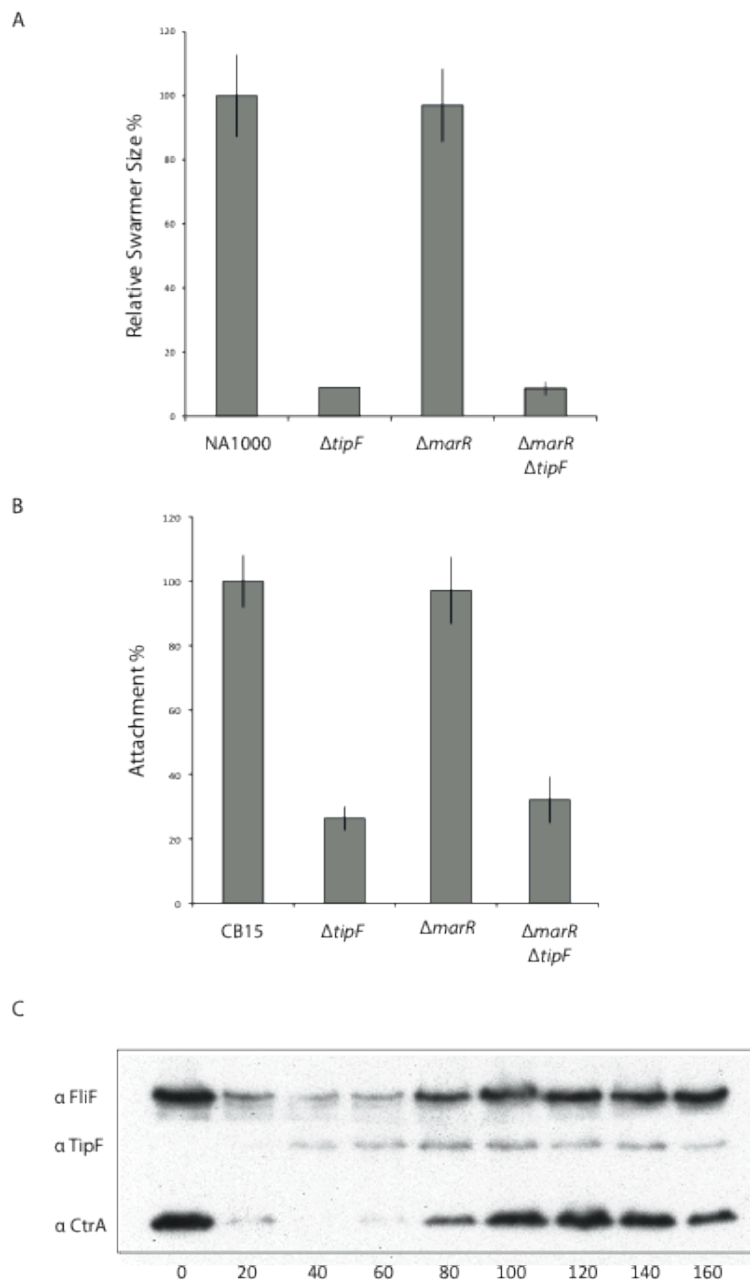
Figure 12: Phenotypes of *marR* deletion mutants. The *marR* deletion mutants were tested for (A) motility (B) attachment and (C) the effect on TipF protein levels through the cell cycle. We have noticed no alteration in all of the measured categories compared to the wild-type.

3.2.6 Material and methods

Growth Conditions

Caulobacter crescentus strains were grown in peptone yeast extract (PYE)[150] or minimal media supplemented with 0.2% glucose or 0.3% D-xylose (M2G or M2X)[150] at 30°C with constant shaking (150 rpm). When selection was required antibiotics in the following concentrations were added: (solid/liquid media in µg/ml): gentamycin (5/0.5), kanamycin (20/5), nalidixic acid (20/not used) and oxytetracycline (5/2.5). For synchronization experiments newborn swarmer cells were isolated by Ludox gradient centrifugation[151] and released into fresh minimal medium. When necessary the medium was supplemented with 1 mM Vanillate or 0.3% xylose. For synchronization with inducible constructs, inducer was added to the growth medium 3 h prior to synchrony.

E. coli strains were grown in Luria Broth (LB) at 37 °C. When necessary for selection the following antibiotic concentrations were used: (solid/liquid media in µg/ml) ampicillin (100/50), gentamycin (20/15), kanamycin (50/30) and oxytetracycline (12.5/12.5).

The yeast *Saccharomyces cerevisiae* was grown at 30° either in yeast peptone dextrose containing adenine (YPDA) or synthetic complete (SC) medium[152].

Motility Assays

C. crescentus colonies were spotted onto PYE soft agar plates (0.3% agar) and incubated for 72 h at 30°C. Plates were then scanned (ScanMaker i800 scanner, Microtek, Germany) and analyzed using Photoshop CS3 (Adobe, CA, USA) and ImageJ (NIH, USA) softwares. For all motility experiments the mean of at least three independent colony sizes is shown. Error bar represent standard deviation.

Attachment Assays

C. crescentus strains were grown in PYE in 96-well microtiter plates (Falcon, USA) for 24 h under constant shaking (200 rpm). After crystal violet (0.3% crystal-violet, 5% isopropanol, 5% methanol) staining of the attached biomass it was dissolved with 20% acetic acid and optical density at 600 nm was measured in a photospectrometer (Genesys6, Thermo Spectronic, USA).

Microscopy

DIC and fluorescence microscopy were performed on a DeltaVision Core (Applied Precision, USA)/Olympus IX71 microscope equipped with an UPlanSApo 100x/1.40 Oil objective (Olympus, Japan) and a coolSNAP HQ-2 (Photometrics, USA) CCD camera. Cells were placed on a patch consisting of 1% agarose in water (Sigma, USA). Images were processed ImageJ software (NIH, USA) or Photoshop CS3 (Adobe, USA) software. Bactria cell and signal localization were analyzed with MicrobeTracker (V0.931) and MATLAB software.

Cryo-electron Tomography was preformed as described in[153].

Immunoblots

Blots were incubated with the indicated primary antibodies (diluted to the final concentration of 1:10 000) for 1 hr and for an additional hr with HPR-conjugated swine anti-rabbit secondary antibodies (Dako Cytomation, Denmark). Blots were developed with the ECL detection reagent (Western Lightning, Perkin Elmer, USA).

Yeast two-hybrid analysis

Yeast two hybrid screen was preformed using the system described in[154]. The EAL domain of TipF fused to the Gal4-DBD (DNA binding domain) was expressed in *Saccharomyces cerevisiae* PJ69-4A from plasmid pGBD Kan[R]. YdeH, an active DGC that produces c-di-GMP, was expressed in the same strain from plasmid p426-cm-YdeH. For screening, a *C. cresentus* library was transformed into the strain containing the two afore mentioned plasmids. The library contains *C. crescentus* genomic DNA fragments between 500 to 3000 bp, obtained after partial digest of genomic DNA using the enzymes HinPIL, MspI and Taq1, fused to the *gal4-AD* (activator domain) in three reading frames (J. Luciano). Transformants were screened on SD medium lacking leucine, tryptophan, uracil and either adenine or histidine. Prey-plasmids of positive clones were isolated and retransformed in PJ69-4A containing the YdeH expressing plasmid and either the Gal4-DBD-TipF-EAL or the Gal4-DBD only expressing plasmid. Interaction strength and self-activity were tested with these strains on selective medium to check for the activation of reporter genes. Inserts of confirmed interactors were sequenced to ensure in-frame coding sequence with the Gal4-AD.

Strains and plasmids

Strains

Name	Genotype	Description	Source or Reference
NA1000	CB15N	Laboratory strain derived from CB15	Evinger and Agabian, 1977
CB15	CB15	*C. crescentus* wild type ATCC 19089	Poindexter and Cohen-Bazire, 1964
UJ3638	NA1000 Δ*tipF*	Markerless in frame deletion of *tipF*	Huitema et al., 2006
UJ2827	NA1000 Δ*popA*	Markerless in frame deletion of popA	Duerig et al., 2009
UJ3628	NA1000 *rcdA* ::*hyg*	Disruption of the rcdA gene by a Hyg[R] cassette	Iniesta et al., 2006
UJ4373	NA1000 Δ*cpdR*	Markerless in frame deletion of cpdR	M. Laub
UJ5065	cdG°	Markerless in frame deletion of cc1850, cc0740, cc0857, cc3285, cc2462, cc3094, cc0655, cc0896	M. Nicollier
UJ6463	cdG° Δ*tipF*	Markerless in frame deletion of *tipF* In UJ5065	S. Abel
UJ6465	cdG° Δ*tipF* p3064	UJ6463 containing plac290 pXyl::*tipF*$_{E211A/F284L}$	This Study
UJ6466	cdG° Δ*tipF* p3066	UJ6463 containing plac290 pXyl::*tipF*$_{E331A/F280L}$	This Study
UJ6469	cdG° Δ*tipF* p264	UJ6463 containing plac290 pXyl::*tipF*$_{D331A/F284L}$	This Study

SoA667	NA1000 cdG° sokA	cdG° with acquired mutation in *ctrA* (*sokA*)	S. Abel
UJ1981	*sokA*	Mutation in *ctrA*	Wu et al., 1998
UJ4456	CB15 Δ*tipF*	Markerless in frame deletion of *tipF*	S. Abel
UJ5396	NA1000 cdG° sokA Δ*tipF*	Markerless in frame deletion of *tipF* in SoA667	This Study
UJ6476	NA1000 Δ*marR*	Markerless in frame deletion of *marR*	This Study
UJ6477	CB15 Δ*marR*	Markerless in frame deletion of *marR*	This Study
UJ6478	NA1000 Δ*marR* Δ*tipF*	Markerless in frame deletion of *marR* in UJ3638	This Study
UJ6479	CB15 Δ*marR* Δ*tipF*	Markerless in frame deletion of *marR* in UJ4456	This Study
UJ6474	NA1000 Δ*celD* Δ*tipF*	Markerless in frame deletion of *celD* UJ3638	This Study
UJ6473	CB15 Δ*celD*	Markerless in frame deletion of *celD*	This Study
UJ6473	CB15 Δ*celD*	Markerless in frame deletion of *celD*	This Study
UJ6475	CB15 Δ*celD* Δ*tipF*	Markerless in frame deletion of in *celD* UJ4456	This Study
UJ5292	Yeast pJ69-4A	trp1-901 leu2-3,112 ura3-52 his3-200 GAL4 ΔGAL80 ΔLYS2::GAL1-HIS3 GAL2-ADE2 met2::GAL7-lacZ	James et al., 1996
UJ5649	pJ69-4A	UJ5292 containing pGBD-tipF-kan, p426-TEF-ydeH-cm	This Study
UJ5646	pJ69-4A	UJ5292 containing pGBD-tipF-kan, p426-TEF-cm	This Study

Plasmids

Name	Description	Source or Reference
p208	pLac290 pXyl::*tipF*$_{WT}$	P. Viollier
p3066	pLac290 pXyl::*tipF*$_{E331A/F280S}$	P. Viollier
p3064	pLac290 pXyl::*tipF*$_{E211A/F284L}$	P. Viollier
p264	plac290 pXyl::*tipF*$_{D331A/F284L}$	P. Viollier
pGBD-kan	pGAD with Kan[R] cassette	J. Nesper
pGBD-tipF-kan	pGAD with Kan[R] cassette and tipF-gaL4 fusion	J. Nesper
p426-TEF-cm	p426 with Cm[R] cassette	J. Nesper
p426-TEF-ydeH-cm	p426 with Cm[R] cassette *pTEF*::*ydeH*	J. Nesper

3.3   The flagellin modification protein FlmA interacts with cyclic diguanosine monophosphate in an unspecific manner

3.3.1   Introduction

The bacterial second messenger cyclic diguanosine monophosphate (c-di-GMP) is a wide spread signal molecule among bacteria. High cellular levels of c-di-GMP are associated with sessile, biofilm associated life forms whereas low cellular levels of c-di-GMP are shown to promote motility and a solitary life form. There is much known about the biosynthesis and degradation mechanisms that govern the intracellular levels of this second messenger. However the processes of signal transduction i.e. how the cellular levels of c-di-GMP induce cellular responses as well as the identity of c-di-GMP effector molecules in particular are mainly unknown. In order to address this subject Christen and colleges have developed a biochemical approach for the identification of putative c-di-GMP binders [33]. One of the proteins that they were able to enrich was FlmA.

