A Moonlighting Enzyme Links *Escherichia coli* Cell Size with Central Metabolism

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Abstract

Growth rate and nutrient availability are the primary determinants of size in single-celled organisms: rapidly growing *Escherichia coli* cells are more than twice as large as their slow growing counterparts. Here we report the identification of the glucosyltransferase OpgH as a nutrient-dependent regulator of *E. coli* cell size. During growth under nutrient-rich conditions, OpgH localizes to the nascent septal site, where it antagonizes assembly of the tubulin-like cell division protein FtsZ, delaying division and increasing cell size. Biochemical analysis is consistent with OpgH sequestering FtsZ from growing polymers. OpgH is functionally analogous to UgtP, a *Bacillus subtilis* glucosyltransferase that inhibits cell division in a growth rate-dependent fashion. In a striking example of convergent evolution, OpgH and UgtP share no homology, have distinct enzymatic activities, and appear to inhibit FtsZ assembly through different mechanisms. Comparative analysis of *E. coli* and *B. subtilis* reveals conserved aspects of growth rate regulation and cell size control that are likely to be broadly applicable. These include the conservation of uridine diphosphate glucose as a proxy for nutrient status and the use of moonlighting enzymes to couple growth rate-dependent phenomena to central metabolism.

Introduction

Cell size control is a fundamental aspect of the cell cycle. Coordinating cell growth with division is essential to ensure that daughter cells have sufficient room for cytoplasmic and genetic material and are the correct size for a given condition or developmental fate. Despite the universal requirement for size control, how cells are able to detect the achievement of a particular size and communicate this information to the division apparatus remains an unresolved question in cell biology [1].

Nutrient availability is a primary determinant of cell size for single-celled organisms. In their seminal 1958 studies Schaechter, Maaloe, and Kjeldgaard determined that *Salmonella* cell size is coupled to growth rate, which is itself a function of nutrient availability [2]. Later work established that growth rate and nutrient availability are conserved determinants of cell size. *Escherichia coli* and *Bacillus subtilis* both coordinate cell size with nutrient availability, as do single-celled eukaryotes including the classic cell cycle model organism *Schizosaccharomyces pombe* [3,4,5].

Coordinating cell growth rate and nutrient availability with size, cells must have a mechanism to transmit information about growth rate and metabolic status to the division machinery. In *B. subtilis*, cell size is coordinated with central metabolism in part through uridine diphosphate glucose (UDP-glucose)-dependent changes in the oligomerization potential of the glucosyltransferase, UgtP [6,7]. During growth in nutrient-rich medium UDP-glucose, synthesized in a two-step pathway from glucose-6-phosphate, stimulates interaction between UgtP and the highly conserved tubulin-like cell division protein FtsZ, delaying assembly of the division machinery and increasing cell size. Conversely, during growth in nutrient-poor medium and/or in the absence of UDP-glucose, UgtP favors self-interaction. This permits division to proceed unimpeded yielding a smaller size. UgtP is a moonlighting enzyme also required for synthesis of the diglucosyl-diacylglycerol (di-glc-DAG) anchor for lipoteichoic acid (LTA), an anionic polymer that is a major component of the Gram-positive cell wall. Loss-of-function mutations in *ugtP* disrupt synthesis of the Di-glc-DAG moiety, but does not impact LTA synthesis [8].

Previous reports have indicated that inactivating UDP-glucose synthesis by inactivating the phosphoglucomutase, *pgm*, results in a ∼25% reduction in *E. coli* cell size under nutrient-rich conditions [9,10]. This phenotype suggests that *pgm* mutant cells are unable to properly coordinate cell division with nutritional conditions and, furthermore, implicate UDP-glucose as a widely conserved intracellular proxy for nutrient-dependent size control. Interestingly, despite the apparent conservation of UDP-glucose as a signaling molecule, the identity of the effector is less obvious. As a Gram-negative bacterium, *E. coli* does not synthesize LTA and computational analysis does not reveal a *ugtP* homolog within its ∼4.6 MB genome.

Here we report the identification and characterization of the integral inner-membrane protein OpgH as a UDP-glucose-activated inhibitor of FtsZ ring formation in *E. coli*. Genetic and biochemical data indicate that OpgH interacts directly with FtsZ via its N-terminal domain to inhibit division in a UDP-glucose-dependent manner. OpgH is the functional homolog of UgtP, a sugar transferase in *B. subtilis* that modulates cell size in response to carbon availability. While both moonlighting enzymes serve as
membrane-associated glucosyltransferases, they share no homology, have distinct enzymatic activities, and inhibit FtsZ assembly through different mechanisms thereby exposing a remarkable instance of convergent evolution. Moreover, this work significantly advances the understanding of nutrient-dependent cell size control in E. coli, the predominant model organism for studying bacterial physiology.

Results

E. coli utilizes UDP-glucose to couple cell size with nutrient availability

Previous work from our lab and others suggests that E. coli may employ UDP-glucose as an intracellular proxy for nutrient availability in the regulatory circuit responsible for coupling cell size with growth rate [10,11]. To test this we examined the size of wild type cells and pgm null mutants that are defective in the first step of UDP-glucose biosynthesis (Figure 1A). If UDP-glucose is central to the nutrient-dependent control of E. coli cell size, the size differential between wild type and pgm mutant cells should be greatest under nutrient-rich conditions and least under nutrient-poor conditions.

Measurements of the cross-sectional area of cells cultured under a range of nutrient conditions indicate that pgm mutants are indeed defective in the growth-rate-dependent control of cell size (Figure 1B). In LB+0.2% glucose (LB-glucose), the average cross-sectional area of wild-type E. coli was 5.66 μm² while pgm mutants were 4.24 μm², a difference of over 25%. However, the size inequity dissipated under nutrient-poor conditions. pgm mutants were only 4% smaller than wild type when cultured at a growth rate 4-times slower in a minimal growth medium supplemented with 0.4% succinate (AB-succinate).

