Coupling prokaryotic cell fate and division control with a bifunctional and oscillating oxidoreductase homolog

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Abstract

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Coupling Prokaryotic Cell Fate and Division Control with a Bifunctional and Oscillating Oxidoreductase Homolog

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SUMMARY

NAD(H)-binding proteins play important roles in cell-cycle and developmental signaling in eukaryotes. We identified a bifunctional NAD(H)-binding regulator (KidO) that integrates cell-fate signaling with cytokinesis in the bacterium Caulobacter crescentus. KidO stimulates the DivJ kinase and directly acts on the cytoticin tubulin, FtsZ, to tune cytokinesis with the cell cycle. At the G1→S transition, DivJ concomitantly signals the ClpXP-dependent degradation of KidO and CtrA, a cell-cycle transcriptional regulator/DNA replication inhibitor. This proteolytic event directs KidO and CtrA into oscillatory cell-cycle abundance patterns that coordinately license replication and cytokinesis. KidO resembles NAD(P)H-dependent oxidoreductases, and conserved residues in the KidO NAD(H)-binding pocket are critical for regulation of FtsZ, but not for DivJ. Since NADPH-dependent regulation by a KidO-like oxidoreductase also occurs in humans, organisms from two domains of life exploit the enzymatic fold of an ancestral oxidoreductase potentially to coordinate cellular or developmental activities with the availability of the metabolic currency, NAD(P)H.

INTRODUCTION

Cells that divide asymmetrically possess intricate regulatory mechanisms to tie the implementation of progeny-specific developmental programs with cytokinesis. In bacteria, the essential FtsZ tubulin is typically the cytokinetic target of regulation (Margolin, 2005). The earliest and defining event of cytokinesis is the organization of FtsZ into circumferential arcs that girdle the division site with a ring- or band-like appearance (Z-ring) and that provide a scaffold on which the other components of the division apparatus assemble.

Asymmetric division in Caulobacter yields a stalked (S phase) cell that replicates its genome and assembles a medial Z-ring (Figure 1A), and a swarmer (G1 phase) cell that is replication and division incompetent (Goley et al., 2007; Skerker and Laub, 2004). The stalked cell is characterized by a cylindrical extension of the cell envelope (the stalk) at the old cell pole. By contrast, the swarmer cell features a flagellum and several adhesive pili at the old pole. While the stalked cell is terminally differentiated, the swarmer cell retains developmental potency and is destined to morph into a stalked cell. During this swarmer-to-stalked cell transition (also known as the G1→S transition), the flagellum is shed, pili are retracted, and a stalk is elaborated from the vacated pole while replication competence is acquired.

A complex phosphor-signaling cascade determines which of the two developmental fates is implemented (Figure 1B) (Biondi et al., 2006; Paul et al., 2008; Quon et al., 1996; Wu et al., 1998). This cascade ultimately targets the activity and abundance of CtrA, a DNA-binding response regulator that, in response to phosphorylation, directly regulates cell-cycle transcription and also functions as an inhibitor of DNA replication (Laub et al., 2000; Quon et al., 1996, 1998). In the G1-cell, phosphorylated CtrA (CtrA→P) accumulates to high levels to directly repress the origin of replication (ori) while also activating transcription of developmental promoters, such as that of the pilA gene, which encodes the structural subunit of the pilus filament (Skerker and Shapiro, 2000). Coincident with the G1→S transition, phosphorylation of CtrA subsides and a proteolytic pathway to degrade CtrA is activated. As a result, ori is derepressed and transcription of many genes that are active in G1 is disabled (Figure 1B).

CtrA→P levels are modulated by the DivK cell fate determinant, a single-domain response regulator that is itself regulated by phosphorylation (Hecht et al., 1995). Phosphorylated DivK (DivK→P) interrupts the phosphate flux to CtrA, and stimulates the ClpXP-dependent proteolytic pathway that degrades CtrA (see Figure 3A) (Biondi et al., 2006; Chien et al., 2007; Duerig et al., 2009; Iniesta et al., 2006; Iniesta and Shapiro, 2008; McGrath et al., 2006). The DivJ kinase catalyzes the phospho-transfer to DivK, and the PleC phosphatase reverses this phosphorylation event by removing the phosphate group from DivK→P (Hecht et al., 1995; Matroule et al., 2004; Wheeler and Shapiro, 1999). Thus, DivJ and PleC antagonistically influence the ratio of phosphorylated versus dephosphorylated DivK (DivK→P/DivK) in the cell. DivK→P also feeds back to directly modulate the activity of DivJ and PleC (Paul et al., 2008).

In the G1 cell, active PleC is localized to the flagellated pole to maintain a low DivK→P/DivK ratio. At the G1→S transition, PleC is dispersed and substituted for active DivJ at the pole where the stalk elaborates. This phosphatase-to-kinase substitution...
results in a surge of DivK~P/DivK, which signals the removal of CtrA~P, thus liberating ori and allowing S phase entry. Polar DivJ is retained at the stalked pole henceforth, while, in mid-S phase, PleC is again deployed to the nascent flagellated pole, directly or indirectly, by the PodJ localization factor (Hinz et al., 2003; Viollier et al., 2002).

The SpmX muramidase homolog was recently identified as the elusive localization factor for the DivJ kinase (Radhakrishnan et al., 2008). SpmX localizes to the nascent stalked pole ahead of DivJ, and positively regulates DivJ activity. Although ΔpleC cells do not express SpmX, they exhibit high DivK~P/DivK (Radhakrishnan et al., 2008), suggesting that DivJ activity can also be supported by a SpmX-independent pathway. Here, we describe a genetic screen to identify regulators of this alternate pathway. We uncovered kinase and division-regulating oxidoreductase homolog (KidO), a previously uncharacterized bifunctional oxidoreductase homolog that integrates cell fate and cytokinesis through regulation of DivJ and FtsZ (Figures 1A and 1B).