FlmA (CC_0322) is a 38kD protein encoded in a cluster with other flagellar modification (*flm*) genes. It was reported that the *C. crescentus* gene cluster *flmA-flmH* is involved in motility[155]. Mutant strains lacking any of the *flm* genes express a normal hook-basal body structure but are failing to assemble the flagellar filament[156]. FlmA contains a C-terminal epimerase domain usually involved in a variety of chemical reactions utilizing nucleotide-sugars as substrates. Moreover, homolog proteins from *Helicobacter plyori* were shown to be involved in the glycosylation of flagellin subunits[157]. Both the putative c-di-GMP binding and the proposed association with motility regulation make FlmA a good candidate as a c-di-GMP effector protein. Based on this knowledge we conducted several experiments to test this hypothesis.

3.3.2 Results and Discussion

3.3.2.1 A *flmA* mutant is non-motile and produces a low molecular mass flagellin

We created a clean *flmA* deletion strain and tested it for various phenotypes. The *flmA* mutant displayed severely impaired motility, leading to a colony size on semi-solid agar plates of only about 5 % compared to the wild type (Fig. 1A). In order to pinpoint the cause of this motility defect we tested the *flmA* deletion mutant for its ability to express flagellar elements of class II and III (i.e. the proteins FliM, FliF and FlgH). We observed no change in the expression levels of those proteins (data not shown). We also tested the *flmA* deletion mutant for the expression of flagellin (class IV) and for extracellular flagellin levels (secretion). Both were reduced compared to the wild-type and more importantly we also observed reduction of the molecular mass of the flagellin subunit (Fig. 1B). Similar results were reported earlier[155,156].
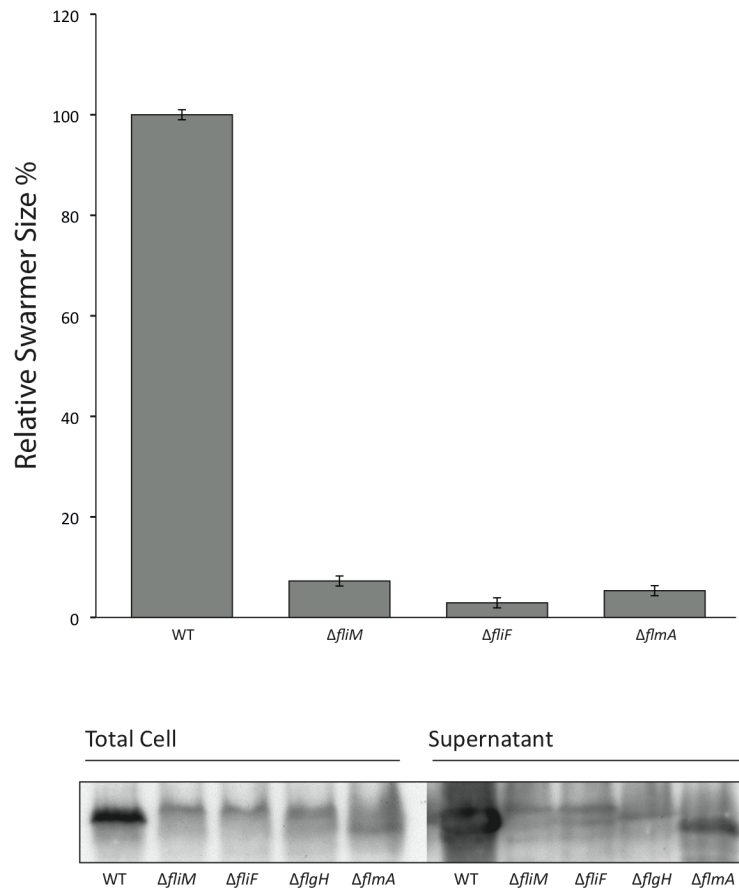
Figure 1: The *flmA* mutant is non-motile and produces a low molecular mass flagellin. (A) Motility assay demonstrating the non-motile phenotype of the *flmA* deletion mutant. The colony diameter on semi-solid agar plates of the *flmA* mutant was compared to wild type. The values for non-motile mutants were included for comparison. (B) The intracellular (total cell) and secreted (supernatant) levels of flagellin analyzed by immunoblots with an anti-Flagellin antibody revealing a reduction in the amount and mass of flagellin in the *flmA* mutant compared to the wild-type. Further flagellar mutants (*flim-*, *fliF-* and *flgH-*) were included for comparison.

3.3.2.2   FlmA interaction with c-di-GMP

Purified FlmA was analyzed for interaction with c-di-GMP by a UV cross-linking assay with ^{33}P labeled c-di-GMP. An increasing amount of labeled c-di-GMP was added to a constant amount of purified protein and after a short incubation on ice the mixture was exposed to UV radiation. The obtained autoradiographs indicated that the protein interacted with c-di-GMP. Moreover, an apparent saturation was observed and the kD of the interaction was determined to be ~25µM (Fig. 2). Although this

measured kD lays above the affinity range of reported c-di-GMP effectors (50nM-5µM), we could not rule out a biological relevance of the interaction between FlmA and c-di-GMP.
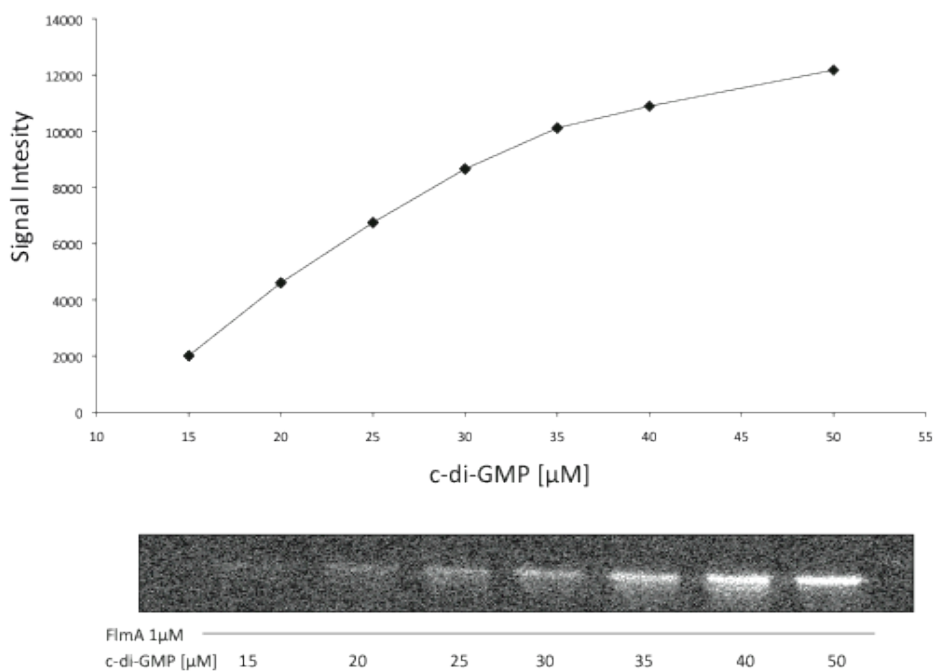
Figure 2: FlmA interaction with ^{33}P labeled c-di-GMP. increasing amount of ^{33}P labeled c-di-GMP wes incubated with purified FlmA and exposed to UV radiation, thereafter samples were separated on SDS-PAGE and bound c-di-GMP was visualized by autoradiography. The signal intensity was plotted revealing an apparent saturation.

In order to attain a better characterization of the FlmA interaction with c-di-GMP we conducted isothermal titration calorimetry (ITC). First we performed a control experiment in which instead of c-di-GMP a non-hydrolysable version of GTP (GTPαS) was titrated into FlmA (5µM) containing solution (Fig. 3). A constant heat release as a result of the dilution process was observed indicating that the molecules did not interact this experimental setup.
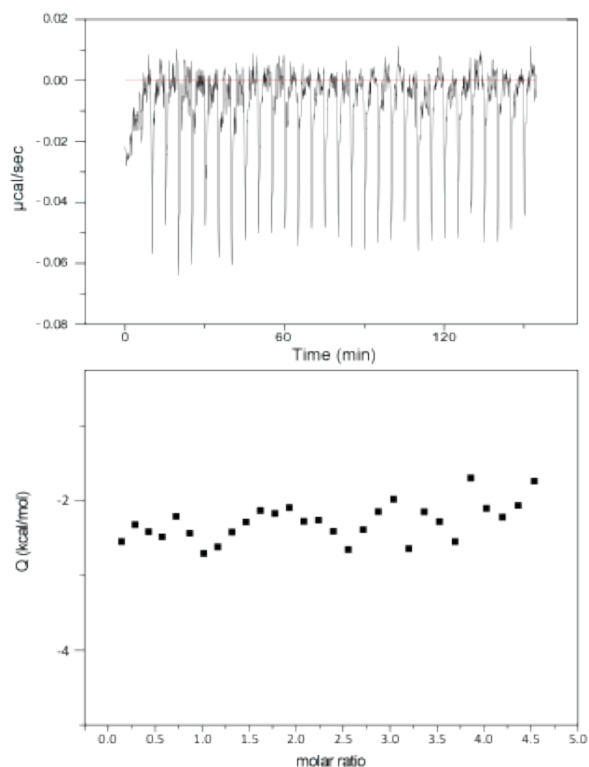
Figure 3: FlmA interaction with GTPαS. Pattern of the heat release during the titration of GTPαS (100 µM) into a 5 µM FlmA solution at 15°C (upper panel). The integrated titration peaks are also shown (lower panel). Since the values remained constant we concluded that there is no interaction of GTPaS with the protein in the concentration range tested.

The calorimetric trace obtained when c-di-GMP was titrated into a protein solution with purified FlmA at 15°C showed that each injection caused an exothermic reaction (Fig. 4). A constant decrease in heat release with each injection, as less free protein was available for binding, was also observed. Once the molecular ratio within the cell reached 1:2 (after ~10 injections) all FlmA molecules seemed to be bound and further injections caused no additional heat release besides dilution effects.
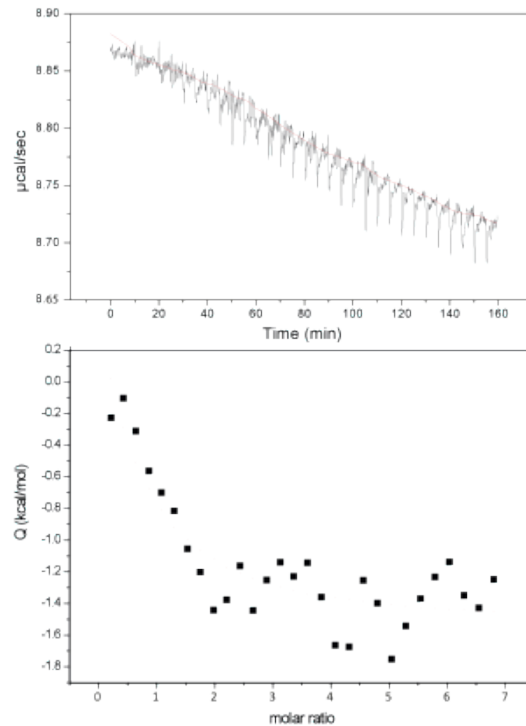
Figure 4: FlmA interaction with c-di-GMP. The titration pattern of d-ci-GMP (150 µM) injected into 5 µM FlmA at 15°C (upper panel). The integrated titration peaks are shown as well (lower panel). The titration curve shows a constant decrease in heat release until a molar ration of 1:2. From then on, only constant heat release (dilution effect) is observed.