Defects in UDP-glucose biosynthesis increase the frequency of FtsZ rings over incompletely segregated nucleoids

Rapidly growing cells are not only longer, but also have more total DNA, a consequence of multikfer replication. Multikfer replication is a phenomenon that allows E. coli and other bacteria to sustain mass doubling times shorter than the period required to complete chromosome replication and division. In previous work we determined that growth rate-dependent increases in cell size help prevent aberrant assembly of the division machinery over unsegregated bacterial chromosomes (nucleoids) in B. subtilis [6]. To determine the impact of a reduction in E. coli cell size on the frequency of division rings formed over nucleoids, we visualized FtsZ ring formation in rapidly growing pgm mutant cells with an inducible copy of ftsZ fused to gfp [12].

Consistent with growth rate-dependent increases in cell size ensuring that E. coli cells have sufficient room for DNA segregation during multifork replication, we observed a near two-fold increase in the frequency of division rings positioned over nucleoids in pgm mutants relative to wild type. In accordance with previous studies [13], approximately 20% (130/634) of wild type cells had an FtsZ ring over chromosomal material. The frequency increased to 35% (246/662) in diminutive pgm::kan cells (Figure 1C; Table S1). This suggests that the growth rate-dependent size increase is, in part, a mechanism to spatially coordinate the excess DNA generated by multifork replication.

Defects in UDP-glucose biosynthesis stabilize FtsZ assembly at midcell

To coordinate size with growth rate, cells must evaluate nutrient availability and subsequently transmit that information to the division machinery. To assess the impact of UDP-glucose on FtsZ assembly in E. coli, we determined if defects in UDP-glucose biosynthesis were sufficient to suppress the conditional lethality of the heat-sensitive ftsZ84 allele. FtsZ84 (G105S) supports assembly of the division ring at 30°C, but is unable at 42°C, leading to extensive filamentation and cell death [14,15].

Consistent with increases in UDP-glucose activating an inhibitor of FtsZ assembly, the viability of pgm::kan ftsZ84 double mutants was ~4.5-fold higher than ftsZ84 alone under restrictive conditions (Figure 1D). The plating efficiency (CFU restrictive/CFU permissive) of ftsZ84 cells grown at 30°C versus 42°C was just 0.013% (±0.001). In contrast, pgm::kan ftsZ84 double mutants exhibited essentially no reduction in viability under identical conditions 99.2% (±0.3). Notably, this is the first example of a loss-of-function mutation that suppresses the heat sensitivity of ftsZ84 without increasing the intracellular concentration of FtsZ84 (Figure S1). Together, the data presented in Figure 1 provides evidence for a UDP-glucose-activated factor coupling cell division directly to carbon metabolism.

Defects in the glucosyltransferase OpgH reduce E. coli cell size

To identify the UDP-glucose-dependent regulator of E. coli cell size, we systematically screened kanamycin resistance cassette insertions in genes predicted to be associated with UDP-glucose synthesis or utilization for defects in cell size, taking advantage of the Keio mutant collection (Figure 1A) [16]. Importantly, mass doubling rates in these mutants were indistinguishable from wild type in both nutrient-rich and nutrient-poor conditions (Table S2).

Of the 16 mutants we screened, knockouts in only three genes pgm, galU encoding a pyrophosphorylase required for synthesis of UDP-glucose from glucose-1-phosphate, and opgH (ubsH, b1049, EcoCyc:EG11886, UniProt:P62517) encoding a Family II glucosyltransferase, resulted in a statistically significant reduction in cell size (Figure 2A, 2B; Figure S3). Wild-type MG1655 cells had an average area of 5.66 μm². The galU::kan and opgH::kan mutants were 18% (4.66 μm²) and 12% (5.01 μm²) smaller than wild type, respectively. As previously shown in Figure 1B, pgm null cells were 25% smaller at 4.24 μm². Importantly, of the six known E. coli enzymes that utilize UDP-glucose (GalE, GalT, Ugd, OpgG, OpgH, and OtsA) mutations in only one, opgH::kan, led to a statistically significant size reduction (p>0.05). (Length and width
data of the various mutants is presented in Table S2.) Why mutations in \( \text{galU} \) and \( \text{opgH} \) do not reduce cell size to the same extent as the \( \text{pgm}::\text{kan} \) mutation is not readily apparent, however it is reminiscent of what we have observed for mutations in the analogous genes in \( \text{B. subtilis} \) [6].

**OpgH inhibits division in a UDP-glucose-dependent fashion**

Based on our study of the parallel pathway in \( \text{B. subtilis} \), we speculated that OpgH, rather than Pgm or GalU, was the UDP-glucose-dependent regulator of cell division in \( \text{E. coli} \). To test this hypothesis, we measured the average size of cells expressing either \( \text{pgm} \), \( \text{galU} \), or \( \text{opgH} \) (fused with an N-terminal thioredoxin and a C-terminal polyhistidine) from a high-copy plasmid cultured in nutrient-rich media.

Consistent with our prediction, induction of \( \text{thio-opgH-his} \) disrupted UDP-glucose production by disrupting the \( \text{pgm}::\text{kan} \) cells culture more than \( \text{thio-galU-his} \) expression, leading to severe filamentation indicative of a block in cell division (Figure 2C, 2D). Cells expressing \( \text{thio-opgH-his} \) grew at a lower rate than the \( \text{wt} \) strain, with an average cell size of \( \sim 2.5 \mu m \) after 4 h of induction. Cells expressing either \( \text{pgm}::\text{kan} \) or \( \text{thio-galU-his} \) grew at a similar rate to the \( \text{wt} \) strain, with an average cell size of \( \sim 13.2 \mu m \) after 4 h of induction.
Expression of either the *thio-pgm-his* or *thio-galU-his* fusion had a negligible impact on cell size.