KidO binds the nicotinamide adenine dinucleotide (NAD+) cofactor, NAD(H), and an intact NAD-binding pocket is important for the regulation of FtsZ, but not for DivJ. Thus, the Caulobacter cell-division cycle is regulated by an NAD(H)-binding protein, potentially allowing for the integration of a metabolic cue into the cell-cycle regulatory circuitry.

**RESULTS**

**An Oxidoreductase Homolog Implicated in Regulation of a Cell Fate Pathway**

To confirm that DivJ is indeed active in the absence of PleC, we measured the relative intracellular level of DivJ auto-phosphorylation (DivJ~P/DivJ), a direct metric for kinase activity, by in vivo phosphorylation analysis in wild-type (WT) and ΔpleC cells. As DivJ~P/DivJ is comparable in both strains (see Figure 2B), we conclude that ΔpleC cells have near-WT DivJ activity, despite the absence of SpmX. To identify an SpmX-independent activator of DivJ, we genetically screened for ΔpleC mutants with reduced DivJ activity (see Figures S1A–S1D available online) with a pilA-nptII transcriptional reporter. In ΔpleC cells, DivJ activity interferes with CtrA~P accumulation and, hence, pilA transcription (Figures 1B and 2A). We thus screened for mini-Tn5 insertions that restore pilA transcription to ΔpleC cells,
an ancillary subunit and regulator of voltage-gated K\textsuperscript{+} ion channels in metazoans that has NAD(P)H-dependent oxidoreductase activity, and the structure of which has been solved (Gulbis et al., 1999; Liu et al., 2001; Tipparaju et al., 2008). While a conserved tyrosine residue is responsible for the catalytic activity in Kvbeta and other oxidoreductases, CC3576 features a phenylalanine residue at this position (Figure 1D, asterisk). An analogous Phe—Tyr mutation in Kvbeta impedes catalysis, but does not affect cofactor binding (Gulbis et al., 1999; Liu et al., 2001; Tipparaju et al., 2008). On these grounds, we hypothesize that CC3576 (henceforth referred to as KidO) binds NAD(P)H without or with little catalytic activity. Biochemical experiments revealed that radiolabeled (\textsuperscript{32}P)-NAD\textsuperscript{+} can be UV cross-linked to TAP-tagged KidO (KidO-TAP) that was purified from Caulobacter (Figure 1E), and competition experiments indicate that NADH is preferred over NADPH as cofactor (Figure 1F). Thus, the predicted NAD\textsuperscript{+}-binding pocket in KidO is indeed functional.

KidO Is Required for Optimal DivJ Kinase Activity

To explore if KidO regulates DivJ activity, we measured DivJ\textsuperscript{−}/P and DivJ\textsuperscript{−}/DivK in WT and ΔpleC strains as well as mutant derivatives harboring an in-frame deletion in the kidO gene (ΔkidO [Figure 1C]). The ΔkidO deletion recapitulates the effects of the kidO::Tn5 mutation, and is not polar on downstream genes, as revealed by complementation experiments (Figure 2C). In vivo phosphorylation analysis showed that KidO is required for efficient DivJ activity, as loss of KidO results in a significant decrease in DivJ autophosphorylation and diminished accumulation of DivJ\textsuperscript{−}/P in WT and ΔpleC cells (Figure 2B). Moreover, DivJ autophosphorylation is elevated when KidO is overexpressed in WT cells from a low-copy plasmid with the xylose-inducible promoter (pP\textsubscript{xy}:KidO [Figure S2A]). Impaired DivJ signaling is predicted to result in elevated CtrA\textsuperscript{−}/P levels (Figure 1B), and we determined that CtrA\textsuperscript{−}/P levels are indeed elevated in the absence of KidO in WT and PleC cells (Figure 2B). The corollary, that this increase in CtrA\textsuperscript{−}/P should restore pilA expression and pilation to PleC\textsuperscript{−} cells, was confirmed by transcriptional analysis, immunoblotting (Figures 2A and 2C), and transmission electron microscopy (Figures S2B–S2C).

Immunoblotting also revealed that SpmX levels are elevated in KidO\textsuperscript{−} cells (Figure S1D and data not shown), raising the possibility that extra SpmX can dampen the effect on DivJ activity caused by the loss of KidO. To test this idea, we sought to create a KidO\textsuperscript{−} SpmX\textsuperscript{−} double mutant. While repeated attempts to generate a ΔkidO ΔspmX double mutant by the standard sucrose-induced counter-selection method (see Experimental Procedures) were unsuccessful, we generated KidO\textsuperscript{−} SpmX\textsuperscript{−} strains by two alternative procedures. First, we transduced the kidO::Tn5 allele into ΔspmX cells, thus bypassing the standard counter-selection step that might compromise growth of the double mutant. The resulting ΔspmX kidO::Tn5 cells are severely elongated (data not shown), indicating that the combined absence of KidO and SpmX results in a severe cell-cycle and/ or division defect (see Figure 2D). Second, prompted by the evidence that the DivJ\textsuperscript{−} phenotype is partially mitigated when PleC is inactivated (Wheeler and Shapiro, 1999), we engineered a ΔspmX ΔkidO ΔpleC mutant strain by the counter-selection method. The triple mutant exhibits a mild cell filamentation since such mutations should reduce the activity of the DivJ signaling pathway. A total of 21 Tn5 mutants with insertions in divJ, in divL, or in the uncharacterized CC3576 gene were isolated (Figure S1B). While Dict and DivL are both known to influence CtrA\textsuperscript{−}/P and DivK (Pierce et al., 2006; Reisinger et al., 2007), CC3576 has not previously been implicated in regulation of the DivJ pathway. CC3576 is predicted to encode a 324 residue protein (34.5 kDa; isoelectric point at pH 10.4 [Figure 1C]) resembling (26% identity, 36% similarity) members of the TAS family of NAD(P)H-dependent oxidoreductases (pfam00248/COG0667 [Figure 1D]). This family includes Kvbeta,
In vivo occupancy of CtrA and a control protein CpaE (a pilus structural protein not known to bind DNA) at the were monitored over time in 20 min intervals by immunoblotting. Y denotes a cross-reacting protein.