However these preliminary results were inconclusive due to the low values of heat release and a drifted base line. We therefore conducted an additional measurement in which a higher concentrated solution of c-di-GMP (350 µM) was titrated into the FlmA solution (19.1 µM) at both 25°C and 15°C (Fig. 5). We noticed that under these conditions a constant heat release per injection step was already reached at a molar ratio of around 1:1, opposing the observations made in the initial experiment. In addition due to the temperature changes we have expected a modification in heat release, as the accessible surface area (ASA) is temperature dependent. However, we did not observe any change in heat release in the measurement made at 15°C in comparison to the measurement made at 25°C (Fig. 5).
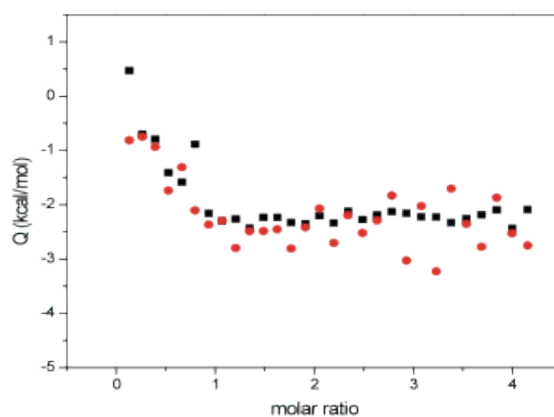
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Figure 5: FlmA interaction with c-di-GMP at different temperatures. c-di-GMP (350 µM) was injected into a solution containing 19.1 µM FlmA at 15°C (red circles) and at 25°C (black squares). The integrated titration peaks are shown. Constant heat release is reached at a 1:1 ratio opposing the observation made in the initial experiment (Fig 4).

This led us to believe that the apparent heat releases measured in the initial experiments were not a result of specific interaction. We tested this assumption by performing an experiment in which we titrated c-di-GMP into a solution without protein and we found that the c-di-GMP dilution process had a very similar heat release profile like the one observed when c-di-GMP was titrated into a FlmA solution (Fig. 6). This unusual dilution profile is most probably due to an oligomeric form transition of c-di-GMP as a cause of the decrease of concentration in solution upon titration[158].
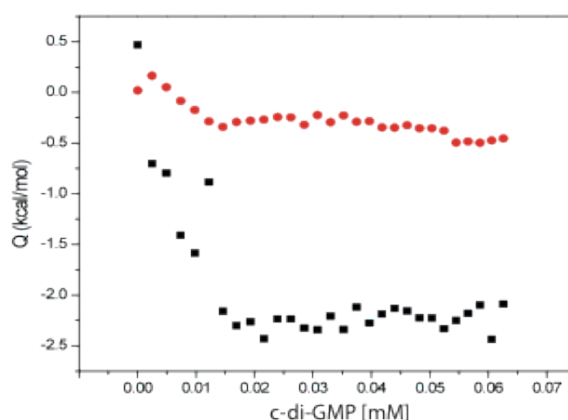

Figure 6: c-di-GMP dilution profile. The integrated titration peaks of the heat release during dilution, obtained from c-di-GMP (350 µM) injection into the buffer (red dots), in comparison to the ones

obtained when c-di-GMP was titrated into FlmA solution (black squares). The constant heat release is reached at the same concentration regardless the presence or absence of the protein.

3.3.2.3   The FlmA interaction to c-di-GMP is unspecific

We conducted a competition experiment in which we measured the ability of small molecules to compete with the c-di-GMP bound to FlmA. In this assay we added a constant amount of ^{33}P labeled c-di-GMP to a constant amount of protein. Thereafter we added the competitor molecules (GTP, GMP, or pGpG) in increasing concentrations. All competitors were able to compete the ^{33}P labeled c-di-GMP (Fig. 7).
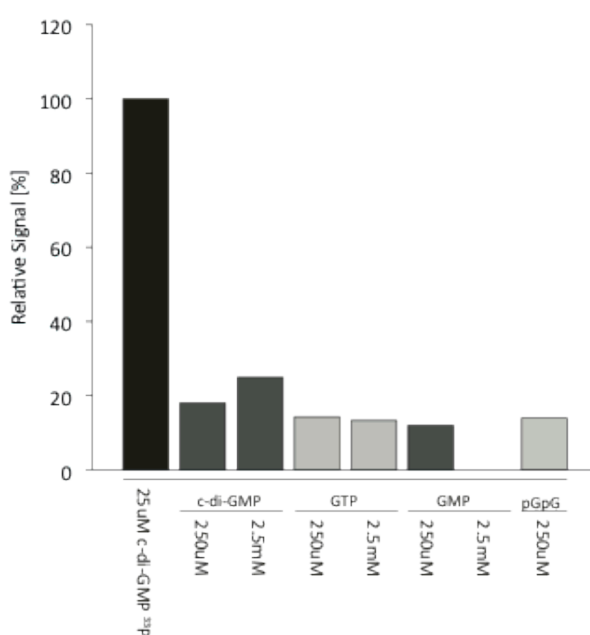
Figure 7: c-di-GMP competition assay. FlmA (1 μM) was incubated with ^{33}P labeled c-di-GMP. Thereafter competitors (unlabeled c-di-GMP, GTP, GMP and pGpG) molecules were added to the indicated final concentrations**.** Samples were separated on SDS-PAGE and the relative signal intensities obtained from the autoradiograph were plotted.

Taken together our data indicates that FlmA might interact with c-di-GMP however this interaction is unspecific and in a range that is biologically non-relevance. This eliminates the possibility that FlmA acts as c-di-GMP effector protein. It is likely that epimerase domain of FlmA that enables interaction of the protein with nucleotide-sugar substrates is the base of the observed unspecific interaction.

3.3.2.4 Material and methods

Growth Conditions

Caulobacter crescentus strains were grown in peptone yeast extract (PYE)[150] or minimal media supplemented with 0.2% glucose or 0.3% D-xylose (M2G or M2X)[150] at 30°C with constant shaking (150 rpm). When selection was required antibiotics in the following concentrations were added: (solid/liquid media in μg/ml): gentamycin (5/0.5), kanamycin (20/5), nalidixic acid (20/not used) and oxytetracycline (5/2.5). For synchronization experiments newborn swarmer cells were isolated by Ludox gradient centrifugation[151] and released into fresh minimal medium. When necessary the medium was supplemented with 1 mM Vanillate or 0.3% xylose. For synchronization with inducible constructs, inducer was added to the growth medium 3 h prior to synchrony.

E. coli strains were grown in Luria Broth (LB) at 37 °C. When necessary for selection the following antibiotic concentrations were used: (solid/liquid media in μg/ml) ampicillin (100/50), gentamycin (20/15), kanamycin (50/30) and oxytetracycline (12.5/12.5).

Motility Assays

C. crescentus colonies were spotted onto PYE soft agar plates (0.3% agar) and incubated for 72 h at 30°C. Plates were then scanned (ScanMaker i800 scanner, Microtek, Germany) and analyzed using Photoshop CS3 (Adobe, CA, USA) and ImageJ (NIH, USA) softwares. For all motility experiments the mean of at least three independent colony sizes is shown. Error bar represent standard deviation.

Attachment Assays

C. crescentus strains were grown in PYE in 96-well microtiter plates (Falcon, USA) for 24 h under constant shaking (200 rpm). After crystal violet (0.3% crystal-violet, 5% isopropanol, 5% methanol) staining of the attached biomass it was dissolved with 20% acetic acid and optical density at 600 nm was measured in a photospectrometer (Genesys6, Thermo Spectronic, USA).

Immunoblots

Primary antibodies were diluted to the final concentration 1:10000 and detected by HPR-conjugated swine anti-rabbit secondary antibodies (Dako Cytomation, Denmark). Western blots were developed with ECL detection reagents (Western Lightning, Perkin Elmer, USA).

Synthesis and Purification of [33P]cyclic-di-GMP

[33P] labelled c-di-GMP was produced enzymatically using α–labelled [33P]GTP as described in[33].

UV Cross-linking of proteins with [33P]c-di-GMP

UV-crosslinking of proteins with radio labelled c-di-GMP was performed as described in[33]. Samples were irradiatedat 254 nm for 20 min on an ice-cooled, wrapped 96-well aluminium block in an RPR-100 photochemical reactor with a UV lamp RPR-3500 (The Southern New England Ultraviolet

Company). After irradiation, samples were mixed with 2x SDS PAGE sample buffer and heated for 5 min at 95 °C. Labelled Proteins were separated by SDS-PAGE and quantified by autoradiography.

Protein purification

Rosetta BL21pLysS E.coli strain was transformed with respective plasmids and used for protein overexpression after induction of cultures with 0.4 optical density at 600nm with 1mM isopropyl-b-D-thiogalactopyranoside (IPTG) for 4h at RT (LB-broth). After induction the cells were collected by centrifugation. The cell pellets were resuspended in buffer consisting of 20mM Tris-HCl pH7, 250mM NaCl, 5mM imidazole, 10mM MgCl$_2$, 1% glycerol and Complete mini cocktail of protease inhibitors, EDTA free at the concentrations specified by manufacturer (Roche). The cells were lysed using French press, and the crude extracts were centrifuged at 15,000xg for 40 min at 4^{0}C. The cleared lysates were incubated with preequillibrated Profinity IMAC Ni-resin (Bio-Rad) for 1h at 4^{0}C and washed consequently with lysis buffer and washing buffer with 20 mM imidazole. The proteins were eluted using 350mM imidazole.

Isothermal titration calorimetry

The interaction of FlmA with cyclic-di-GMP was measured with a VP-ITC isothermal titration calorimeter from MicroCal (Northampton, MA), with the condition specified in the text. All solutions were degassed and equilibrated at desired temperature before using. The delay between the injections was set to 5 min to ensure re-equilibration between injections. Data were evaluated using Origin software (OriginLab) provided by Microcal (Northampton, MA).

Strains and plasmids

Name	Genotype	Description	Source or Reference
CB15	CB15	*C. crescentus* wild type ATCC 19089	Poindexter and Cohen-Bazire, 1964
NA1000	CB15N	Laboratory strain derived from CB15	Evinger and Agabian, 1977
UJ6480	NA1000 Δ*flmA*	Markerless in frame deletion of *flmA*	This Study
UJ1793	NA1000 Δ*flif*	Markerless in frame deletion of *flif*	Jenal and Shapiro, 1995
UJ413	NA1000 *fliM::Tn5*	Disruption of the *fliM* gene by *Tn5*	P. Aldridge
UJ3257	NA1000 Δ*flgH*	markerless in frame deletion of *flgH*	A. Levi

Plasmids

Name	Description	Source or Reference
pET21*flmA*	pET21::His6-*flmA*	M. Christen

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5 Appendices

5.1 De- and repolarization mechanism of flagellar morphogenesis during a bacterial cell cycle

De- and repolarization mechanism of flagellar morphogenesis during a bacterial cell cycle

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Eukaryotic morphogenesis is seeded with the establishment and subsequent amplification of polarity cues at key times during the cell cycle, often using (cyclic) nucleotide signals. We discovered that flagellum de- and repolarization in the model prokaryote *Caulobacter crescentus* is precisely orchestrated through at least three spatiotemporal mechanisms integrated at TipF. We show that TipF is a cell cycle-regulated receptor for the second messenger—bis-(3′–5′)-cyclic dimeric guanosine monophosphate (c-di-GMP)—that perceives and transduces this signal through the degenerate c-di-GMP phosphodiesterase (EAL) domain to nucleate polar flagellum biogenesis. Once c-di-GMP levels rise at the G1 → S transition, TipF is activated, stabilized, and polarized, enabling the recruitment of downstream effectors, including flagellar switch proteins and the PflI positioning factor, at a preselected pole harboring the TipN landmark. These c-di-GMP-dependent events are coordinated with the onset of *tipF* transcription in early S phase and together enable the correct establishment and robust amplification of TipF-dependent polarization early in the cell cycle. Importantly, these mechanisms also govern the timely removal of TipF at cell division coincident with the drop in c-di-GMP levels, thereby resetting the flagellar polarization state in the next cell cycle after a preprogrammed period during which motility must be suspended.

Cell polarity is fundamental to intracellular organization and morphogenesis in all forms of life, yet little is known about how polarity-dependent cues are implemented, reinforced, and removed during the bacterial cell cycle. Eukaryotic cells interpret (cyclic) nucleotide signals to regulate polarity-dependent processes via effectors such as kinases and/or GTPases dictated in response to spatiotemporal cues provided by prepositioned "landmark" proteins and cell cycle regulators (Slaughter et al. 2009; Charest and Firtel 2010; St Johnston and Ahringer 2010; Amato et al. 2011; Ji and Tulin 2012). Here we unearth a polarized cyclic nucleotide signaling pathway cued by bis-(3′–5′)-cyclic dimeric guanosine monophosphate

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(c-di-GMP) that first triggers and reinforces and later resets flagellar polarity during a bacterial cell cycle.