UDP-glucose was absolutely required for OpgH-mediated division inhibition. Expression of wild-type *thio-opgH-his* in a *pgm::kan* background had no significant impact on cell size (Figure 2C, 2D; Figure S4). Cell size was similarly wild-type in cells expressing a mutant allele of *opgH* (PIC249AIA) defective in residues of the putative UDP-glucose binding site [17]. Strains with this mutation were also unable to complement normal glucosyltransferase function (Figure S5). Together, the data presented in Figure 2 suggests OpgH functions as a UDP-glucose-dependent antagonist of cell division.

**Figure 2.** OpgH acts as a nutrient-dependent division antagonist. (A) Cell area measurements of mutants in UDP-glucose synthesis or utilization cultured in LB-glucose. WT is set to 100%. >250 cells were measured per sample, error bars are standard deviation (n = 3). The *opgH* null is complemented with a plasmid encoding *Plac::opgH-gfp* and was cultured with 0.08 mM IPTG. This fusion is shown to be functional for glucosyltransferase ability (Figure S5). * denotes p < 0.001, ** signifies p < 0.05 as judged by chi² analysis. (B) Micrographs of WT and knockouts of genes involved in UDP-glucose pathway grown in LB-glucose and stained with the membrane dye FM4-64. Bar = 5 μm. (C) Representative micrographs and (D) cell length measurements of strains cultured in LB overexpressing genes in the UDP-glucose pathway from an arabinose inducible promoter. Uninduced cells are on the upper panel. Induced constructs are on the bottom panel. Cells are stained with FM4-64, Bar = 5 μm. Error bar denotes standard deviation (n = 3). See also Table S2 (information of length, width, and growth rate), Figure S3 (measurements of cells defective in factors adjacent to the UDP-glucose biosynthesis pathway), and Figure S4 (Thio-OpgH-His overexpression levels).

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Figure 3. OpgH localizes to midcell in a growth rate- and FtsZ-dependent manner. Immunofluorescence localization of FtsZ and OpgH in various growth conditions or genetic backgrounds. (A) OpgH localizes at midcell with FtsZ only at fast growth rates. Wild type cells were grown in either LB-glucose (τ = 21'), AB-glucose + casamino acids (τ = 38'), or AB-glucose (τ = 60'). (B) OpgH is unable to localize to midcell in the absence of FtsZ. A strain encoding a sodium salicylate inducible copy of ftsZ (PL3180) was grown to mid-log phase and back-diluted into LB broth ± inducer (2.5 μM sodium salicylate) for 2.5 h. (C) The frequency of OpgH at midcell is independent of UDP-glucose. Congenic strains either encoding a deletion of a key gene in UDP-glucose biosynthesis (pgm::kan) or a mutation in OpgH's putative UDP-glucose binding site. (A–C) DNA is stained by DAPI. Bar = 5 μm. White arrowheads indicate OpgH midcell localization. OpgH is green in wild type and FtsZ is in red in the overlays. The percent covariance of FtsZ and OpgH at midcell is indicated below the micrographs.

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OpgH regulates cell size independent from its role in OPG synthesis

OpgH is an inner-membrane glucosyltransferase that synthesizes osmoregulated periplasmic glucans (OPGs); branched glucans consisting of 5 to 13 glucose residues joined by β-1–2 linkages and branched by β-1–6 branches [18,19]. OpgH is responsible for the synthesis and subsequent periplasmic delivery of β-1–2 poly-glucose chains from UDP-glucose. opgH is co-transcribed with opgG, which encodes a periplasmic protein required for generating the β-1–6 branches [20].

The loss of OPG synthesis has been implicated in a range of abnormal phenotypes including deficiencies in: envelope stability, flagellar synthesis and motility, phage infectivity, biofilm formation, and pathogenicity [21,22,23,24,25,26,27,28,29]. While some of these phenotypes can be attributed to an altered cell envelope, the majority are tied to aberrant activation of the Rcs phosphor-ecrely [22,30,31]. To eliminate the possibility that the cell size defect was a secondary consequence of either lacking OPGs and/or Rcs-mediated gene expression, we examined the size of cells defective for OPG synthesis or Rcs activity.

Our data shows that OpgH’s role in size modulation is independent of OPG synthesis or activation of the Rcs system. Inactivating opgG, thereby eliminating OPG production either by a non-polar mutation in opgG or by complementing opgH in trans in an opgGH double mutant, had no impact on cell size (Figure 2A; Figure S6A).

Based on previous reports indicating that one of the six promoters driving ftsZ is positively regulated by Rcs [32,33], we were particularly concerned that induction of Rcs in the absence of UDP-glucose or OPGs might lead to overexpression of ftsZ, which in turn would reduce cell size. However, FtsZ levels are unperturbed in either a background unable to synthesize UDP-glucose (pgm::kan) or a mutation in the putative UDP-glucose binding region (Figure 3C). Midcell covariance of OpgH and FtsZ localization at midcell in the pgm::kan cells was 91% (178/196) and 93% (212/227) in the nucleotide sugar-binding mutant when cultured in LB-glucose. Together, this data supports a model in which OpgH dynamically localizes to the division machinery in a growth rate-dependent, but UDP-glucose-independent manner.

The N-terminus of OpgH is both necessary and sufficient for division inhibition

OpgH is an 848 amino acid (97 kDa) integral inner-membrane protein. Reporter fusion analysis indicates that OpgH is composed of eight transmembrane domains with both N- and C-termini residing in the cytoplasm (Figure 4A) [39]. OpgH has three significant cytoplasmic domains referred to here as OpgHα (1–138), OpgHβ (211–514), and OpgHγ (702–848). OpgHβ, the largest domain, contains the protein’s putative UDP-glucose binding domain based upon sequence homology.