WT SW cells were isolated and suspended in M2G supplemented with novobiocin (100 μg/ml) to block DNA replication. KidO and CtrA steady-state levels were monitored over time in 20 min intervals by immunoblotting. Y denotes a cross-reacting protein.

KidO and CtrA Are Interverwoven into the Cell-Cycle Circuitry

To determine if KidO is cell-cycle regulated, we monitored KidO abundance in synchronized cells by immunoblotting with polyclonal antibodies to KidO. As shown in Figure 3B, KidO oscillates over the cell-cycle with a remarkably similar periodicity to that of the CtrA replication inhibitor (Domian et al., 1997). KidO is present in G1 phase, absent during S phase, and reaccumulates at the time of division. The levels of WT or mutant KidO (D82A, see below) still cycle when their synthesis is driven by the transcriptional and translational signals from the regulatory proteolytic removal at the G1/S transition by the pathy of KidO and CtrA during the cell cycle of WT, ΔcdCA, ΔcdpR, popA::himar1, divKCS (cold-sensitive allele of divK), and ΔkidO cells harboring either a Pxyl-kidO (KidO) or Pxyl-kidO-DD (ΔkidO) plasmid integrated at the xy1X locus. In addition, a WT strain harboring the Pxyl-kidO-DD plasmid integrated at the xy1X locus was enriched for a suppressor (sup) mutation that allowed synchronization, because KidO-DD (DD in red, with C-terminal Asp-Asp residues in lieu of Val-Ala) -expressing cells are elongated (see Figure 4A) and cannot be synchronized. The cell-cycle abundance of untagged KidO (WT in red) and KidO-DD was subsequently determined in the sup mutant. All strains were grown in M2G at 30 °C, except divKCS cells that were grown at 32 °C (permissive) or 25 °C (nonpermissive).

The left panel shows immunoblots of KidO and CtrA steady-state levels in WT cells carrying pMO88, a low-copy plasmid encoding dominant-negative ClpX under the control, 2 hr after induction (0.3% xylose, X) or without induction (0.2% glucose, G). The right panel shows immunoblots of KidO levels in WT cells expressing WT KidO (pPxyl-kidO) or KidO-DD (pPxyl-kidO-DD) from a plasmid integrated at the xy1X locus. A strain harboring the empty vector (pXGFP4) integrated at xy1X served as control. Y denotes a cross-reacting protein.

KidO steady-state levels in WT cells carrying pMO88, a low-copy plasmid encoding dominant-negative ClpX under the control, 2 hr after induction (0.3% xylose, X) or without induction (0.2% glucose, G). The right panel shows immunoblots of KidO levels in WT cells expressing WT KidO (pPxyl-kidO) or KidO-DD (pPxyl-kidO-DD) from a plasmid integrated at the xy1X locus. A strain harboring the empty vector (pXGFP4) integrated at xy1X served as control. Y denotes a cross-reacting protein.

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Figure 3. Cell-Cycle Fluctuation of KidO and CtrA Caused by the Same Proteolytic Pathway that Is Engaged at the G1 → S Transition

(A) Schematic of the components for the proteolytic pathway (depicted in green). Fine dashed lines denote direct transcriptional regulation; coarse dashed lines point to indirect relationships.

(B) Immunoblotting to determine the relative abundance of KidO and CtrA during the cell cycle of WT, ΔcdCA, ΔcdpR, popA::himar1, divKCS (cold-sensitive allele of divK), and ΔkidO cells harboring either a Pxyl-kidO or Pxyl-kidO-DD plasmid integrated at the xy1X locus. In addition, a WT strain harboring the Pxyl-kidO-DD plasmid integrated at the xy1X locus was enriched for a suppressor (sup) mutation that allowed synchronization, because KidO-DD (DD in red, with C-terminal Asp-Asp residues in lieu of Val-Ala) -expressing cells are elongated (see Figure 4A) and cannot be synchronized. The cell-cycle abundance of untagged KidO (WT in red) and KidO-DD was subsequently determined in the sup mutant. All strains were grown in M2G at 30 °C, except divKCS cells that were grown at 32 °C (permissive) or 25 °C (nonpermissive).

(C) The left panel shows immunoblots of KidO and CtrA steady-state levels in WT cells carrying pMO88, a low-copy plasmid encoding dominant-negative ClpX under the control, 2 hr after induction (0.3% xylose, X) or without induction (0.2% glucose, G). The right panel shows immunoblots of KidO levels in WT cells expressing WT KidO (pPxyl-kidO) or KidO-DD (pPxyl-kidO-DD) from a plasmid integrated at the xy1X locus. A strain harboring the empty vector (pXGFP4) integrated at xy1X served as control. Y denotes a cross-reacting protein.

(D) Immunoblotting showing the difference in stability of KidO (WT in red), KidO-DD (DD in red), and CtrA. Expression of KidO or KidO-DD in ΔkidO cells harboring a Pxyl-kidO-DD plasmid integrated at the xy1X locus was induced by the addition of xylose (0.3%) before translation was inhibited by the addition of chloramphenicol (2 μg/ml). The abundance of KidO, KidO-DD, and CtrA was monitored over time. Black and white arrowheads point to proteins that cross-react with the anti-KidO antibodies. In the bottom panel, note the production of a faster-migrating KidO-DD derivative expressed from an internal start codon.
KidO and CtrA proteolysis is coregulated, and KidO clearly indicates that their proteolytic removal is temporally coordinated with, but not contingent on, DNA replication (Figure 3E).