The Gram-negative α-proteobacterium *Caulobacter crescentus* (henceforth, *Caulobacter*) is an ideal model system to study how polarization is coordinated with cell cycle progression. At the predivisional cell stage, *Caulobacter* is overtly polarized, bearing a cylindrical extension of the cell envelope (the stalk) at the old pole and a newly assembled flagellum whose rotation is activated at cytokinesis at the opposite pole (Tsokos and Laub 2012). Cytokinesis yields a motile swarmer cell that resides in a G1-like, nonreplicative state and a dividing stalked cell engaged in S phase (Fig. 1A). Two master transcriptional regulators of the cell cycle, CtrA and GcrA, reinforce the transcriptional program at sequential stages of the cell cycle (Quon et al. 1996; Laub et al. 2000; Holtzendorff et al. 2004). CtrA is present in G1 phase, proteolytically removed during the G1 → S transition, and reappears later in S phase (Figs. 1A, 7D [below]). In
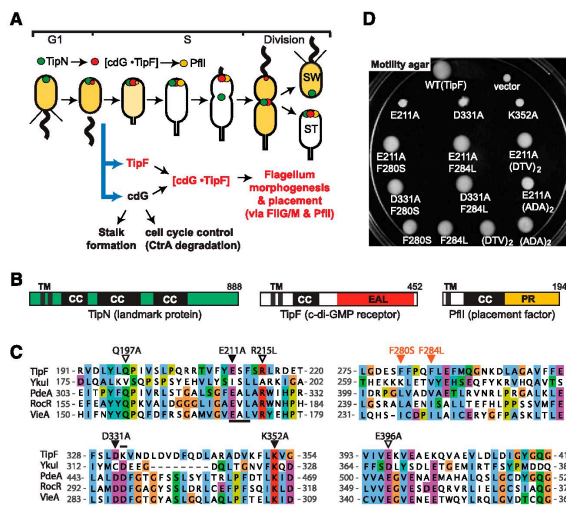
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Figure 1. Localization of TipN, TipF, and PflI to the flagellated pole. (A) Dynamic protein localization of TipN, TipF, and PflI and its coordination with other cell cycle events in *C. crescentus*. TipN (green) localized at the nonflagellated pole recruits newly synthesized TipF (red), which in turn recruits the PflI flagellar positioning protein (yellow). Thick blue arrows indicate the synthesis of TipF (by GcrA) and c-di-GMP. (B) Domain organization of TipN, TipF, and PflI. Shown are the predicted transmembrane domains (TM; gray), coiled-coil domains (CC; black), PDE domain (EAL; red), and proline-rich domain (PR; yellow). (C) Alignment of the EAL domains from TipF and the c-di-GMP PDEs PdeA of *C. crescentus*, VieA of *Vibrio cholerae*, RocR of *Pseudomonas aeruginosa*, and YkuI of *Bacillus subtilis*. Key residues that are predicted to be required for PDE activity (empty arrowheads) or required for c-di-GMP binding (filled black) are marked. Residues that bypass the requirement of E211 or D331 for motility (red triangles), the defining EAL motif, and the K332 residue of the conserved Asp–Asp loop (underlined) are indicated. (D) Motility assay of Δ*tipF* mutants harboring plasmids encoding wild-type (WT) or single- or double-mutant TipF. DTV and ADA denote the triple duplications of DTV or ADA residues at positions 121–124 or 128–130, respectively. Overnight cultures were spotted on PYE swarm agar plates and incubated for 60 h at 30°C. Compact swarms indicate the motility defect caused by mutations in the c-di-GMP-binding site, whereas the suppressive mutations yield diffuse and enlarged swarms.

contrast, GcrA accumulates during the G1 → S transition, inducing the synthesis of CtrA along with polarity and other cell cycle proteins (McAdams and Shapiro 2011), and is then eliminated from G1 progeny cells along with c-di-GMP (Paul et al. 2008; Christen et al. 2010).

During the G1 → S transition, the flagellum is shed to permit cells to immobilize to surfaces using an adhesive polar structure. The flagellum is then resynthesized at the new pole in late S phase following this preprogrammed nonmotile period. CtrA induces the synthesis of early flagellar structural constituents, including the FliF MS ring protein and two components of the switch complex (FliG and FliM) (see Fig. 3A, below). The newly synthesized flagellar parts are assembled from the inside of the cell outward, first with an organizational platform in the inner membrane (the MS ring) to which the switch complex on the cytoplasmic side of the membrane is tethered (Chevance and Hughes 2008; Brown et al. 2011). The assembly of subsequent structures follows suit, ultimately ending with the elaboration of the external parts, such as the hook and the flagellar filament.

Superimposed on these temporal patterns are spatial cues that guide flagellar proteins to the appropriate site of assembly at the new cell pole opposite the stalk (Fig. 1A). The new cell pole harbors TipN, a polytopic coiled-coil protein that is deposited at the new pole as it is generated during cytokinesis (Huitema et al. 2006; Lam et al. 2006). At cytokinesis, TipN redistributes to the division plane from the poles, ensuring that cell polarity cues are avail-

able for the flagellar polarization in the next cell cycle. Indeed, flagella are mispositioned in the absence of *tipN*, indicating that TipN is required for the proper placement, but not the assembly, of the flagellum (Huitema et al. 2006; Lam et al. 2006). A flagellar misplacement defect has also been observed in mutants lacking the PflI positioning factor, a bitopic membrane protein with a proline-rich domain at the C terminus facing the cytoplasm that is localized to the future flagellated pole before the synthesis of early flagellar components (Obuchowski and Jacobs-Wagner 2008). While the spatial relationship between TipN and PflI remains unexplored, TipN recruits TipF, a polytopic membrane protein that positively regulates flagellum biogenesis (Huitema et al. 2006). In the absence of TipN, TipF localizes to sites of misplaced flagella, raising the possibility that mislocalized TipF is responsible for the flagellar misplacement phenotype of a *tipN* mutant. This is supported by the observation that *tipF* deletion mutants lack external flagellar structures such as the hook and filament (Huitema et al. 2006).

TipF features a C-terminal and cytoplasmic EAL (also known as DUF2) domain that, in related proteins, confers c-di-GMP-specific phosphodiesterase (PDE) activity (Hengge 2009; Schirmer and Jenal 2009; Boyd and O'Toole 2012). The levels of ci-di-GMP are modulated by diguanylate cyclases (DGCs) that synthesize c-di-GMP from GTP and PDEs that hydrolyze c-di-GMP into linear pGpG. In *Caulobacter*, multiple DGCs and PDEs determine c-di-GMP levels during the cell cycle to promote develop-

ment and cell cycle progression (Paul et al. 2004, 2008; Abel et al. 2011). c-di-GMP levels are low in G1 cells but peak at the G1 → S transition and decline thereafter (Paul et al. 2008; Christen et al. 2010). While c-di-GMP binds to various receptor proteins to curb flagellar rotation (Wolfe and Visick 2008; Boehm et al. 2010; Fang and Gomelsky 2010; Paul et al. 2010), the spatiotemporal mechanism underling c-di-GMP-induced flagellum morphogenesis has not been resolved.

Here we show that c-di-GMP controls the activation, polarization, and stabilization of the flagellar regulator TipF (Huitema et al. 2006). We found that TipF is a receptor for c-di-GMP that peaks at the G1 → S transition and show that TipF•c-di-GMP seeds polar flagellar assembly by recruiting the PflI placement factor (Obuchowski and Jacobs-Wagner 2008) and components of the flagellar switch into a complex with TipN, a landmark protein that is prepositioned at the newborn pole (Huitema et al. 2006; Lam et al. 2006). Importantly, we found that c-di-GMP-induced stabilization and polarization of TipF is amplified with the coordinated transcription of the *tipF* gene and thus is tuned to other c-di-GMP-dependent developmental events that occur at the G1 → S transition (Paul et al. 2004, 2008; Duerig et al. 2009).

Results

c-di-GMP binds and activates TipF

Initial hints into the mechanism of TipF-mediated flagellum biogenesis came from comparisons of the TipF primary structure with known PDEs. TipF features a Lys at position 332 instead of an Asp in related proteins (Fig. 1C), and a Glu–Ser–Phe (ESF, residues 211–213) triplet replaces the defining Glu–Ala–Leu (EAL) motif. The highly conserved Asp331–Asp332 motif is a hallmark of active PDEs, with both residues being involved in coordinating the two metal ions in the catalytic center (Barends et al. 2009). To explore whether the sequence conservation reflects a functional requirement for the residues at these positions, we engineered alanine mutations at several conserved positions implicated or not in coordinating the cofactor (Mg^{2+} or Mn^{2+}), the substrate, and/or the nucleophile (E211A, D331A, and K352A) (Fig. 1C, filled triangles). Neither of these mutant proteins could support TipF function (motility on 0.3% soft agar plates) when expressed in a Δ*tipF* background from P_{xyl} on a low-copy plasmid, indicating that these residues play a key role in TipF function (Fig. 1D).

To test whether TipF is an active PDE, we purified the soluble hexa-histidine (His_6)-tagged form of TipF lacking the first 115 residues, including the two predicted membrane-spanning segments, and the E211A mutant derivative from *Escherichia coli*. Circular dichroism (CD) spectra suggested that the secondary structures of purified TipF wild type and E211A mutant were unchanged (Supplemental Fig. S1A). We then assayed both proteins for PDE activity by high-pressure liquid chromatography (HPLC) analysis with the c-di-GMP substrate (Fig. 2A). No cleavage of c-di-GMP to the linear form pGpG was observed with either wild-type TipF or the E211A mutant (Fig. 2A, right panel) even after extended incubation times of up to 24 h (Supplemental Fig. S1B,C). In contrast, the control PDE YahA from *E. coli* converted all of the c-di-GMP to pGpG in <5 min (Fig. 2A, left panel).
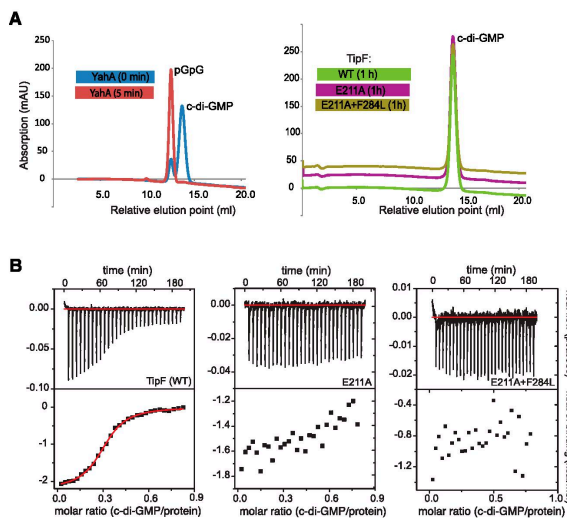

Figure 2. TipF is enzymatically inactive but binds c-di-GMP. (*A*) c-di-GMP hydrolytic activity is not detected in recombinant (soluble) TipF but is in the control PDE YahA. (*Left* panel) Purified proteins (1 μM) were incubated with c-di-GMP for 5 min and then separated on a ResourceQ column to observe the cleavage product pGpG. No pGpG was detected after 1 h of incubation with wild-type (WT) or mutant (E211A or E211A/F284L) TipF with c-di-GMP. (*B*) ITC experiments showing that c-di-GMP binds to the soluble portion of wild-type TipF but not to the mutant derivatives E211A or E211A/F284L. The *top* panels show the raw ITC data curves collected at 25°C in binding buffer (50 mM Tris/HCl, 50 mM NaCl at pH 8.0). The *bottom* panels show the integrated titration peaks fitted to a one-site binding model (solid line). The average dissociation constant (K_D) of TipF to c-di-GMP was estimated at 0.4 (±0.2) μM, the stoichiometry of binding (*n*) was estimated at 0.35 (±0.1), and the enthalpy of reaction was estimated at −2.1 (±0.3 kcal/mol).