To identify determinants within OpgH required for division inhibition, we expressed thioredoxin/polyhistidine fusions to OpgHα, OpgHβ, or OpgHγ from an arabinose inducible promoter on multicopy vectors in an opgH::kan mutant background. Induction of thi-o-pgHβ*-his, but not thi-o-pgHα*-his or thi-o-pgHγ*-his, resulted in a substantial increase in cell size relative to an uninduced control (Figure 4B, 4C). The area of cells expressing Thio-OpgHβ*-His increased more than two-fold ±3 h post-induction (from 2.9 μm to 5.9 μm), indicative of a partial block in division. The differential in division inhibition efficacy between OpgH and OpgHβ may be the consequence of the former being at the membrane where it can more readily access the division apparatus. Expression of an OpgHγ deletion mutant (thio-o-pgHγ1-210)-his) had no impact on cell size (Figure 4C). These data are consistent with a model in which UDP-glucose binding to OpgHβ leads to a conformational change that promotes OpgHβ mediated division inhibition.

Expression of thioredoxin fusions to a set of nested deletions within the OpgHβ domain identified an 18 amino acid peptide (residues 83–101) that was sufficient for division inhibition (Figure 4C; Figure S7). However, systematic alanine substitutions to this region or deletion of all 18 residues had no significant impact on the ability of OpgHβ to mediate division inhibition (Figure 4C; data not shown). This finding suggests the presence of at least one additional determinant within OpgHβ that is sufficient for its role in division inhibition.
Consistent with a role in cell division, OpgHN localized to midcell independent of the rest of the protein. Immunofluorescence microscopy using antibodies against the His tag, indicated that of the three cytoplasmic domains, only Thio-OpgHN-His exhibited medial localization on its own. During growth in LB-glucose, 84% (379/452) of cells expressing thio-opgHN-his displayed colocalization with FtsZ at midcell (Figure 4D). Strikingly, a Thioredoxin fusion to the 18 residue fragment of OpgHN (residues 83–101) that was sufficient for division inhibition, also colocalized with FtsZ in 82% (169/205) of cells (Figure S7B). Although OpgHN was sufficient for medial localization, it was not necessary. A thioredoxin fusion to the OpgH(D1–210), the N-terminal deletion mutant, colocalized with FtsZ 67% of the time (148/221) during growth in LB-glucose, implying the N-terminal domain is not necessary for medial localization of OpgH (Figure S7B). Given the inability of the OpgHM or OpgHC domains to localize on their own, we hypothesize that the second localization determinant is situated in a transmembrane and/or periplasmic region of the protein (see Discussion).

OpgH inhibits division by blocking FtsZ ring formation

In light of our finding that cytoplasmic OpgH N was capable of blocking division in vivo (Figure 4B, 4C), as well as genetic data suggesting that the loss of UDP-glucose positively impacts FtsZ assembly at midcell (Figure 1D), we speculated that OpgH might modulate cell division through direct interactions with FtsZ. To test this possibility, we measured the proportion of cells with FtsZ rings following induction of either thio-opgH-his or thio-opgHN-his. If OpgH inhibits division by antagonizing FtsZ polymerization dynamics, then induction of thio-opgH-his or thio-opgHN-his should reduce the number of FtsZ rings.

For this experiment, dilutions were calibrated to ensure cells would be in early exponential growth at each time point. Samples were taken at 25-minute intervals for ~3 h, fixed, and the percentage of cells with an FtsZ ring were scored using immunofluorescence microscopy. Induction of both thio-opgH-his as well as thio-opgHN-his nearly abolished cells with FtsZ rings by ~2.5 h post-induction (Figure 5A).

Figure 4. The N-terminal cytoplasmic region of OpgH is necessary and sufficient to inhibit cell division. (A) A schematic representation of the inner-membrane glucosyltransferase OpgH [39]. (B) Overexpression of OpgH N increases cell size in the opgH::kan strain. Micrographs of cells encoding arabinose inducible N-terminal thioredoxin fusions to each of OpgH's cytoplasmic domains cultured in LB±0.5% arabinose. Cells are stained with FM4-64. Bar = 5 μm. (C) Cell area measurements of cells with the various thio-opgH-his constructs. Cells were cultured in LB with either 0% arabinose (dark red bars) or 0.5% arabinose (light red bars). Error bars equal standard deviation (n = 3). (D) Immunofluorescence micrographs of various Para::thio-opgH-his constructs following 2 h of induction in LB±0.5% arabinose. Overlays of OpgH (green) and FtsZ (red) localization are on the bottom row. Bar = 3 μm. See also Figure S7 (localization data on additional deletion constructions).

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As an additional test of the impact of UDP-glucose and OpgH on FtsZ assembly dynamics in vivo, we evaluated the effects of overexpressing the spatial cell division regulator MinD. The precise temporal and spatial regulation of cell division is achieved through a concert of factors that modulate FtsZ assembly. Overexpression of an FtsZ inhibitor, and the associated lethality, can be balanced by deletion of another inhibitor [6,40].

Consistent with defects in UDP-glucose synthesis or deletion of opgH enhancing FtsZ assembly, inactivating genes in the metabolic pathway suppressed lethality associated with minD overexpression. MinD is overexpressed by ~2-fold, which is at the threshold of lethality in WT. Error bars equals standard deviation (n = 3). (See Figure S2 for relative MinD expression levels.) (C) A representative 90° angle light scattering plot of FtsZ assembly ± OpgH. FtsZ is at 5 μM, OpgH is at 10 μM. Arrow indicates addition of 1 mM GTP. (D) Concentration-dependent inhibition of FtsZ polymerization by OpgH. The ratio of FtsZ to OpgH is listed below. FtsZ is at 5 μM in all cases. (See Figure S9A, S9B for additional controls.)