Because KidO also features two hydrophobic residues (Val-Ala) at the same location, we explored whether KidO degradation occurs through the same proteolytic pathway that degrades CtrA. Four lines of evidence support this view. First, when CipXP protease activity is crippled by overexpression of a dominant-negative version of CipX (CipX*), the abundance of KidO and CtrA increases (Figure 3C). Second, a KidO variant (KidO-DD) harboring purified FtsZ and BSA (each 6.4 μg) was incubated with purified KidO-TAP (25 nM) and finally probed with a polyclonal antiserum to KidO. Note that FtsZ and BSA comigrate on 10% SDS-PAGE. (E) SPR experiments to measure the K_i of recombinant FtsZ for surface-immobilized KidO-TAP. FtsZ was injected into the flow chamber at concentrations [FtsZ] ranging from 0.5 to 10.0 μM.

KidO and CtrA proteolysis is coregulated, and KidO clearly influences the activity of CtrA. Remarkably, quantitative chromatin immunoprecipitation (qChIP) assays disclosed an additional layer by which KidO and CtrA are interwoven into the underlying cell-cycle circuitry. The KidO promoter region features a CtrA-binding consensus motif (McGrath et al., 2007), and CtrA interacts efficiently and specifically with this region in vivo, as revealed by qChIP experiments (Figure 3F), suggesting that CtrA regulates kidO transcription. The reaccumulation of CtrA in late-predivisional cells precedes that of KidO (Figure 3B), suggesting that CtrA positively regulates KidO expression.

Cell Division Control by KidO
Several results suggest that KidO has an additional function in regulating the cell-division cycle independently of its effect on DivJ. First, as outlined above, the division defect of KidO^-SpmX^- cells raised the possibility that KidO regulates division. Second, ΔkidO cells expressing stable KidO-DD are markedly elongated (Figure 4A), indicating that the failure to degrade

CipXP protease (Chien et al., 2007; Domian et al., 1997; Jenal and Fuchs, 1998). The two penultimate hydrophobic residues (Ala-Ala) at the C terminus, and the regulatory factors, DivK, CpdR, RcdA, and PopA (Figure 3A), are required for efficient proteolysis of CtrA in vivo (Biondi et al., 2006; Domian et al., 1997; Duerig et al., 2009; Iniesta et al., 2006; McGrath et al., 2006). Because KidO also features two hydrophobic residues (Val-Ala) at the same location, we explored whether KidO degradation occurs through the same proteolytic pathway that degrades CtrA. Four lines of evidence support this view. First, when CipXP protease activity is crippled by overexpression of a dominant-negative version of CipX (CipX*), the abundance of KidO and CtrA increases (Figure 3C). Second, a KidO variant (KidO-DD) harboring Asp-Asp in lieu of Val-Ala at the extreme C terminus: (1) does not oscillate during the cell cycle (Figure 3B); (2) accumulates to higher steady-state levels (Figure 3C); and (3) exhibits dramatically increased stability in vivo compared with WT KidO (Figure 3D). The comparable mutation in CtrA—CtrA-DD—also abrogates cell-cycle proteolysis and oscillation (Domian et al., 1997). Third, like CtrA, KidO is stabilized at the G1→S transition in cells that are mutant for
KidO at the G1→S transition perturbs division. Third, overexpression of KidO from pP$_{xyr}$kidO (a low-copy number plasmid) in WT cells disrupts division (Figure 4B) and is lethal, manifested by a ~30-fold reduction in plating efficiency (data not shown).

**FtsZ is the Direct Target of KidO**

To identify the cyto-kinetic target of KidO, a combination of cytological, biochemical, and genetic approaches were used. Collectively, four lines of evidence show that KidO acts directly on the FtsZ tubulin.

First, live-cell fluorescence microscopy revealed that KidO overexpression disperses Z-rings. Overexpression of KidO from pP$_{xyr}$kidO in cells expressing FtsZ-mCherry and native FtsZ (from the vanillate-inducible vanA locus and the ftsZ locus, respectively) lowered the percentage of cells with medial Z-rings (Figure 4B). At the time of induction, 78% (115/146) of cells had Z-rings, while 60 and 120 min later, Z-rings were only observed in 57% (99/175) and 22% (55/255) of cells, respectively. Thus, KidO interferes with the formation and/or the maintenance of the medial Z-ring. Time-lapse analysis revealed that KidO overexpression disrupts the formation of de novo Z-rings (Figure 4C) and disperses pre-existing Z-rings (Figure 4B). Control experiments showed that this effect of KidO on Z-rings is not due to an inhibition of DNA replication initiation or a consequence of FtsZ degradation that occurs in the wake of Z-ring disassembly (Figures S4C–S4D).

Second, numerous biochemical experiments show that KidO interacts directly with FtsZ. KidO-TAP expressed from a low-copy plasmid in a kidO strain was pulled down by the TAP procedure (Puig et al., 2001) and probed for the presence of FtsZ by immunoblotting with polyclonal antibodies to FtsZ. A signal presumed to correspond to FtsZ was detectable in the KidO-TAP purification, but not in a mock-purified control sample from kidO cells with the empty vector (Figure 4D). To confirm that this signal stemmed from FtsZ rather than a protein reacting nonspecifically with the anti-FtsZ polyclonal antiserum, we analyzed the corresponding silver-stained gel slices from both purifications by mass spectrometry (LC/MS/MS). Two peptides containing residues 10–16 (‘TTTEKPR’) and residues 460–474 (‘QRYEQQASAPQAQR’) of FtsZ were detected only in the KidO-TAP sample. Neither DivJ nor DivK or CtrA were detected in the pulldown samples, either by immunoblotting or by mass spectrometry (data not shown). To determine if the interaction between KidO and FtsZ is direct, we first conducted far-Western analysis with purified proteins (Figure 4E). We found that KidO-TAP binds to FtsZ blotted onto a membrane, but not to a control protein (BSA).