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In the absence of detectable PDE activity, we asked whether wild-type TipF or the E211A mutant can bind c-di-GMP using an isothermal titration calorimetry (ITC)-based binding assay (Fig. 2B). Successive injections of 10 µL of a 118 µM solution of c-di-GMP solution into the ITC reaction chamber containing 32.5 µM wild-type TipF was accompanied by the characteristic heat release, reflecting specific and high-affinity binding of c-di-GMP (Fig. 2B, left panel). The resulting integrated titration peaks were fitted to a sigmoidal enthalpy curve, and a dissociation constant (K_D) of 0.4 ($\pm$0.2 µM) for c-di-GMP was derived for wild-type TipF. In contrast, only background (nonspecific) heat release was observed when an equimolar amount of TipF (E211A) was injected into the chamber (Fig. 2B, middle panel [note the different scales in the panels]). Together, these results demonstrate that TipF does not have PDE activity under the conditions tested and that wild-type TipF, but not the E211A derivative, binds c-di-GMP with high affinity. On the basis of these results, we hypothesize that c-di-GMP binding is necessary for TipF to signal motility and that the E211A mutant is nonfunctional because it can no longer bind and be activated by c-di-GMP. In support of this view, we found that depleting *Caulobacter* cells of c-di-GMP phenocopies the localization and flagellar assembly defect caused by the absence TipF or the "c-di-GMP-blind" E211A mutant (Fig. 6E, below).

If TipF is activated without hydrolyzing c-di-GMP, then mutations that are known to specifically cripple catalytic activity of PDEs, but not c-di-GMP binding, should not affect TipF function. To test this prediction, we engineered mutants Q197A, R215L, and E396A (Fig. 1C, open triangles) and found that the mutant proteins R215L and E396A confer motility in Δ*tipF* cells indistinguishable from that of wild-type TipF, while Q197A also supported motility but with reduced efficiency (data not shown). Taken together, we conclude that TipF binds c-di-GMP and that binding is required for flagellar functions.

TipF acts early in polar flagellum assembly

Since previous transmission electron micrographs showed the external structures, the flagellar hook, and the filament (Fig. 3A) to be missing from Δ*tipF* cells (Huitema et al. 2006), we wondered whether the assembly of internal flagellar (sub)structures also requires TipF. To this end, we conducted electron cryotomography (ECT) of wild-type and Δ*tipF* cells by ECT (Fig. 3B; Supplemental Fig. S2A,B; see the Supplemental Movies), a high-resolution imaging method that preserves cellular structures in their native (cryogenically vitrified) state and reveals them owing to the natural contrast from electron density. We subjected two-dimensional (2D) projections of three-
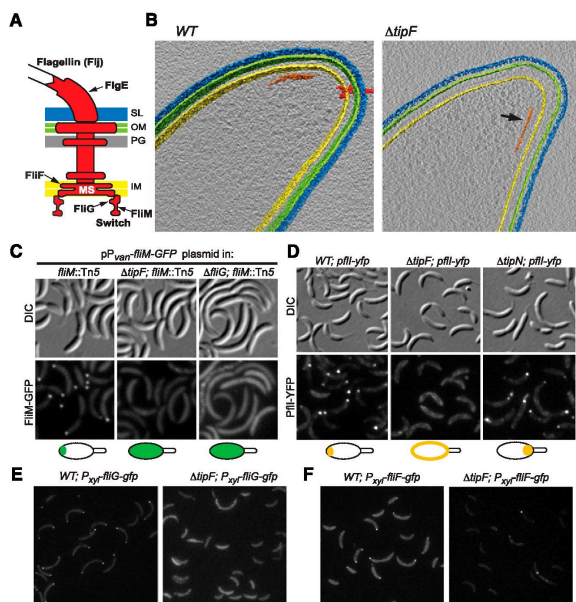

Figure 3. TipF mediates the localization of flagellar proteins FliM, FliG, FliF, and PflI to the cell pole. (*A*) Schematic of the flagellum. The relative position of the MS ring (FliF), the switch complex (including FliG and FliM), the hook (FlgE), and the filament (flagellins) are indicated, as are the envelope layers: inner (cytoplasmic) membrane (IM), peptidoglycan layer (PG), outer membrane (OM), and S layer (SL). (*B*) ECT imaging reveals densities corresponding to the intact flagellum, including the MS ring and the switch complex in or near the inner membrane of the wild type (WT) that are absent in the Δ*tipF* mutant. The color-coding of the segmented structures corresponds to the color scheme in *A*. Flagellar structures (red) are shown in the *left* panel and in the segmentation of wild type. The black arrow points to the chemoreceptor array (orange) in Δ*tipF* cells that is also present in wild type. Bars, 200 nm. (*C*) The subcellular localization of FliM-GFP from the P_{van} promoter on a medium copy number plasmid in a *fliM* mutant (*fliM*::Tn5), a *tipF* mutant (Δ*tipF*; *fliM*::Tn5), or a *fliG* mutant derivative (Δ*fliG*; *fliM*::Tn5) analyzed by live-cell fluorescence microscopy. (*D*) Localization of PflI-YFP expressed from P_{pflI} at the *pflI* locus in the presence (*pflI-yfp*) or absence of TipF (Δ*tipF*; *pflI-yfp*) or TipN (Δ*tipN*; *pflI-yfp*). (DIC) Differential interference contrast images. (*E,F*) Localization FliG-GFP (*E*) and FliF-GFP (*F*) expressed from P_{xyl} at the *xylX* locus in a Δ*tipF* mutant.

dimensional (3D) tomograms to segmentation algorithms to reveal the densities of the flagellar MS ring and switch complex at the cytoplasmic membrane at the poles of wild-type cells but could not detect these structures at $\Delta tipF$ poles (Fig. 3A,B). The fact that chemoreceptor arrays that normally assemble at the same pole (Alley et al. 1992) and at the same time as the flagellum were seen in $\Delta tipF$ cells confirms that the correct site was imaged (Fig. 3B, right panel).

Live-cell fluorescence imaging was used next to underscore the ECT results. To this end, we localized the FliM switch protein (Fig. 3C) as a C-terminal protein fusion to GFP (FliM-GFP) expressed from P$_{van}$ in $fliM$::Tn5 cells harboring or not harboring a $\Delta fliG$ or $\Delta tipF$ in-frame deletion. FliM is predicted to interact with the FliG switch protein that is tethered to the inner membrane via the FliF MS ring protein (Fig. 3A; Chevance and Hughes 2008). Consistent with this notion, FliM-GFP is diffuse in the cytoplasm of $\Delta fliG$; $fliM$::Tn5 cells but is sequestered to the flagellated pole (opposite the stalk) in $fliM$::Tn5 cells (Fig. 3C). Moreover, FliM-GFP is delocalized in $\Delta tipF$; $fliM$::Tn5 cells (Fig. 3C). Consistent with the dependence of polar localization of FliM on FliG, we confirmed that a derivative of the FliG switch protein fused C-terminally to GFP (FliG-GFP) also depends on TipF for polar localization (Fig. 3E; Supplemental Fig. S2C).

Since two switch proteins (FliG and FliM) are no longer polarized in the absence of TipF, we asked whether the MS ring protein FliF localizes in a TipF-dependent fashion using a partially functional FliF-GFP fusion expressed in the presence of endogenous FliF from the xylose–inducible promoter at the chromosomal $xylX$ locus. Akin to FliG-GFP, only few polar FliF-GFP foci were seen in $\Delta tipF$ cells (Fig. 3F; Supplemental Fig. S2B), providing further evidence that neither the MS ring nor the switch complex assembles in $\Delta tipF$ cells. Interestingly, we found that FliF-GFP required FliG to be sequestered to the pole (Supplemental Fig. S2D), suggesting that the mislocalization of FliF-GFP seen in $\Delta tipF$ cells is a secondary effect of FliG dispersion. In support of this notion, we identified FliG as an interaction partner of soluble TipF in a yeast two-hybrid assay, suggesting that TipF and FliG interact (see below).

Based on the result that FliF and FliG are dispensable for polar localization of TipF (data not shown), we conclude that TipF acts at the earliest known (nucleation) step in flagellum biogenesis, switch, and MS ring formation, respectively.

The PflI flagellar positioning factor is recruited into a polar complex by TipF

PflI is required for proper positioning of the flagellum and is recruited to the future flagellum assembly site before flagellar structural proteins are expressed by an unknown mechanism (Obuchowski and Jacobs-Wagner 2008). Like TipF, PflI is still polar in the absence of the MS ring protein FliF, raising the possibility that PflI localization is dependent on TipF. In support of this, we found TipF-mCherry to colocalize with PflI-GFP (PflI fused C-terminally to GFP) (Supplemental Fig. S3C) and observed that PflI-YFP or PflI-GFP are delocalized in $\Delta tipF$ cells (Fig. 3D; Supplemental Fig. S3A, respectively). Conversely, PflI does not noticeably affect TipF localization, as indicated by the apparent normal localization of TipF-GFP (expressed in lieu of endogenous TipF from the $tipF$ locus) in $\Delta pflI$ compared with wild-type cells (Supplemental Fig. S3B).

Since TipF is recruited to this site by the TipN landmark protein (Supplemental Fig. S3B), we predicted that PflI localization should also be dependent on TipN. Indeed, PflI-YFP or PflI-GFP foci are mispositioned near or within the stalk in $\Delta tipN$ cells, a pattern resembling the misplacement of TipF-GFP in the absence of TipN (Fig. 3D; Supplemental Fig. S3A,B). The formation of these (mis)-localized PflI foci in $\Delta tipN$ cells is still TipF-dependent, as indicated by the dispersion of PflI-GFP in the envelope of $\Delta tipF$; $\Delta tipN$ cells (Supplemental Fig. S3A). A similar dispersion of PflI-GFP occurs when the C-terminal proline-rich domain of PflI is deleted (residues 93–142 or 142–194) (Fig. 1B; Supplemental Fig. S3D), suggesting that this domain responds to the presence of TipF. In contrast, the $\Delta pflI$ mutation does not seem to change the mislocalization of TipF-GFP in $\Delta tipN$ cells (Supplemental Fig. S3B). Thus, flagellar assembly and recruitment events at the newborn pole proceed in the order TipN > TipF > [(PflI)(FliF/G/M)].

To test whether this localization dependency is reflected in physical interactions among the proteins, we conducted pull-down experiments from extracts derived from cells harboring an empty vector control or a plasmid expressing either a TipF or PflI derivative with a C-terminal TAP (tandem affinity purification) tag (see the Materials and Methods) from the P$_{van}$ promoter. Owing to the instability of TipF (described below), we conducted the pull-down experiments in mutant backgrounds ($\Delta tipN$ and $flbD$::Tn5, when appropriate) in which TipF abundance is increased (Fig. 4A). This likely results from a post-transcriptional mechanism, as $tipF$ transcription is unchanged in a $tipN$ and $flbD$ mutant (Supplemental Fig. S4A). Immunoblotting of TipF-TAP and PflI-TAP pull-down samples with antibodies to PflI (Fig. 4B) or TipF (Fig. 4C) provided evidence that TipF and PflI interact (directly or indirectly), but the pull down is inefficient. In contrast, coimmunoprecipitation of PflI with TipF-GFP using monoclonal antibodies to GFP was efficient (Supplemental Fig. S4B,C,D). Moreover, immunoblotting of the TipF-TAP (Fig. 4D) and PflI-TAP (Fig. 4E) pull-down samples with polyclonal antibodies to TipN provided evidence that TipN is in a complex with TipF and PflI.