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Figure 5. OpgH{\textsuperscript{N}} is an inhibitor of FtsZ assembly. (A) Induction of OpgH inhibits FtsZ assembly in vivo. Cells were sampled and imaged for immunofluorescence microscopy at 25 minute intervals following the induction of either thio-his (black squares), thio-opgH-his (dark grey diamonds), or thio-opg{\textsuperscript{N}}-his (light grey circles). Cells were cultured in LB. >200 cells were evaluated per sample. Representative α-FtsZ immunofluorescence micrographs from time points 0° and 150° are shown in the lower left. (B) Mutations that disrupt synthesis of UDP-glucose or OpgH itself suppress the lethality of MinD overexpression. MinD is overexpressed by ~2-fold, which is at the threshold of lethality in WT. Error bars equals standard deviation (n = 3). (See Figure S2 for relative MinD expression levels.) (C) A representative 90° angle light scattering plot of FtsZ assembly ± OpgH{\textsuperscript{N}}. FtsZ is at 5 μM, OpgH{\textsuperscript{N}} is at 10 μM. Arrow indicates addition of 1 mM GTP. (D) Concentration-dependent inhibition of FtsZ polymerization by OpgH{\textsuperscript{N}}. The ratio of FtsZ to OpgH{\textsuperscript{N}} is listed below. FtsZ is at 5 μM in all cases. (See Figure S9A, S9B for additional controls.)

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As an additional test of the impact of UDP-glucose and OpgH on FtsZ assembly dynamics in vivo, we evaluated the effects of overexpressing the spatial cell division regulator MinD. The precise temporal and spatial regulation of cell division is achieved through a concert of factors that modulate FtsZ assembly. Overexpression of an FtsZ inhibitor, and the associated lethality, can be balanced by deletion of another inhibitor [6,40].

Consistent with defects in UDP-glucose synthesis or deletion of opgH enhancing FtsZ assembly, inactivating genes in the metabolic pathway suppressed lethality associated with minD overproduction (Figure 5B). Two-fold overexpression of MinD reduced the plating efficiency of wild-type E. coli to 0.03%. However, overexpression of MinD to identical levels in pgm null cells resulted in a plating efficiency of 3.9%, >100-fold higher than in the wild-type background (Figure 5B; Figure S2).

Consistent with OpgH interacting directly with FtsZ to delay division, an opgH::kan mutation increased the viability of cells overexpressing minD by ~1.5 fold (Figure 5B; Figure S2). Cells expressing opgH::PIC249.4A phenocopied the opgH null results, further corroborating that UDP-glucose binding is essential for OpgH mediated inhibition of FtsZ assembly. In contrast to a loss-of-function mutation in pgm, opgH::kan was unable to suppress the heat-sensitivity of ftsZ84 allele.

Genetic data suggests that OpgH reduces the pool of FtsZ available for assembly into the cytokinetic ring.

To position OpgH within the regulatory hierarchy responsible for the temporal and spatial control of cell division, we examined the phenotypes of cells defective in either UDP-glucose synthesis (Δpgm) or opgH with defects in one of three previously characterized FtsZ antagonists: minCDE, sinA, or clpX. We reasoned that if OpgH shared a role in nucleoid occlusion, ring constriction, or preventing immediate division ring reassembly,
then the double knockouts might exacerbate defects in division rings bisecting nucleoids, augmented cell size, or increased rate of minicells.

None of the double mutants had defects in growth rate, increased rates of division rings over unsegregated nucleoids, or a higher rate of inaccurately placed FtsZ rings during growth in LB-glucose (Table S1, data not shown). However, supporting a model in which OpgH reduces the effective concentration of FtsZ, a loss-of-function mutation in either opgH or pgm reduced the length of the abnormally long midCDE null mutants by approximately half (Figure S8; Table S1). This finding suggests that the pools of FtsZ available for assembly into the cytokinetic ring are elevated in the absence of either opgH or pgm.

OpgH<sup>N</sup> interacts directly with FtsZ to inhibit assembly

Based on the genetic evidence suggesting that OpgH antagonizes division through direct interactions with FtsZ, we next determined if purified OpgH<sup>N</sup> was sufficient to inhibit FtsZ assembly in vitro. For this experiment we employed 90° angle light scattering, a functional assay for FtsZ assembly [41,42]. All experiments were performed with OpgH<sup>N</sup> (residues 1–138) and <i>E. coli</i> FtsZ in their native (untagged) form.

Consistent with our genetic data, OpgH<sup>N</sup> inhibited FtsZ assembly in a dose-dependent fashion (Figure 5C, 5D). At a 1:1 ratio of FtsZ to OpgH<sup>N</sup>, FtsZ assembly was reduced by more than 30%. At higher ratios of FtsZ:OpgH<sup>N</sup>, 1:1.5 and 1:2, FtsZ assembly was reduced further by ~60% and ~80%, respectively. Heat-inactivating OpgH abolished its ability to inhibit FtsZ assembly (Figure S9A, S9B). This level of inhibition is slightly less potent than other <i>E. coli</i> FtsZ inhibitors [43,44,45,46], however it is consistent with a model in which OpgH functions to delay division, not prevent it.

OpgH raises the apparent critical concentration of FtsZ required for GTP hydrolysis

Although 90° angle light scattering is valuable for measuring gross FtsZ assembly, it does not provide insight into the particular mechanism by which FtsZ assembly is obstructed. To elucidate the mechanism by which OpgH inhibits FtsZ assembly, we next determined the impact of OpgH<sup>N</sup> on FtsZ’s intrinsic GTPase activity. FtsZ binds to GTP as a monomer, however dimerization (which binding of UDP-glucose by OpgH promotes a conformational change revealing an FtsZ interaction site on the OpgH<sup>N</sup> domain) and/or maturation of the cytokinetic ring, delaying division and increasing cell size (Figure 7A). During slow growth in nutrient-poor conditions or defective for UDP-glucose production, OpgH assumes a conformation that obscures the interaction between OpgH<sup>N</sup> and FtsZ. FtsZ assembly is able to proceed unimpeded, reducing cell size (Figure 7B). OpgH, specifically OpgH<sup>N</sup>, is well conserved in the order Enterobacteriales, suggesting functional conservation in close relatives of <i>E. coli</i> (Figure S10).