Next, we confirmed this direct interaction by surface plasmon resonance (SPR) and determined the apparent dissociation constant (K$_D$) of FtsZ for surface-immobilized KidO-TAP. The K$_D$ value (2.3 ± 0.61 μM) derived from the concentration-dependent response curves shown in Figure 4F is indicative of a high affinity of FtsZ for KidO-TAP in vitro. We conclude that KidO acts directly on FtsZ, but that DivJ is likely influenced through an unknown intermediary.

Third, suppressor analyses demonstrated that FtsZ and KidO interact genetically. In an unbiased approach to identify the cyto-kinetic target of KidO, we sought extragenic suppressor mutations that escape division inhibition by KidO. UV-induced mutagenesis led to the recovery of six mutants that tolerate KidO overexpression (see Supplemental Information). Genetic mapping by transposon linkage and cotransduction, followed by DNA sequencing, revealed that each mutant harbored a different single or double nucleotide substitution in the ftsZ gene, encoding mutations F55G, I68N, T69N/Q70E, E79K, G225S, or S257A (Figure 5A).

Two experiments demonstrated that these mutations in ftsZ, rather than a mutation in a gene nearby ftsZ, confer resistance to KidO overexpression. First, we conducted a series of complementation experiments with the ftsZ(E79K) mutant strain and plasmids expressing WT FtsZ in trans. In the presence of WT FtsZ, the mild division defect of ftsZ(E79K) cells was corrected (Figure 5B). Second, when FtsZ(E79K) was expressed from a plasmid in an FtsZ$^{-}$ background, cells tolerated the compatible KidO overexpression plasmid, pCWR464 (encoding KidO-DD).

By contrast, when WT FtsZ was expressed in the FtsZ$^{-}$ background, no transformants were obtained with pCWR464 (data not shown). Third, we isolated additional mutant ftsZ plasmids conferring resistance to pCWR464 in the FtsZ$^{-}$ background (see Experimental Procedures). These plasmids encode FtsZ mutants A56T, A74T, M82V, and K262E (Figure 5A). In summary, the isolation of 10 independent suppressor mutations in ftsZ that confer resistance to KidO shows that ftsZ is a target of KidO in vivo.

Fourth, live-cell fluorescence microscopy revealed that KidO localizes to the division site in an FtsZ-dependent manner. When KidO-mCherry was expressed from the vanA locus in ΔkidO cells, fluorescence was enriched at or near the constriction Z-ring in 73% (193/265) of late-predivisional (pinched) cells (Figure 5C). KidO-mCherry still inhibits cytokinesis upon overexpression (data not shown), demonstrating that mCherry-tagged KidO is functional. Importantly, while KidO-mCherry is still at the septum in rcdA mutants (data not shown), it is either delocalized (T69N/Q70E, E79K, and G225S) or mislocalized (I68N) in four of the six chromosomal ftsZ point-mutant strains (Figure 5D and data not shown), indicating that the recruitment of KidO to the Z-ring depends on FtsZ, but not on the RcdA proteolytic regulator. Complementation with a WT copy of FtsZ restored septal localization of KidO-mCherry to ftsZ(E79K) cells (Figure 5E). In ftsZ(F55G) or ftsZ(S257A) cells, KidO-mCherry is at the septum (data not shown), indicating that more than one mechanism of FtsZ resistance to KidO exist: one interfering with the recruitment of KidO to the Z-ring, and a later-acting event in which KidO might act on FtsZ polymer stability (see below).

**Multiple Mechanisms Confer FtsZ Resistance to KidO**

If KidO destabilizes FtsZ polymers, then FtsZ mutants that assemble into polymers with increased stability might confer resistance to KidO. GTP binding and hydrolysis promote the polymerization and depolymerization of FtsZ, respectively (Margolin, 2005). Notably, the KidO-resistant mutation, A74T, in Caulobacter FtsZ is synonymous with a mutation (A70T) in Escherichia coli FtsZ that confers resistance to the DNA damage-inducible division inhibitor, SulA. The E. coli A70T mutation impairs hydrolysis, but not binding of GTP, likely increasing the stability of FtsZ polymers in vivo (Dai et al., 1994). To explore whether the Caulobacter A74T mutant has similar biochemical properties, we measured GTP hydrolysis and binding of WT

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As shown in Figure 5H, mutations I68N and T69N/Q70E that impair FtsZ mutants for binding to KidO-TAP by far-Western assay. Interestingly, we used purified components to test selected KidO-resistant FtsZ mutants for binding to KidO in vitro. This suggests that residues 68–70 contribute to the recruitment of KidO to the Z-ring. Mutations in these residues can disturb the interaction of FtsZ monomers with KidO and, possibly compounded by other effects on FtsZ polymerization and GTPase activity, impair the localization of FtsZ to the Z-ring. As a result, KidO cannot induce disassembly of the mutant Z-ring, and cells are rendered resistant to KidO overexpression.

In summary, FtsZ can become immune to KidO through mutations that: (1) interfere with binding to KidO; (2) appear to increase the stability of FtsZ filaments; or (3) affect other properties.