Finally, using an unbiased approach for interaction partners of TipF, we conducted a yeast two-hybrid screen using soluble TipF (lacking transmembrane segments, residues 1–90) as bait to probe a prey library of *Caulobacter* genomic fragments. Two positive clones, each encoding Gal4-AD fusions to a C-terminal fragment of FliG (FliG$_C$)—residues 192–340 and residues 237–340 (Fig. 4F,G)—emerged from this screen. This result shows that FliG can directly interact with the soluble part of TipF and accounts for the localization dependency seen in vivo.
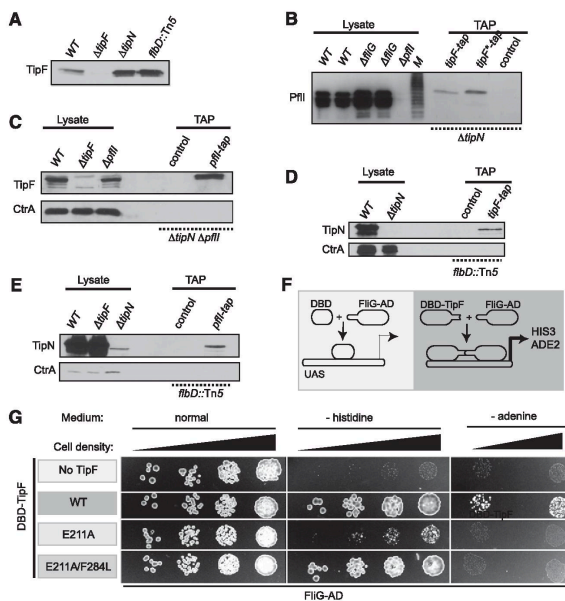
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Figure 4. TipF directly interacts with FliG and forms a complex with TipN and PflI. (*A*) TipF steady-state levels as determined by immunoblotting using polyclonal antibodies to TipF in lysates from wild-type, Δ*tipF*, Δ*tipN*, and *flbD::Tn5*. (*B–E*) TAP pull-down of TAP-tagged proteins expressed from P$_{van}$ on a medium copy plasmid followed by immunoblotting using polyclonal antibodies to TipF, PflI, TipN, and CtrA. The cell lysates derived from boiled cells shown in the lanes at the *left* of the panels provide negative and positive controls for the specificity for the antisera. CtrA immunoblots are shown as a control for loading. (*B*) TipF-TAP and TipF*-TAP (the E211A/F284L mutant is referred to as TipF*) were expressed from the P$_{van}$ promoter on a medium copy number plasmid in Δ*tipN* mutants. (M) Protein marker lane. (*C*) TipF is present in PflI-TAP purifications of Δ*tipN* Δ*pflI* double-mutant lysates. (*D,E*) TipN is present in pull-downs of TipF-TAP (*D*) or PflI-TAP (*E*) from *flbD::*Tn5 lysates. (*F*) Schematic showing the yeast two-hybrid (Y2H) assay using the C terminus of FliG (residues 237–340) as prey and the soluble part of TipF (residues 92–452) as bait to induce transcriptional activation of HIS3 and ADE2 (dark-gray box) as readout of the interaction. In the presence of DNA-binding domain (DBD)-TipF (TipF fused to the GAL4 DBD), transcriptional activation is seen, but not when DBD is used without a TipF fusion (light-gray box). (*G*) Growth of the yeast strains expressing the DBD-only (i.e., without TipF), wild-type, and mutant DBD-TipF variants along with FliG-AD (activation domain) on normal medium and selective medium lacking histidine (−histidine) or adenine (−adenine). The latter condition is more stringent. Gray scales from dark to light indicate the level of interaction corresponding to the growth readout on selective medium. Dark gray represents the strongest interaction as seen for wild-type DBD-TipF with FliG-AD.

Collectively, our results show that TipF interacts directly with FliG$_C$ and assembles a flagellar organizational center comprising FliG, FliN, PflI, and TipN at the new pole.

c-di-GMP induces TipF polarization and signaling

If TipF is activated upon binding c-di-GMP, then cells expressing TipF variants that are unable to interact with c-di-GMP or cells depleted for c-di-GMP should also be unable to localize PflI. Neither the E211A derivative nor the D331A or K352A mutants were able to direct PflI into polar assemblies (Fig. 5A). Moreover, these inactive TipF mutants were themselves no longer sequestered to the pole (Fig. 5B). Furthermore, the *tipF(E211A)* mutation phenocopies the flagellar assembly defect of the Δ*tipF* deletion, as reflected in the absence of the FlgE hook protein (see Fig. 6F).

To test whether c-di-GMP depletion mimics the effects of the (c-di-GMP-binding defective) E211A mutant, we heterologously expressed the potent *Pseudomonas aeruginosa* PDE PA5295 in strains carrying the *pflI-yfp* reporter and imaged the resulting cells. Expression of PA5295, but not the catalytically inactive mutant PA5295-AAL [carrying an analogous mutation to TipF(E211A) at position E328], was previously shown to reduce c-di-GMP levels beyond detection (Duerig et al. 2009). We found that induction of

PA5295 (depletion of c-di-GMP) dispersed PflI-YFP from the pole (Fig. 6A). Consistent with these results, we observed that c-di-GMP depletion completely delocalized TipF-GFP from the pole (Fig. 6B), while TipN-GFP was still polar under these conditions (Fig. 6C). c-di-GMP depletion also prevented FlgE expression, akin to the Δ*tipF* or *tipF(E211A)* mutation (Fig. 6F). Interestingly, however, c-di-GMP depletion had a much stronger effect on transcription of the *flgE* gene than the Δ*tipF* or *tipF(E211A)* mutation (Fig. 6E), indicating that c-di-GMP modulates another step in flagellum biogenesis independently of TipF.

We conclude that c-di-GMP binding is a critical step in TipF activation and polarization. Interfering with this activating step by either introducing mutations in TipF that prevent c-di-GMP binding or depleting cells of c-di-GMP locks TipF in a conformational state that prevents its activation and polarization, the recruitment of PflI, and flagellar assembly at the newborn pole.

Bypassing the requirement of c-di-GMP for TipF signaling

To illuminate how c-di-GMP activates and polarizes TipF, we isolated intragenic suppressor mutations that restore
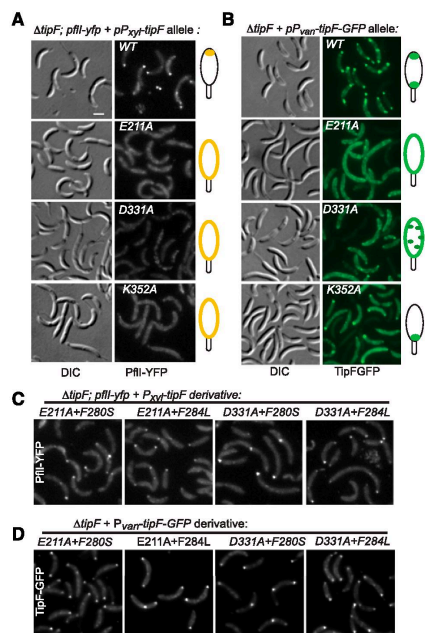
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Figure 5. Suppressor mutations render TipF and PflI localization c-di-GMP-independent. (A–D) Localization of PflI-YFP (A,C) or TipF-GFP (B,D) mutants in ΔtipF cells harboring plasmids encoding TipF loss-of-function mutants expressed from the P$_{xyl}$ (A,C) or the P$_{van}$ (B,D) promoter a low copy number plasmid. (DIC) Differential interference contrast images. Note that TipF(K352A)-GFP can cluster at the stalked pole but not at the newborn pole and that the ectopic signals at the stalked pole of wild-type (WT) TipF-GFP are due to (constitutive) expression of TipF from P$_{van}$ on the low-copy plasmid compared with constructs expressing TipF-GFP or YFP from the chromosome (cf. B vs. Fig. 6B and Supplemental Fig. S3B).

motility to tipF(E211A) or tipF(D331A) cells. The suppressor alleles carry either a missense mutation in one of two proximal codons (F280S or F284L) within the c-di-GMP-binding (EAL) domain or a duplication of a codon triplet (either ADA or DTV, residues 121–124 or 128–130, respectively) within the coiled-coil motif preceding the EAL domain (Fig. 1C,D). Importantly, the F280S or F284L missense mutations suppress either the tipF(E211A) or tipF(D331A) allele. As F280S or F284L are not allele-specific mutations, we considered the possibility that they also mitigate the localization defects of the D331A and E211A mutants. Indeed, C-terminal GFP fusions of the four double mutants (E211A/F280S, E211A/F284L, D331A/F280S, and D331A/F284L) revealed the proteins to be polarized and able to recruit PflI-YFP to the new pole (Fig. 5C,D).

If the suppressor mutations lock TipF in an activated state, then they might allow TipF to remain polarized under conditions of c-di-GMP depletion. We tested this idea by localizing YFP-tagged derivatives of wild-type, E211A/F280S, and E211A/F284L versions of TipF expressed in ΔtipF cells. While all three fusion proteins were polarized in the presence of c-di-GMP (Supplemental Fig. S5A), depletion of c-di-GMP dispersed wild-type TipF-YFP, but polar foci of the E211A/F280S and E211A/F284L versions persisted (Fig. 6D). Biochemical and biophysical analyses showed that the E211A/F284L double mutant is still unable to bind or hydrolyze c-di-GMP in vitro (Fig. 2A,B) and that the secondary structure is preserved (Supplemental Fig. S1). Interaction studies of wild-type and mutant TipF by yeast two-hybrid (Fig. 4G) and coimmunoprecipitation (Supplemental Fig. S4C,D) assays showed that TipF(E211A) associates poorly with FliG and PflI, but the suppressor mutations attenuated this effect of the E211A mutation.

To test whether these c-di-GMP bypass mutants can also support motility in the absence of c-di-GMP, we tested whether cells harboring suppressor mutations could swim in broth after depletion of c-di-GMP. As this was not the case (data not shown), we wondered whether c-di-GMP also promotes flagellar assembly or function at a later, TipF-independent step. We examined this possibility using the accumulation of the FlgE hook protein as a convenient proxy for defects in flagellar gene expression that can arise when assembly is blocked. Immunoblotting (Fig. 6F) revealed that FlgE steady-state levels (cell-associated and the hook accumulating in the supernatant) are reduced in c-di-GMP-depleted cells even in the presence of the E211A/F280S and E211A/F284L TipF versions. Transcription of flgE (probed using a P$_{flgE}$-lacZ transcriptional reporter) attained only 30% ± 1% (E211A/F280S) and 30% ± 1% (E211A/F284L) of wild-type activity and was much below the activity level observed for the ΔtipF single mutant (57% ± 1% of wild type) (Fig. 6E). Moreover, P$_{flgE}$-lacZ reporter activity is further reduced (to 21% of wild type) when c-di-GMP is depleted in ΔtipF cells, indicating that this c-di-GMP control step is required for efficient flgE transcription. A similar c-di-GMP-dependent effect was seen on transcription of the fljL flagellin gene using the P$_{fljL}$-lacZ transcriptional reporter (Supplemental Fig. S5B). In this case, however, c-di-GMP depletion reduces fljL transcription in ΔtipF cells from near-full wild-type levels (fljL transcription is not affected by ΔtipF mutation) to 32% ± 2%.

We conclude that TipF localization can be partially uncoupled from c-di-GMP control by the F280S and F284L suppressor mutations, but c-di-GMP influences at least one additional event in flagellum assembly, gene expression, localization, and/or (possibly affects) TipN polarization.

TipF abundance coincides with and depends on the cell cycle burst in c-di-GMP

Monitoring the steady-state levels of wild-type TipF and TipF(E211A) by immunoblotting suggested that (untagged)
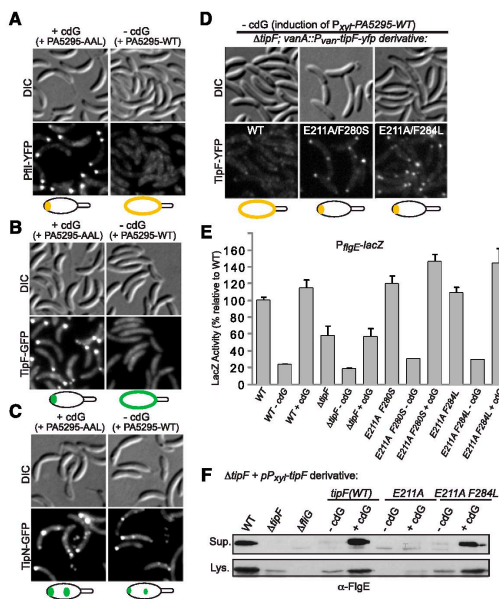
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Figure 6. Localization of TipF and PflI as well as expression of FlgE is c-di-GMP-dependent. (*A–D*) Localization of PflI-YFP (*A*), TipF-GFP (*B*), TipN-GFP (*C*), and YFP-tagged TipF suppressor mutants (*D*) under normal (PA5295-AAL, +cdG) or c-di-GMP-depleted (PA5295-WT, −cdG) conditions. PA5295 variants were expressed from the P_{xyl} promoter on a medium copy number plasmid in cells expressing the GFP fusion proteins from their respective promoters at the endogenous locus. In *D*, TipF-YFP variants were expressed from P_{van} at the *vanA* locus in Δ*tipF* cells. (DIC) Differential interference contrast images. (*E*) Effects of c-di-GMP concentration on transcription of the *flgE* hook gene by β-galactosidase assays of cells (wild-type [WT], Δ*tipF*, or Δ*tipF* cells expressing mutant TipF from P_{van} at the *vanA* locus) harboring a P_{flgE}-lacZ transcriptional reporter fusion. (*F*) Effects of c-di-GMP depletion on FlgE steady-state levels in supernatants (Sup.) or cell lysates (Lys.), determined by immunoblotting using anti-FlgE antibody (α-FlgE). TipF mutants were expressed from the P_{xyl} promoter on a low copy number plasmid in Δ*tipF* cells.