Based on our genetic and biochemical data, we propose that nutrient-dependent activation of OpgH leads to a reduction of FtsZ available for assembly into the cytokinetic ring. FtsZ levels are constant regardless of growth rate [57]. Nutrient-dependent increases in OpgH activity should translate into proportional reductions in the pool of available FtsZ subunits. Cells increase in size until they have accumulated sufficient FtsZ to overcome OpgH-mediated inhibition and support assembly of a mature cytokinetic ring [1]. This model is consistent with data from the Vicente lab demonstrating that modest reductions in FtsZ pools lead to a transient increase in cell size, but do not impact the timing of division under steady-state conditions [58].

Multiple determinants ensure OpgH localization to the division machinery

OpgH’s localization to the division apparatus only occurs in nutrient-rich conditions (Figure 3). Intriguingly, this growth rate-dependent localization is not controlled by interaction with its substrate, UDP-glucose, unlike its functional <i>B. subtilis</i> homolog UgpP [6]. Thus, the mechanism behind OpgH’s dynamic nutrient-dependent localization remains to be elucidated.

Analysis of OpgH deletion constructs suggests the presence of at least two determinants within the OpgH polypeptide are required for localization to the cytokinetic ring (Figure 4D; Figure S7B). One such determinant is within the soluble OpgH<sup>N</sup> domain. The other determinant resides somewhere in residues 211–848. Intriguingly, residues 651–713, encoding OpgH<sup>N</sup>’s 7th and 8th transmembrane domains, share homology to the SEDS (shape,
Figure 6. OpgH<sup>H</sup> appears to function as an FtsZ monomer sequestering protein. (A) Concentration-dependent inhibition of FtsZ’s GTPase activity by OpgH<sup>H</sup>. The GTP hydrolysis rate of 5 μM FtsZ is shown at differing ratios of OpgH<sup>H</sup>. OpgH<sup>H</sup> alone is at 5 μM. Error bars equal standard deviation (n = 3). (B) FtsZ GTPase rates at increasing concentrations of OpgH<sup>H</sup>. The critical concentration for assembly of FtsZ was determined at OpgH<sup>H</sup> concentrations of 0 μM, 2.5 μM, 5 μM, or 10 μM. (See Figure S9C, S9D for additional controls.)

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A remarkable example of convergent evolution

When viewed in the context of the parallel pathway in *B. subtilis*, this study highlights an exceptional example of convergent evolution. *E. coli* and *B. subtilis*, organisms separated by a greater evolutionary distance than *Homo sapiens* and *Saccharomyces cerevisiae*, both utilize UDP-glucose and unrelated glucosyltransferases in the regulatory circuit coupling cell size with growth rate and nutrient availability (Figure 7C).

Why UDP-glucose-utilizing glucosyltransferase enzymes were chosen to coordinate this phenomenon is an intriguing question. In general, moonlighting enzymes tend to be more valuable if the multiple functions are both beneficial at the same time [61]. Thus, the question arises: Aside from growth in rich nutrient conditions, are there circumstances in which an increased need for their sugar transferase activity coincides with a need to prevent division?

Both effectors have previously described roles in envelope biogenesis: OpgH<sup>H</sup> produces periplasmic glucans and UgtP synthesizes the Di-gl-DAG anchor for LTA. These products are particularly important during conditions that prompt the osmotic stress response [8,62,63]. Curiously, shifting the osmotic or turgor pressure has been reported to cause a temporary arrest of cell division that is alleviated only after cells equilibrate to the new osmotic conditions [64,65]. Thus, while our work describes a role for OpgH<sup>H</sup> and UgtP in nutrient-dependent size control, we speculate that both proteins may also function to inhibit division during osmotic stress or other cell envelope perturbations.

UDP-glucose is an ideal signaling molecule for transmitting information to the division apparatus under both conditions of rapid growth and cell envelope stress. Its accumulation is directly coupled to central carbon metabolism and is likely to accumulate primarily under nutrient-rich conditions, the same conditions that support multifork replication and necessitate an increase in cell size. Likewise, consistent with a role in the osmotic stress response pathway, genes involved in UDP-glucose synthesis are up-regulated during cell envelope stress and UDP-glucose is directly incorporated into the cell envelope [66,67].

Curiously, synthesis of both OPG and LTA is the primary source of diacylglycerol in *E. coli* and *B. subtilis* [68], raising the possibility that diacylglycerol may serve as a secondary messenger in the regulatory circuit governing bacterial cell size. (FabH, an enzyme involved in key steps of fatty acid biosynthesis, has recently been implicated in the nutrient-dependent control of cell size, suggesting another potential role for lipids in cell size homeostasis [69].)

In closing, our findings represent a major advance in our understanding of cell size control in *E. coli*, the predominant model system for the study of bacterial physiology. Moreover, through comparison with a parallel pathway in *B. subtilis*, our work reveals conserved aspects of growth rate regulation and cell size control – including conservation of UDP-glucose, a molecule common to all domains of life, as a proxy for nutrient availability and the use of moonlighting enzymes to couple growth rate-dependent phenomena to central metabolism – that are likely to be broadly applicable.

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Materials and Methods

**Strains and media**

*E. coli* strains and plasmids and their construction are described in Supporting Information (Text S1, Table S3, and Table S4). Cells were cultured in Luria-Bertani (LB) supplemented with 0.2% glucose or AB defined media [70] supplemented with 10 μg/ml thymine, 0.5% casamino acids (CAA), and either 0.2% glucose or 0.4% succinate as a carbon source. Cells were grown at 37°C and used for experimentation at early exponential growth phase (an OD of 0.15–0.3) unless otherwise stated. Standard techniques were employed for cloning, P1vir transductions, and other genetic manipulations.