**Temporal Regulation of Z-Ring Formation by KidO**

Since KidO is cell-cycle regulated, we wondered if it functions as a temporal regulator of Z-ring formation. Microscopic examination of KidO (ΔkidO) cells revealed a mild chaining phenotype (Figures S6A–S6B), indicating that cells are impaired in completing division. In such ΔkidO cells, Z-rings are absent from some constrictions, while present at a central location in the associated, often unpinched, daughter cell. By contrast, in WT cells, Z-rings are observed at every constriction. Since improper timing of Z-ring formation might underlie this phenotype, we conducted colocalization experiments (Figure 6A) with FtsZ-mCherry and FtsK-GFP (which localizes to the septum after FtsZ [Wang et al., 2006]). In 93% (108/115) of WT cells, FtsZ and FtsK colocalize at deeply constricted sites. By contrast,
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we observed that FtsZ and FtsK colocalized only in 64.4% (85'/ 132) of KidO− cells, yielding predivisional cells with FtsK-GFP at the site of constriction and a Z-ring at an internal position in the daughter compartment (Figure 6B). Since immunoblotting revealed comparable steady-state levels of FtsZ in WT and ΔkidO cells (Figure S6C), Z-ring formation and/or stability might be enhanced in the absence of KidO. To test if ΔkidO cells assemble the Z-ring earlier than WT cells, we monitored Z-ring formation during the cell cycle of WT and ΔkidO cells expressing FtsZ-mCherry from PvanA, and native FtsZ, either from the endogenous ftsZ promoter or from Pxyl (Figure 6C). Consistent with previous results, FtsZ-mCherry was found at a polar site in WT G1 cells (Thanbichler and Shapiro, 2006). This localization pattern was mirrored in ΔkidO cells at the onset of the experiment (data not shown). However, by the 30 min time point, FtsZ-mCherry localization differed in the two strains (Figure 6C). While FtsZ-mCherry was still at a pole in 75% (285/382) of KidO− cells, it already localized to a medial position in 78% (367/474) of KidO− cells. The cell cycle-dependent accumulation of FtsZ is not noticeably altered in the absence of KidO (Figure S6D), arguing that KidO also influences the timing of Z-ring formation by regulating assembly/stability.

Synergistic Division Control of FtsZ by KidO and the Spatial Regulator, MipZ

The result that KidO acts directly on FtsZ prompted us to explore potential redundancies and complementarities between KidO and MipZ (Thanbichler and Shapiro, 2006). The ParA-like ATPase, MipZ, is localized to the cell pole(s), where it inhibits FtsZ polymerization (Figure 1A).

Five lines of evidence argue that KidO and MipZ are complementary, nonredundant division control systems of FtsZ that act at distinct times in the cell cycle and at different subcellular locations. First, the kidO::Tn5 or ΔkidO mutation partially alleviates the growth and division defect of MipZ− cells (Figure 6D and 6E and data not shown), indicating that KidO disturbs division independently of MipZ. Second, FtsZ(E79K) cells that are resistant to KidO overexpression exhibit elevated sensitivity toward MipZ expressed from a multicopy plasmid compared with WT cells (Supplemental Information), providing genetic evidence that KidO does not affect FtsZ through MipZ. Third, the FtsZ(A74T) mutation can partially curtail the genetic evidence that KidO does not affect FtsZ through MipZ (Thanbichler and Shapiro, 2006), providing that KidO and MipZ are active at different times in the cell cycle and at different subcellular locations. Fourth, the FtsZ(E79K) mutation can partially curtail the inhibitory activity of KidO (Figure 2D). This result suggests that KidO acts directly on FtsZ. Finally, the growth and division defect of MipZ− cells in the absence of KidO (kidO::Tn5) seen after growth on PY agar plate for 3 days at 30°C (left) or in PYE broth for 18 hr (right).

Figure 6. The Role of KidO in Temporal Regulation of Z-Ring Formation
(A) DIC and fluorescence micrographs of WT and ΔkidO cells expressing FtsZ-mCherry (red) and FtsK-GFP (green) from PvanA or Pxyl control at the vanA or xyI locus, respectively, following induction with 0.5 mM vanillate and 0.3% xylose for 2 hr.
(B) WT or ΔkidO mutant cells constitutively expressing FtsZ-mCherry from PvanA (at the vanA locus) were grown in M2G, induced with 0.5 mM vanillate for 2 hr, synchronized, and suspended in M2G with 0.5 mM vanillate. Images were acquired after 80 min. The mean intensity of FtsZ-mCherry localization at the septum in ΔkidO cells expressed as an absolute value (in arbitrary units [AU]) or relative to WT (as percentage).
(C) Composite images of KidO+ (left panels) or KidO− (ΔkidO, right panels) cells constitutively expressing FtsZ-mCherry (FtsZ-mCh) from PvanA (at the vanA locus) and native FtsZ from Pxyl (at the xyI locus, upper panels) or from the endogenous promoter at the native ftsZ locus (lower panels). Cells were grown in M2G (lower panel) or M2G with 0.3% xylose (upper panel) and induced with 0.5 mM vanillate for 2 hr, synchronized, and suspended in M2G containing vanillate and xylose or M2G with vanillate alone. Images were acquired after 30 min. (B) and (C) Schematics summarizing the localization results of FtsZ-mCherry (red) are shown below the fluorescence micrographs.
(D) Growth on a PYE agar plate for 3 days of serial dilutions of MipZ− (ΔmipZ) or MipZ+ KidO− (ΔmipZ kidO::Tn5) cells.
(E) Partial relief of the growth and division defect of MipZ− (ΔmipZ) cells in the absence of KidO (kidO::Tn5) seen after growth on PYE agar plate for 3 days at 30°C (left) or in PYE broth for 18 hr (right).
KidO Is Bifunctional and Requires an Intact NAD(H)-Binding Pocket for Division Control

The finding that KidO regulates DivJ and FtsZ, two dissimilar proteins with different cellular functions, raised the possibility that KidO is a bifunctional protein. This possibility prompted us to test whether these two functions could be genetically uncoupled by point mutations. Residues required for division control in KidO were identified in a screen for mutations that uncouple by point mutations. Residues required for division control in KidO were identified in a screen for mutations that intermingle with DivJ stimulation (Figure 7E). Thus, DivJ stimulation activity, is important for FtsZ regulation by KidO.

We also explored the role of other conserved residues in KidO by creating single alanine substitutions at the conserved positions E91, H142, S174, and S224. While overexpression studies indicated that the side chains of E91, H142, S174, and S224 are all dispensable for division control (Figure 7A and data not shown), complementation experiments with the ∆kidO ΔpleC strain showed that the D82A and R223L mutants still stimulate DivJ (Figure 7E), indicating that these mutations specifically impede division control. By contrast, overexpression of KidO(F87Y), in which the putative oxidoreductase catalytic residue is reinstated, inhibits FtsZ in a manner indistinguishable from that of WT KidO (data not shown). In summary, we conclude that NAD(H) binding and/or cross-linking (Figure 7D). Moreover, a complementation assay that KidO is a bifunctional protein. This possibility prompted us to test whether these two functions could be genetically uncoupled by point mutations. Residues required for division control in KidO were identified in a screen for mutations that intermingle with DivJ stimulation (Figure 7E). Thus, DivJ stimulation activity, is important for FtsZ regulation by KidO.