TipF is stabilized upon binding c-di-GMP (Fig. 7A,B). The abundance of wild-type TipF expressed from P_{xyl} (i.e., using the transcriptional and translational regulatory signals of the *xylX* gene) in Δ*tipF* cells is significantly higher than that of the E211A version, suggesting that TipF is regulated at the level of stability by c-di-GMP. Indeed, half-life measurements of (untagged) wild-type TipF and TipF(E211A) under normal (Fig. 7B) or c-di-GMP-depleted (Fig. 7A) conditions (as described above) approximated the half-life of wild-type TipF at >2 h in the presence of c-di-GMP and <20 min in its absence. In contrast, for the E211A version, the half-life values were ∼10–20 min irrespective of the presence or absence of c-di-GMP, demonstrating that the E211A protein is not further destabilized upon c-di-GMP depletion (Fig. 7A,B). Remarkably, the half-life (∼40 min) of the E211A/F284L variant was higher than that of the E211A single mutant but lower than that of wild-type TipF. Again, the half-life of this mutant was largely insensitive to the presence or absence of c-di-GMP, consistent with the persistence of E211A/F284L at the pole in the absence of c-di-GMP (see above). Thus, when c-di-GMP is depleted, TipF is unable to adopt an active conformation and is destabilized. These events can be uncoupled from c-di-GMP with a mutation of F284L, which not only activates the E211A mutant form constitutively, but, at the same time, partially protects it from proteolysis in vivo.

In the search for a candidate protease that destabilizes TipF in vivo, we tested strains with mutations in all ATP-dependent proteases and found that ClpX, the ATP-dependent chaperone component of the ClpXP protease, controls the cell cycle-dependent accumulation of endogenous (untagged) TipF expressed from its native chromosomal locus (Fig. 7C). Immunoblotting revealed TipF to be absent from G1-phase cells, induced during the G1 → S transition (40 min), and diminished at the time of division (Fig. 7D). Poisoning ClpXP activity by expression of a dominant-negative (catalytically inactive) ClpX variant (ClpX*) (Osteras et al. 1999) from P_{xyl} allows TipF accumulation in G1 cells (Fig. 7C), presumably because the normal turnover of TipF in G1 phase is blocked. Since a precipitous drop in c-di-GMP levels occurs in G1 cells (Paul et al. 2008; Christen et al. 2010) and TipF stability is clearly influenced by c-di-GMP abundance/binding, our results are consistent with the notion that the trough in c-di-GMP facilitates the turnover and thus the removal of TipF-dependent polarization during the cell cycle. In support of this view, fusion of GFP to the C terminus of TipF causes the aberrant accumulation of TipF in G1 phase (Fig. 7D) and allows TipF steady-state levels in unsynchronized populations to be maintained in the absence of c-di-GMP (Supplemental Fig. S6A,B). Similarly, comparison of the steady-state levels between untagged wild-type and TipF(E211A/F280S) expressed from
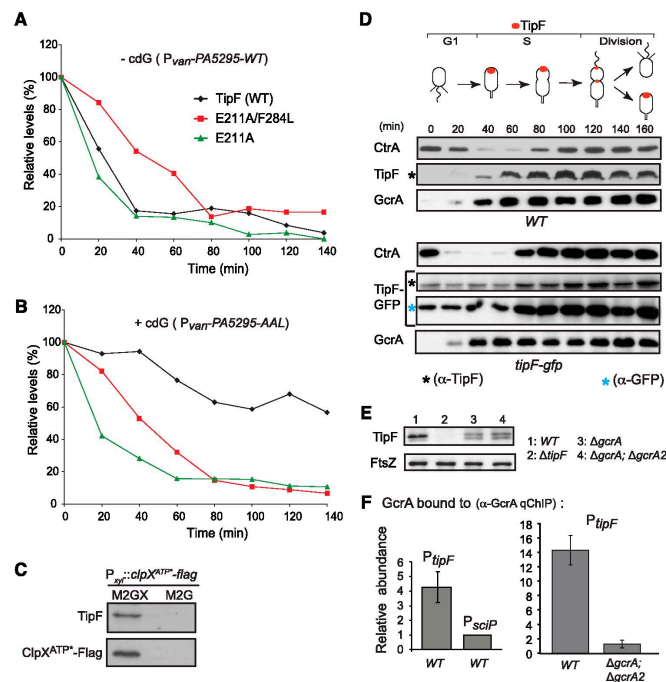
Mechanism of flagellum de- and repolarization

Figure 7. c-di-GMP levels affect TipF protein stability. (*A,B*) Depletion of c-di-GMP by overexpression of a potent PDE reduces TipF steady-state levels. TipF, the c-di-GMP-binding mutant E211A, or the intragenic suppressor mutant E211A/F284L was expressed from P_{xyl} in $\Delta tipF$ cells grown in M2G containing xylose following a shift to M2G containing vanillate to induce the expression of the PDE PA5295 from *P. aeruginosa* or its active site mutant, PA5295-AAL, and repress P_{xyl}. Samples were taken every 20 min, and protein levels were quantified from the immunoblots and plotted as percentages of the highest value. (*C*) The dominant-negative $clpX_{ATP}$ allele was expressed from plasmid in M2G containing xylose (M2GX) or repressed by growing the cells in M2G. Swarmer cells were isolated, and TipF was detected using a polyclonal anti-TipF antibody and ClpX^{ATP*}::Flag using monoclonal anti-M2 antibodies. In the presence of wild-type (WT) ClpXP, TipF was not detectable, while inactivation of ClpXP by the dominant-negative allele encoding ClpX^{ATP*} led to stabilization of TipF. (*D*) The cell cycle abundance of TipF resembles that of GcrA. Synchronized wild-type or *tipF-gfp* (in which endogenous *tipF* is replaced by *tipF-gfp*) swarmer cells were released in fresh medium, and CtrA, TipF, TipF-GFP, and/or GcrA steady-state levels were determined by immunoblotting using antibodies to TipF (black asterisk), GFP (blue asterisk), CtrA, or GcrA at different times during cell cycle progression. (*E*) TipF and FtsZ (control) steady-state levels in wild-type and mutant cells, as determined by immunoblotting using antibodies to TipF and FtsZ. (*F*) GcrA binds the *tipF* promoter, as determined by qChIP experiments using polyclonal antibodies to GcrA. The abundance of the *tipF* and *sciP* promoters was quantified in the immunoprecipitates (*left* panel) and *tipF* (*right* panel) in wild-type and $\Delta gcrA$; $\Delta gcrA2$ double-mutant cells.

the xylose-inducible P_{xyl} promoter on a low-copy plasmid revealed that the stabilized E211A/F280S variant is more abundant than wild-type TipF in G1-phase cells (Supplemental Fig. S6C). Together, these findings support the model that the fluctuation of c-di-GMP during the cell cycle (i.e., its concentration trough in G1 cells) helps restrict TipF abundance to S phase.

Additionally, the cell cycle abundance pattern of TipF is reinforced by timely synthesis, as is evident from the immunoblotting experiments in Figure 7D showing the increase of TipF during the cell cycle. Strikingly, the cell cycle pattern of TipF mirrors that of GcrA, a master transcriptional regulator that is required for the accumulation of a myriad of cell cycle-regulated transcripts, including *tipF*, at the G1 → S transition (Holtzendorff et al. 2004). Consistent with the hypothesis that GcrA directly activates transcription of the TipF-encoding gene at the G1 → S transition, we observed that GcrA is required for efficient TipF protein accumulation (Fig. 7E), and quantitative chromatin immunoprecipitation (qChIP)

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analysis using polyclonal antibodies to GcrA revealed that GcrA binds the *tipF* promoter, but not the *sciP* promoter, in vivo in a GcrA-dependent manner (Fig. 7F). The induction of TipF expression at the transcriptional level by GcrA and the concordant surge in c-di-GMP levels at the G1 → S transition ensure that TipF is active and ready to prime flagellum biosynthesis at the newborn pole, while a drop in c-di-GMP in the incipient G1 daughter cells results in the removal of TipF, thus resetting the flagellar polarity cascade for the next cell cycle.

Discussion

The establishment, amplification, and propagation of polarity cues during the cell cycle are hallmarks of all polarized cells, from bacterial to metazoan origin. Here we elucidate a mechanism that not only implements but also serves to subsequently reset a polarity pathway during the cell cycle in response to cyclic nucleotide signaling. While nucleotide-based signals direct critical events in the establishment and/or reinforcement of polarity cues in eukaryotes (Slaughter et al. 2009; Charest and Firtel 2010; St Johnston and Ahringer 2010; Amato et al. 2011; Ji and Tulin 2012), the underlying mechanisms are not well understood in bacterial systems. Using polar flagellum morphogenesis as a proxy to unravel how and when polarity cues are instated by cyclic nucleotide signaling during the *Caulobacter* cell cycle, we found that the presence of c-di-GMP activates and amplifies the TipF-dependent flagellar polarization pathway, while its removal promotes the elimination of TipF from G1 cells and provides a (possible checkpoint-like) mechanism, allowing the re-establishment of the flagellar polarization pathway to be coordinated with other developmental events at the G1 → S transition.

At least four regulatory factors (TipN, GcrA, ClpX, and c-di-GMP) impart spatiotemporal control on TipF through interwoven mechanisms acting at different levels. c-di-GMP adopts a central position in this regulatory web, implementing its effects by directly binding TipF (Fig. 2). The induction of TipF at the G1 → S transition is reinforced by two concurrent mechanisms: its synthesis triggered by the GcrA master regulator and its stabilization by c-di-GMP (Fig. 1A). Both mechanisms ensure that TipF accumulation is coordinated to other critical morphogenetic events that are activated at the same time. GcrA promotes the expression of genes encoding determinants for polarity (PodJ, controlling morphogenesis of pili), division (MipZ, a division inhibitor), and asymmetry (PleC, a histidine kinase/phosphatase) (Viollier et al. 2002; Holtzendorff et al. 2004; Thanbichler and Shapiro 2006). The concurrent accumulation of c-di-GMP protects TipF from ClpXP-dependent proteolysis and thus reinforces the surge of TipF while also promoting the degradation of CtrA at the G1 → S transition via the PopA effector and stalk biogenesis by an unknown mechanism (Paul et al. 2004, 2008; Duerig et al. 2009; Abel et al. 2011). Importantly, c-di-GMP binding is required not only to activate and polarize TipF, but also for the TipF-dependent recruitment of flagellar assembly factors and regulators such

as PflI, FliG, and FliM to the new pole. The underlying conformational rearrangements that likely occur when TipF binds c-di-GMP can be blocked by mutational destruction of the c-di-GMP-binding site or when c-di-GMP is depleted from cells. While these manipulations abrogate TipF polarization and function, we isolated compensatory missense mutations in the c-di-GMP-binding domain that reverse these effects and also allow TipF localization under conditions of c-di-GMP depletion. The same motility suppressor screen was answered by triplet codon duplications in a predicted coiled-coil-rich region that precedes the c-di-GMP-binding domain (Fig. 1B). Helical wheel analysis of these mutant proteins predicts that the α-helicity is maintained by the duplication while causing a shift of the hydrophobic region that could cause structural rearrangements. This rearrangement likely also underlies the interaction of TipF•c-di-GMP with TipN. During the late stages of constriction of the preceding cell cycle, the TipN landmark signal is deposited at the newborn pole to ensure correct interpretation of the polarity axis in the progeny (Huitema et al. 2006; Lam et al. 2006). Because of this spatial cue, the subsequent events triggered by c-di-GMP at the G1 → S transition are executed at the correct subcellular site (i.e., the newborn pole) (Fig. 1A). In support of this, our pull-down experiments revealed TipN to be part of this flagellar organizational complex that includes TipF, Pfl, and at least one flagellar switch component (Fig. 2A).