Quantitative immunoblotting

Cells were lysed at early to mid-log phase (an OD<sub>600</sub> of 0.2–0.5) using physical or chemical techniques. Lysates were then normalized to either OD or total protein using a bicinchoninic acid (BCA) assay and subjected to SDS-PAGE. Immunoblots were performed using either rabbit α-MinD antibody (the gift of William Margolin), chicken α-His antibody (Millipore), rabbit α-FtsZ (the gift of David Weiss), or rabbit α-DnaA (the gift of Jon Kaguni) with cognate goat α-rabbit or donkey α-chicken secondary antibody conjugated to horseradish peroxidase (Jackson Immunoresearch). Band intensity was determined using ImageJ software and processed in Microsoft Excel [71].

**Immunofluorescence microscopy**

Microscopy performed was essentially similar to [10]. Cells were fixed in paraformaldehyde similar to as described in [72]. His-tagged proteins were detected using a chicken α-polyhistidine tag antibody (Millipore) with cognate goat α-chicken serum conjugated to Alexa488 (Invitrogen). FtsZ was detected using affinity-
purified polyclonal rabbit α-FtsZ serum (the gift of William Margolin) in combination with goat α-rabbit serum conjugated to Alexa546 (Invitrogen). OpgH was detected using mouse α-OpgH polyclonal serum with goat anti-mouse Alexa488 (Invitrogen). Genetic content was stained with DAPI.

Cell size measurements
Cells in early to mid-log phase were stained with FM-464 and adhered to 15-well glass slides using poly-L-lysine. The length and width of cells were calculated using the Openlab software. Only length was measured in cases of severe cell filamentation when width was irrelevant to the interpretation.

ftsZ84 and minD plating efficiency
Plating efficiency was done as previously described [73]. Briefly, strains encoding ftsZ84 or an inducible copy of minD were cultured in permissive conditions to early/mid-log phase (OD_{600} 0.1–0.4), back-diluted to an OD_{600} of 0.01 and grown to an OD_{600} of 0.15–0.3. Cultures were normalized to optical density and then serially diluted from 10^{-1} to 10^{-6}. Equal volumes were plated at permissive conditions (LB with 0.05% NaCl at 30°C for ftsZ84; LB-glucose + 0 mM IPTG for minD) and restrictive conditions (LB with 0.05% NaCl at 42°C for ftsZ84; LB-glucose + 0.15 mM IPTG for minD). Plating efficiency was calculated as the ratio of colony forming units at restrictive versus permissive conditions.

Protein purification
Untagged E. coli FtsZ was purified as described previously [42]. Native OpgH was purified using the IMPACT Protein Purification System (New England Biolabs) as follows. BL21(DE3) cells harboring pH616 (pTYB4 + opgH) was grown in one-liter cultures with 100 μg/ml ampicillin at 37°C to mid-log, then induced with 1 mM IPTG for 4–6 h. (Inducting at temperatures under 37°C did not yield functional OpgH.) Cells were pelleted, washed in 1×PBS (pH 7.4), repelleted, and frozen at −80°C for later use. On the day of purification, cell pellets were thawed and resuspended in 30 ml of ice-cold IMPACT lysis buffer (20 mM Tris-HCl, 500 mM NaCl, 1 mM EDTA, pH 8) containing 1 mM dithiothreitol (DTT) was loaded to stimulate Intein auto-cleavage. The column was left incubating with DTT at 4°C for 20 h). The eluate yielded both the OpgH-Intein fusion (71.9 kDa) and OpgH (15.9 kDa). The fractions were pooled and transferred into dialysis tubing (7,000 MWCO) and concentrated with PEG. Aliquots were flash frozen in liquid nitrogen and stored at −80°C. Protein concentrations were determined by a Coomassie Plus (Pierce) assay using a SPECTRAmax Plus spectrophotometer (Molecular Devices) using a BSA standard.

90° angle light scattering assay
Light scattering assays were performed as described previously using a DM-45 spectrofluorimeter (Olis) [40,73]. Readings were taken every 0.5 s at 30°C. A baseline was established −60 s previous to the addition of 1 mM GTP. The reaction mixtures contained a final concentration of 5 μM FtsZ diluted in polymerization buffer (50 mM morpholinosulfonic acid (MES), 2.5 mM MgCl2, 1 mM EGTA, 50 mM KCl, pH 6.5) ± OpgH or OpgH buffer (20 mM Tris-HCl, 100 mM NaCl, 10% glycerol, pH 8). Data was collected by SpectralWorks (Olis) and exported into Microsoft Excel for processing. Baseline corrections were applied in Microsoft Excel to remove the background signal from unassembled FtsZ.

Regenerative coupled GTPase assay
FtsZ’s GTPase activity was evaluated as previously described [42] using a continuous, regenerative coupled GTPase assay [74]. Brieﬂy, experiments were done in the same buffer conditions as used for light scattering. A 150 μl reaction volume included: 1 mM phosphoenolpyruvate (PEP), 80 units/ml lactose dehydrogenase, 80 units/ml pyruvate kinase, 250 μM NADH, 1 mM GTP then varying amounts of FtsZ and OpgH or equivalent volume of respective buffer. Absorbance at 340 nm was measured at 30°C for 3 min in a quartz cuvette (1 cm path length) using a SPECTRAmax Plus spectrophotometer (Molecular Devices). Raw data from 60–120 s was translated into activity with the extinction coefﬁcient for NADH at 340 nm of 6220 M^−1 cm^−1.

Supporting Information

Figure S1  FtsZ84 levels are not elevated in pgm null strain. A representative quantitative immunoblot of FtsZ84 levels for WT and pgm::kan strains encoding the ftsZ84 allele at both permissive (30°C) and restrictive (42°C) growth conditions (See Figure 1D). The replication protein DnaA is shown as a loading control (LC). Relative expression (below) was calculated using WT grown at 30°C as the reference (n = 3).