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and FtsZ control can be genetically uncoupled by different point mutations, demonstrating that KidO is a true bifunctional regulator.

**DISCUSSION**

**Regulation by Ancestral NAD(P)H-Dependent Enzymes**

Our discovery of KidO, an NAD(H)-binding oxidoreductase homolog that regulates the Caulobacter cell-division cycle, provides precedence that homologs of NAD(P)H-dependent enzymes can be appropriated for regulatory purposes in the bacterial or eukaryotic cell. As descendants of metabolic enzymes, these proteins are perhaps predisposed to evolve into NADP(H)-dependent regulators. Kvbeta, an NADP(H)-dependent oxidoreductase with a TIM-barrel fold, associates with a voltage-gated potassium channel (Kv) in metazoans to regulate channel excitability (Tipparaju et al., 2007; Weng et al., 2006). NADPH-binding residues are critical for the regulatory activity of Kvbeta, and corresponding residues are also required for regulation of FtsZ and efficient NAD(H) binding in KidO. Thus, these residues may play similar structural roles in the cofactor-binding pocket of the TIM-barrel.

In addition to the TIM-barrel, the Rossmann fold has been exploited for NADP(H)-dependent regulation. HSCARG, a regulator of nitric oxide production in humans, uses the Rossmann fold from short chain dehydrogenases/reductases to bind NADP(H) and allosterically influence protein conformation (Zhao et al., 2008). KidO, Kvbeta, and HSCARG do not feature additional conspicuous functional domains. Structural changes are induced by NAD(P)H binding in Kvbeta and HSCARG to influence interactions with other protein(s), and a similar allosteric principle may underlie FtsZ-regulation by KidO.

**Regulation of FtsZ by Homologs of Metabolic Enzymes**

Localization of KidO to the Z-ring is disrupted by mutations in the cofactor-binding pocket that disturb the association with NAD(H), implying that NAD(H) binding is important for the recruitment of KidO to the Z-ring. Evidence for metabolic control of FtsZ was recently provided in the Gram-positive bacterium, Bacillus subtilis. UgtP, a glycosyltransferase homolog, inhibits FtsZ assembly in vitro and localizes to the Z-ring. Uridine-5’-diphosphoglucose (UDP-Glc), a metabolic intermediate from the glycolipid biosynthetic pathway and presumed substrate of UgtP, is required for the recruitment of UgtP to the Z-ring (Weart et al., 2007). KidO could target lateral interactions in between FtsZ filaments to destabilize the Z-ring (Dajkovic et al., 2008). While this predicts that KidO has affinity for a polymeric structure of FtsZ, our far-Western analyses also show that KidO can bind monomeric FtsZ. Thus, KidO could also destabilize FtsZ polymers indirectly through sequestration of monomers, effectively lowering the pool of subunits available for polymerization (Dajkovic et al., 2008).

KidO is wired into the Caulobacter cell-cycle circuitry

Activation of the DivJ kinase by KidO and SpmX at the G1→S transition results in a burst in DivK−P, which ultimately signals the degradation of the CtrA replication inhibitor and that of KidO (Figure 1B). It is possible that SpmX, which remains colocalized with DivJ, can support DivJ-catalyzed phosphorylation of DivK throughout S phase without KidO. This could explain why no apparent trough in DivK−P/DivK is observed during S phase in the wake of the proteolytic removal of KidO. The regulatory interdependence of DivK−P and KidO implies that KidO indirectly influences its own stability and/or, conversely, that DivK essentially modulates its own phosphorylation state. How KidO promotes DivJ activity is currently unknown. Mutations in KidO that impair NAD(H) binding have no apparent effect on DivJ activity, suggesting that KidO stimulates DivJ in an NAD(H)-independent manner. Moreover, pulldown experiments indicate that KidO does not interact with DivJ or DivK, leading us to suspect that KidO modulates the DivJ kinase through another, perhaps currently unknown, regulatory component of the cell-cycle circuitry.

The concomitant and DivK−P-induced proteolytic degradation of KidO and CtrA licenses two critical and intricately coupled S phase events in Caulobacter: the initiation of DNA replication, and the assembly of the medial Z-ring. With the onset of S phase, the ori-proximal region is replicated and segregated to both poles, an event that triggers the redeployment of FtsZ to midcell (Figure 1A). The latter event is mediated by polarly localized MipZ, transported to the poles by way of its association with ori-proximal sequences regulator (Thanbichler and Shapiro, 2006). MipZ abundance is highest at the poles and lowest near midcell. As a consequence, Z-ring assembly is disrupted by MipZ at the poles, and occurs efficiently at midcell, provided that KidO has previously been degraded. If KidO is not eliminated, Z-ring formation is disturbed and, in the absence of KidO, medial Z-ring formation occurs prematurely. Later in S phase, CtrA−P reaccumulates and induces the synthesis of KidO, which then accumulates at the septum to promote Z-ring disassembly (Figure 1A).

It is possible that the availability of NAD(H) determines whether KidO regulates DivJ or FtsZ. At the time of division, KidO is localized to the septum to modulate FtsZ. Since this cytokinetic function of KidO appears to require NAD(H), this cofactor should be present at the time of constriction, and might be maintained in the ensuing G1 phase. Our studies suggest that KidO stimulates DivJ at the G1→S transition, and that this event does not require NAD(H) binding. While other, currently unknown, mechanism(s) could influence whether KidO regulates DivJ and/or FtsZ, a drop in cellular NAD(H) levels at the G1→S transition, and subsequent replenishment when cells constrict, could engage one or the other activity of KidO. Unknown internal cue(s) are thought to regulate the Caulobacter cell-division cycle. Germans to the idea that a change in NAD(H) levels constitutes such an internal signal, biological cycles in eukaryotes are thought to be influenced by oscillations in NAD(P)H levels (Asher et al., 2008; Koch-Nolte et al., 2009; Tu and McKnight, 2006; Tu et al., 2007; Wijnen, 2009).