The flagellar switch complex is best known as a target to regulate flagellar rotation but fulfills a fundamental role in flagellar assembly (Irikura et al. 1993; Chevance and Hughes 2008; Davis and Viollier 2011). Indeed the FliG switch protein interacts with the FliF MS ring protein and the MotA stator protein along with other switch proteins. This central position of FliG in flagellar assembly and function has been exploited to regulate motility in response to specific developmental states or nutritional and systemic cues (Ryjenkov et al. 2006; Blair et al. 2008; Paul et al. 2011; Zarbiv et al. 2012). Despite the dual role of FliG, no mechanism has been described to regulate flagellar assembly through FliG. TipF is the first representative of this class, as the soluble portion of TipF (encompassing the predicted coiled-coil and EAL domains) interacts directly with the C-terminal portion (FliG$_C$). The role of c-di-GMP in flagellar assembly likely goes beyond the TipF–FliG interaction, as indicated by the existence of an apparent TipF-independent, but c-di-GMP-dependent, flagellar assembly step (Fig. 6E,F).

The FlhF GTPase of *vibrios*, *pseudomonads*, and *Campylobacter jejuni* may function akin to the TipF and c-di-GMP-dependent flagellar polarization pathway (Pandza et al. 2000; Salvetti et al. 2007; Balaban et al. 2009; Kusumoto et al. 2009). FlhF is polarized and recruits the MS ring protein FliF to the polar site of flagellation in *Vibrio. cholerae* (Green et al. 2009). However, despite the pervasive polar flagellation found across bacterial lineages, FlhF polarization, activation, and function are poorly understood. Interestingly, the *Myxococcus xanthus* Ras-like GTPases MglA and SofG are used to direct motility pro-

teins required for flagellum-independent (gliding) motility to the pole (Leonardy et al. 2010; Zhang et al. 2010; Bulyha et al. 2013). Moreover, a member of the ParA/MinD family of ATPases positions the chemosensory apparatus at the pole in *V. cholera* (Ringgaard et al. 2011; Yamaichi et al. 2012), and a related protein ensures the medial placement of the chemosensory apparatus in *Rhodobacter sphaeroides* (Thompson et al. 2006), but the temporal relationships with the cell cycle are unexplored in these systems.

c-di-GMP-mediated spatiotemporal control of polar morphogenesis extends far beyond the α-proteobacterial lineage. In the γ-proteobacterium *P. aeruginosa*, an imbalance in c-di-GMP abundance is observed in the two progeny compartments of a dividing cell, with the trough in c-di-GMP levels occurring in the daughter compartment bearing the polar flagellum (Christen et al. 2010). FimX, an EAL-GGDEF domain hybrid protein from *P. aeruginosa*, is polarly localized and promotes the assembly of retracting type IV (polar) pili (Tfp) for twitching motility. Importantly, FimX influences the positioning of Tfp and uses an EAL domain to bind c-di-GMP and induce (long-range) conformational change in the adjacent domain (Huang et al. 2003). However, it is unknown how FimX scouts the pole, how it signals Tfp assembly, and how its localization is coordinated with cell cycle progression. We show that spatiotemporal cues from pre-existing spatial landmarks, the c-di-GMP second messenger, and the cell cycle transcriptional program are integrated at TipF to orchestrate the periodic removal and re-establishment of a polarity pathway controlling morphogenesis during the cell cycle. Thus, small nucleotide-induced polarization pulses that drive morphogenesis at key times in the cell cycle appear to have been invented more than once during evolution from prokaryotic to metazoan cells and may thus even function akin in eukaryotic organelles.

Materials and methods

Coimmunoprecipitation

Cells (50 mL) were grown to mid-log phase in the presence of 50 mM vanillate inducer, harvested, washed three times in buffer I (50 mM sodium phosphate at pH 7.4, 50 mM NaCl, 1 mM EDTA), and resuspended in buffer II (50 mM sodium phosphate at pH 7.4, 50 mM NaCl, 0.5% n-dodecyl-β-D-maltoside, two protease inhibitor tablets [Complete EDTA-free, Roche]) containing 1× Ready-Lyse lysozyme solution (Epicentre), 2 mM MgCl$_2$, 1 mM EDTA, and 30 U of DNase I (Roche). The solution was incubated for 20 min at room temperature and subsequently centrifuged at 10,000g for 3 min at 4°C to remove cellular debris. The supernatant was precleared with 50 µL of Proteinase-G agarose beads (Roche). To the precleared solution, mouse monoclonal anti-GFP antibodies (1:300 dilution; Living Colors A.V. monoclonal antibody [JL-8], Clontech,) was added and incubated for 90 min at 4°C. The antibody–protein complexes were trapped with Proteinase-G agarose beads washed once with buffer I containing 0.5% n-dodecyl-β-D-maltoside, twice with 1% immunoprecipitation buffer (Protein G immunoprecipitation kit, Sigma-Aldrich), and once with 0.1% immunoprecipitation buffer to remove salts and finally resuspended in 70 µL of 1× Laemmli sample buffer. After boiling and centrifugation, precipitated pro-

teins were identified by immunoblotting using polyclonal antibodies to PflI or CtrA.

TAP

The TAP procedure was based on that described by Rigaut et al. (1999). Briefly, cells (1 L) were grown to mid-log phase in the presence of 50 mM vanillate for 3 h and harvested by centrifugation at 6000g for 10 min. The cells were then washed in buffer I (50 mM sodium phosphate at pH 7.4, 50 mM NaCl, 1 mM EDTA) and lysed for 15 min at room temperature in 500 µL of buffer II (buffer I + 10% n-dodecyl-β-D-maltoside, two protease inhibitor tablets [Complete EDTA-free, Roche], 1× Ready-Lyse lysozyme [Epicentre], 500 U of DNase I [Roche], 2 mM MgCl$_2$, 1 mM EDTA). The volume of the solution was brought up to 10 mL with buffer I and incubated for 15 min at room temperature. Cellular debris was removed by centrifugation at 7000g for 15 min at 4°C, and the salt concentration of the supernatant was adjusted by adding 10 µL of 1 M Tris per milliliter sample, 20 µL of 5 M NaCl per milliliter sample, and 25 µL of 10% n-dodecyl-β-D-maltoside per milliliter sample. The supernatant was incubated for 2 h at 4°C with IgG Sepharose beads (GE Healthcare Biosciences) that had been washed once with IPP150 buffer (10 mM Tris-HCl at pH 8, 150 mM NaCl, 0.25% n-dodecyl-β-D-maltoside). After incubation, the beads were washed at 4°C three times with IPP150 buffer and once with TEV cleavage buffer (10 mM Tris-HCl at pH 8, 150 mM NaCl, 0.25% n-dodecyl-β-D-maltoside, 0.5 mM EDTA, 1 mM DTT). The beads were then incubated overnight at 4°C with 1 mL of TEV solution (TEV cleavage buffer with 100 U of TEV protease per milliliter [Promega]) to release the tagged complex. CaCl$_2$ (3 µM) was added to the solution and incubated for 1 h at 4°C with calmodulin beads (GE Healthcare Biosciences) that had been washed once with calmodulin-binding buffer (10 mM β-mercaptoethanol, 10 mM Tris-HCl at pH 8, 150 mM NaCl, 1 mM magnesium acetate, 1 mM imidazole, 2 mM CaCl$_2$, 0.25% n-dodecyl-β-D-maltoside). After incubation, the beads were washed three times with calmodulin-binding buffer and eluted with IPP150 calmodulin elution buffer (calmodulin-binding buffer substituted with 2 mM EGTA instead of CaCl$_2$). The eluate was then concentrated with Biomax centrifugal filter tubes (Millipore).

Protein overexpression and purification for PDE assay and ITC analyses

E. coli BL21 cells carrying the respective wild-type and mutant TipF overexpression plasmids were grown in LB medium, and expression was induced by adding isopropyl 1-thio-β-d-galactopyranoside at A600 = 0.4 to a final concentration of 1 µM. Cells were centrifuged and resuspended in buffer containing 20 mM Tris-HCl (pH 7), 250 mM NaCl, 5 mM imidazole, 10 mM MgCl$_2$, 1% glycerol, and Complete minicocktail of EDTA-free protease inhibitors at the concentrations specified by the manufacturer (Roche). Cells were lysed by passage through a French pressure cell, and the suspension was clarified by centrifugation at 15,000g for 40 min. The supernatant was incubated with pre-equilibrated Profinity IMAC Ni-resin (Bio-Rad) for 1 h at 4°C. The resins were washed, and the proteins were eluted with 350 mM imidazole. The eluate was loaded on a HiLoad 16/60 Superdex 75 prep-grade gel filtration column (GE Healthcare; FPLC system: AEKTA Purifier, Amersham Biosciences) for the next purification step using 50 mM Tris-HCl (pH 8) and 50 mM NaCl buffer. The monomer fractions were collected and concentrated (Amicon Ultra 10,000-MW centrifugal filters, Millipore) for further assays. The protein concentrations were determined using the Bradford assay (Bio-Rad).

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PDE assay

Wild-type and mutant TipF were purified as His6-tagged variants using standard conditions (see the Supplemental Material) and tested for c-di-GMP PDE activity. As a control, PDE class A YahA from *E. coli* was used. For each protein, a 10 μM final concentration was incubated with 100 μM c-di-GMP (synthesized enzymatically and purified via FPLC reverse-phase chromatography) in 50 mM Tris-HCl (pH 8), 50 mM NaCl, 5mM MgCl$_2$, and 1 mM DTT at room temperature. Samples were taken at time points 0 h, 1 h, 2 h, and overnight. The reactions were stopped by heating for 5 min at 99°C, diluted with 5 mM NH$_4$HCO$_3$ (pH 8), filtered (0.22 μM), and analyzed on a Resource Q ion-exchange chromatography column (GE Healthcare).

ITC

The interaction of TipF with c-di-GMP was measured with a VP-ITC isothermal titration calorimeter from MicroCal, with TipF (32.5 μM) in the calorimeter cell and c-di-GMP (118 μM) in the injection syringe (buffer: 50 mM Tris/HCl, 50 mM NaCl at pH 8 at 25°C). All solutions were degassed and equilibrated at the desired temperature (25°C or 35°C) before using. The delay between the injections was set to 5 min to ensure re-equilibration between injections. Data were evaluated using Origin software (OriginLab).

Cryoelectron microscopy sample preparation

C. crescentus grown in PYE medium were flash-frozen (plunge-frozen) onto glow-discharged, 200 mesh, Quantifoil copper grids (Quantifoil) in liquid ethane using a Vitrobot Mark II system (FEI). BSA-treated colloidal gold (10 nm) was applied to the grids and air-dried before sample application and plunge-freezing.

Cryoelectron microscopy data collection

Cryoelectron tomography data collection was performed with an FEI Tecnai G^2 F30 at the University of Minnesota Characterization Facility. Images were acquired with a Gatan 4 k × 4 k Ultrascan charged-couple device (CCD camera) with a pixel size of 0.372 nm (at 31,000×) or 0.495 nm (at 23,000×) on the specimen. A total electron dose of 150 electrons per squared angstrom (e-/Å^2) was used to collect a full-tilt series ranging from −65° to +65° (131 images). Data were collected at a defocus of 8.0 μm under focus (first CTF 0: 1/4 nm^{-1}). Images were acquired automatically with 1° tilt steps using the predictive University of California at San Francisco tomography package (Zheng et al. 2007).

Cryoelectron image processing and analysis

Tomographic reconstructions were generated using IMOD (Kremer et al. 1996). The Amira visualization software package (Visage Imaging Systems, Inc.) was used for volume rendering and material labeling (segmentation). A visualization and segmentation toolbox (Pruggnaller et al. 2008) developed specifically for 3D electron microscopy data analysis in Amira was used to achieve a more objective segmentation of the data by avoiding manual contouring (entirely subjective tracing of edges or features). After contrast inversion, slices were filtered using a 3D Gaussian smoothing with a kernel size of 3 × 3 × 3. Subsequently, the IslandLabel module was applied to binarize the data with a threshold and tag connected regions of the binary image with a label.

Construction of strains and plasmids, immunoblotting, yeast two-hybrid analyses, and fluorescence microscopy

Descriptions of the construction of strains and plasmids, immunoblotting, yeast two-hybrid analyses, and fluorescence microscopy can be found in the Supplemental Material.

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De- and repolarization mechanism of flagellar morphogenesis during a bacterial cell cycle

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In the above-mentioned article, Nicole J. Davis and Yaniv Cohen contributed equally. The authors acknowledge there should not be a second notation of equal contribution between Yaniv Cohen and Stefano Sanselicio.

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To my family

5.3   Curriculum Vitae

Curriculum Vitae

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