Figure S2  MinD overexpression and FtsZ levels are congruent in wild type and UDP-glucose pathway mutants. A representative quantitative immunoblot of MinD overexpression and FtsZ levels in WT and the UDP-glucose pathway mutants encoding a plasmid with P::minD. Cells were cultured in LB ± 0.15 mM IPTG for ~5 h. Average fold overexpression level displayed below (n = 3).

Figure S3  The cell size defect is conﬁned to the UDP-glucose biosynthesis pathway. Cell area measurements of loss-of-functions mutations in genes adjacent to the UDP-glucose (see Figure 1A). Cells were grown in LB-glucose. >250 cells per strain were evaluated per replicate. Error bar equals standard deviation (n = 3).

Figure S4  Thio-OpgH-His expressed to similar levels. Expression levels for wild-type Thio-OpgH-His, a putative UDP-glucose binding mutant (PIC249AIA), or wild-type OpgH in a pgm::kan background. Cells were grown in LB ± 0.5% arabinose.

Figure S5  opgH-gfp and thio-opgH-his fusion constructs complement for size and glucosyltransferase activity. (A) Cell area distribution of WT (black), opgH::kan (white), opgH::kan + P::opgH-gfp (green), and opgH::kan + P::thio-opgH-his (red) grown in LB-glucose. The P::opgH-gfp is induced with 0.08 mM IPTG, while P::thio-opgH-his is induced with 0.25% arabinose. >250 cells were evaluated. Averages shown in the inset. (B) Swarm phenotypes are a proxy for glucosyltransferase activity (see Text
Figure S6 The ΔopgH cell size defect is independent of osmoregulated periplasmic glucans and Rcs activation. (A) Cell area measurements of an opgH null complemented with either \( P_{\text{rpsL}}-\text{opgG-gfp} \) or \( P_{\text{rpsL}}-\text{opgF-gfp} \). Cells grown in LB-glucose with 0.1 mM IPTG. (B) A representative quantitative immunoblot of FtsZ levels for WT and the UDP-glucose null strains. Lysates were normalized to total protein using a BSA assay. Relative expression (below) was calculated using WT as the reference \((n = 3)\). (C) Loss-of-function mutations in the UDP-glucose-pathway genes combined with null mutations in either the Rcs response regulator \( rcsB \) (left) or the alternative sigma factor \( rpoS \) (right). >250 cells per strain were evaluated three times. Error bars equal standard deviation \((n = 3)\).

Figure S7 An 18-amino acid peptide of OpgH is sufficient, though unnecessary, to inhibit division and localize to the division ring. (A) Cell area measurements of \( P_{\text{rpsL}}-\text{opgH}^{\text{Hi}} \)-his deletion constructs in a \( \text{opgH}^\text{kan} \) background cultured in LB±0.5% arabinose. Cells were cultured with inducer for ~3 h. >250 cells were assessed for cell area in each replicate. (B) Immunofluorescence of Thio-OpghH(83–101)-His and Thio-OpghH(211–248)-His after being cultured in LB+0.5% arabinose for 2 h. OpgH localization using an α-His antibody is shown in the top panel, α-FtsZ in the middle panel, and the colocalization displayed on the bottom panel (Opgh in green, FtsZ in red). The covariance of Opgh/FtsZ midcell localization is denoted below. Bar = 3 μm. (C) Quantitative immunoblot of the various Thio-OpghH constructs in the \( \text{opgH} \) null cultured in LB±0.5% arabinose. FtsZ is used as a loading control (below).

Figure S8 UDP-glucose pathway mutants reduce filamentation of \( \Delta \text{minCDE} \), \( \text{minCDE}:\text{kan} \), \( \Delta \text{pgm} \minCDE:\text{kan} \), \( \Delta \text{opgH} \minCDE:\text{kan} \) strains grown in LB-glucose, stained with FM4-64. The average area is listed below \((>150 \text{ cells counted, } n = 3)\). Bar = 5 μm. A representative immunoblot of FtsZ levels of the mutants is shown below.

Figure S9 Heat-treated OpghH\(^N\) loses ability to inhibit FtsZ. (A) A representative 90° angle light scattering plot and (B) percent assembly of FtsZ, assembly ± heat-treated OpghH\(^N\). Arrow indicates addition of 1 mM GTP. (C) The rate of GTP hydrolysis of FtsZ ± heat-treated OpghH. (A–C) FtsZ is at 5 μM, heat-treated OpghH\(^N\) is at 10 μM. OpghH was subjected to 30 minutes at 90°C to act as the heat-treated control. (D) The GTPase activity of calf intestinal phosphatase (CIP) was measured with either OpghH\(^N\) buffer (black) or 10 μM OpghH\(^N\) (blue). CIP’s activity is able to hydrolyze GTP is unaltered with the addition of OpghH\(^N\). This demonstrates that OpghH\(^N\)’s activity is specific to FtsZ and is not inhibiting a different reaction in the regenerative NADH-coupled assay. Error bars equal standard deviation \((n = 3)\).

Table S1 Phenotypes of combining defects in UDP-glucose synthesis (\( \Delta \text{pgm} \)) or \( \Delta \text{opgH} \) with inactivating characterized \( \text{E. coli} \) division inhibitors.

Table S2 Detailed cell size measurements of mutants associated with the UDP-glucose synthesis.

Table S3 Bacterial strains used in this study.

Table S4 Bacterial plasmids used in this study.

Text S1 A description of media conditions, strain construction, and auxiliary methods.

Author Contributions

Conceived and designed the experiments: NSH PAL. Performed the experiments: NSH YS PJB. Analyzed the data: NSH YS PJB. Contributed reagents/materials/analysis tools: NSH PJB PAL. Wrote the paper: NSH PJB.
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