**EXPERIMENTAL PROCEDURES**

**Immunoblotting and Far Western**

His6-KidO was purified under denaturing conditions from E. coli Rosetta (DE3)/pLYSs (Novagen, Madison, WI) with Ni-NTA agarose, excised from a 12.5% SDS-PAGE gel, and visualized under UV light. The purity of the His6-KidO sample was assessed by Coomassie-staining SDS-PAGE gels. Western blotting was performed using a ChemiDoc MP imaging system (Bio-Rad Laboratories, Hercules, CA). The molecular weight of the KidO protein was determined using a series of molecular weight standards including the following: Staphylococcus aureus lysozyme, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor, and human IgG from Sigma-Aldrich. The identity of the KidO protein was confirmed by mass spectrometry.
SDS polyacrylamide gel and used to immunize New Zealand white rabbits (Josman LLC, Napa, CA). Immunoblotting with the anti-KidO antiserum (1:2000 dilution) was as previously described (Radhakrishnan et al., 2008).

For far-Western analysis, blots with purified WT and mutant FtsZ proteins were blocked in a TBST solution (200 mM Tris-HCl [pH 7.9], 150 mM NaCl, 0.5% Tween-20, 5% nonfat dried milk) for 1 hr and then incubated for 3 hr at room temperature in a TBST solution with 25 nM KidO-TAP purified from Caulobacter. The blot was then washed several times with TBS (200 mM Tris-HCl [pH 7.9], 150 mM NaCl), probed with the polyclonal antiserum to KidO (1:2000 dilution), and detected with a donkey anti-rabbit antibody conjugated to hors eradish peroxidase (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA).

**FtsZ Purification and Assays**

FtsZ proteins were purified with an N-terminal, UlpI-cleavable His6-SUMO tag in E. coli Rosetta(DE3)pLysS cells. After cleavage of His6-SUMO (Bendezu et al., 2009), an N-terminal histidine residue remains on FtsZ. GTPase assays were as previously described (Mukherjee et al., 1993; Thanbichler and Shapiro, 2006). GTP-binding was determined by UV-induced cross-linking (de Boer et al., 1992) of [α-32P]GTP (PerkinElmer, Boston, MA) at 4°C with a UV Stratallinker 2400 (Stratagene, La Jolla, CA).

**NAD⁺ Cross-Linking**

KidO-TAP was purified from Caulobacter NR6138 (ftsZ789N/Q25E) cells harboring pCWRS89 (pTAP-KidO-TAP) by tandem affinity purification (Puig et al., 2001) (see Supplemental Information) and dialyzed against 50 mM KCl, 50 mM Tris-HCl (pH 7.9), and 10% glycerol. KidO-TAP or BSA was mixed with 20 Ci of P32-labeled NAD⁺ (800 Ci/mmol; PerkinElmer, Boston, MA) in a 20 μl reaction buffer containing 20 mM Tris-acetate, 100 mM potassium-acetate, 1 mM EDTA, 0.5 mM DTT, and 50 mM magnesium-acetate to give a final concentration of 3 μM. For the mock sample, four times the volume as that from the KidO-TAP sample was mixed with radiolabeled NAD⁺ (20 Ci) in the 20 μl reaction buffer. All reactions were then UV cross-linked as described above.

**SPR**

SPR was performed on a Biacore 3000 system at 25°C with a flow rate of 10 μl/min in HBS-P buffer (Biacore AB, Uppsala, Sweden) containing 5 mM MgCl₂. KidO-TAP was covalently immobilized by amine coupling to Sensor Chip CM5. FtsZ was injected for 4 min at various concentrations, and the surface was regenerated between injections by washing with 0.01% SDS for 30 s, followed by HBS-P buffer for 4 min. BIAevaluation software (Biacore) corrected for nonspecific binding by subtracting the signal obtained from a control cell lacking KidO, and derived the KD with data from three independent SPR experiments.

**KidO Stability Analysis**

KidO or CtrA stability was monitored by immunoblotting under two conditions. PYE-grown ΔkidO cells expressing KidO or KidO-DD from the xy/O locus on the chromosome were induced with 0.3% xylose (3 hr), washed, and suspended into PYE with chloramphenicol (2 μg/ml). Additionally, WT (NA1000) swarmers were treated with DNA gyrase inhibitor, novobiocin (100 μg/ml), in M2G (Jensen et al., 2001).

**Mass Spectrometric Analyses**

Gel slices stained with silver (SilverQuest Silver Staining Kit; Invitrogen, Carlsbad, CA) were analyzed by LC/MS/MS (Taplin Mass Spectrometry Facility, Harvard Medical School, Boston, MA).

**β-Galactosidase Assays, In Vivo Phosphorylation Assays, qChIP, and Microscopic Analyses**

β-Galactosidase assays, in vivo phosphorylation assays, qChIP, and microscopic analyses were as previously described (Radhakrishnan et al., 2008).

A Pnuc fragment comprising nt 3,857,810–3,858,141 of the NA1000 genome sequence was assayed in qChIP experiments. The SEM shown in the figures was derived with Origin 7.5 software (OriginLab Corporation, Northampton, MA) from data of three independent experiments.

**Tandem Affinity Purification, Strain, and Plasmid Constructions**

Detailed descriptions are in the Supplemental Information.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes six figures and Supplemental Experimental Procedures and can be found with this article online at doi:10.1016/j.devcel.2009.10.024.

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