FisB mediates membrane fission during sporulation in *Bacillus subtilis*

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How bacteria catalyze membrane fission during growth and differentiation is an outstanding question in prokaryotic cell biology. Here, we describe a protein (FisB, for fission protein B) that mediates membrane fission during the morphological process of spore formation in *Bacillus subtilis*. Sporulating cells divide asymmetrically, generating a large mother cell and smaller forespore. After division, the mother cell membranes migrate around the forespore in a phagocytic-like process called engulfment. Membrane fission releases the forespore into the mother cell cytoplasm. Cells lacking FisB are severely and specifically impaired in the fission reaction. Moreover, GFP-FisB forms dynamic foci that become immobilized at the site of fission. Purified FisB catalyzes lipid mixing *in vitro* and is only required in one of the fusing membranes, suggesting that FisB–lipid interactions drive membrane remodeling. Consistent with this idea, the extracytoplasmic domain of FisB binds with remarkable specificity to cardiolipin, a lipid enriched in the engulfing membranes and regions of negative curvature. We propose that membrane topology at the final stage of engulfment and FisB–cardiolipin interactions ensure that the mother cell membranes are severed at the right time and place. The unique properties of FisB set it apart from the known fission machineries in eukaryotes, suggesting that it represents a new class of fission proteins.

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The remodeling of lipid bilayers is essential to all organisms. These remodeling events include membrane fusion, wherein two membranes merge to become one, and membrane fission, where a single lipid bilayer divides into two. Our understanding of the molecular mechanisms underlying these processes comes from extensive studies in eukaryotes and the enveloped viruses that infect them. In eukaryotes, fusion and fission play central roles in a broad array of cell biological processes, including exocytosis, endocytosis, cytokinesis, and intracellular membrane trafficking (Chernomordik and Kozlov 2003; Südhof and Rothman 2009; Kozlov et al. 2010). They are also critical for the entry and exit of enveloped viruses into (and out of) their host (Kielian and Rey 2006; Harrison 2008). In contrast, virtually nothing is known about how bacteria remodel their membranes

during growth and differentiation despite the fact that they undergo membrane fission in every cell division cycle. Here, we describe the identification and characterization of a newly identified protein (FisB, for fission protein B) that functions in membrane fission during the developmental process of spore formation in the bacterium *Bacillus subtilis*.

From a topological perspective, fusion and fission can be seen as opposed processes. However, at the molecular level, both involve local disruption, bending, and bridging of lipid bilayers. Achieving lipid mixing during either fusion or fission requires forcing bilayers to within a critical distance of a few nanometers (Rand and Parsegian 1986; Wong et al. 1999; Kozlovsky and Kozlov 2003; Bashkirov et al. 2008; Kozlov et al. 2010). For both processes, hydration forces are thought to provide the major obstacle against lipid merger, which is alleviated by bending coupled to lipid tilting that allows hydrophobic attraction between the hydrocarbon chains of the lipids (Chernomordik and Kozlov 2003; Kozlov et al. 2010). Studies in eukaryotes and the viruses that infect them have revealed that specialized proteins generate the work

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required for these lipid rearrangements. In the case of eukaryotic membrane fusion, SNARE proteins anchored in apposed membranes form *trans*-complexes that overcome repulsive forces to bring bilayers into intimate contact. In enveloped viruses, membrane fusion proteins that are anchored in the viral membrane insert into host cell membranes and, through a series of conformational changes, bring the lipid bilayers into close proximity, leading to fusion. As for fission, the only factors described to date that participate in it are dynamin (Ferguson and De Camilli 2012) and the endosomal sorting complex required for transport (ESCRT-III complex) (Hurley 2010; Wollert and Hurley 2010; Henne et al. 2011). Dynamin and dynamin-like proteins are involved in endocytosis, multivesicular membrane budding, cytokinesis in chloroplasts, and mitochondrial genesis. These proteins are thought to function as “pinching machines”: They polymerize around the outside of the neck that connects two parts of a contiguous membrane, constricting the neck to a critical radius. GTP hydrolysis then produces conformational changes that result in fission (Ferguson and De Camilli 2012). In contrast, the ESCRT-III complex appears to assemble on the inside or at one of the openings of the neck to induce membrane constriction and, ultimately, fission (Hurley 2010; Henne et al. 2011). The ESCRT-III complex has been implicated in the biogenesis of multivesicular bodies (Gruenberg and Stenmark 2004; Hurley 2008; Wollert et al. 2009; Wollert and Hurley 2010), membrane abscission during cytokinesis (Elia et al. 2011), and budding of some enveloped viruses from the host membrane (Strack et al. 2003; Morita and Sundquist 2004; Bieniasz 2006). Interestingly, a homologous set of proteins appears to function in cytokinesis in the Crenarchaea *Sulfolobus* (Lindås et al. 2008; Samson et al. 2008).

The molecular mechanism underlying membrane fission during cytokinesis in bacteria remains enigmatic. It has been proposed that protein components of the cell division machinery could catalyze fission (Sharp and Pogliano 1999; Liu et al. 2006; de Boer 2010; Fleming et al. 2010). Alternatively, it has been suggested that cell wall synthesis on the outside of the cell could force the constricting membranes into close proximity, ultimately leading to fission (Weiss 2004; Judd et al. 2005; Meyer et al. 2010). To date, no protein has been directly implicated in this fission reaction.

Here, we investigated a specialized membrane fission event that occurs during sporulation in *B. subtilis* (Fig. 1). In response to starvation, *B. subtilis* differentiates into a dormant spore (Stragier and Losick 1996; Errington 2003). Upon initiation of this developmental process, the cell divides asymmetrically, generating two cells of unequal size and dissimilar fate. The smaller cell is the prospective spore and is referred to as the forespore. The larger cell is called the mother cell. Initially, these two cells lie side by side, separated by a double-membrane septum. However, shortly after polar division, the mother cell membranes migrate around the forespore in a phagocytic-like process called engulfment. In the last stage of this process, the leading edges of the migrating membranes meet at the cell pole and, upon membrane fission,

Figure 1. Membrane fission during spore development. Schematic representation of the morphological stages of the engulfment process. After polar division, the mother cell membranes migrate around the forespore. When they meet at (or near) the cell pole, membrane fission releases the forespore into the mother cell cytoplasm. Enlarged schematics highlight the engulfing membranes pre- and post-fission. (Middle panel) At the final stage, the membranes form a tube that is severed in the fission reaction. Fluorescent images show sporulating cells (strain BKM015) before and after membrane fission. Prior to fission, the lipophylic dye FM4-64 labels the peripheral membranes of the mother cell and the double membrane surrounding the forespore. After fission, the dye cannot access the membranes surrounding the forespore. The presence of a forespore within the mother cell is assessed by a fluorescent forespore reporter (P_{spoIIQ} -*Cfp*).

the forespore is released into the mother cell cytoplasm (Fig. 1). The forespore is thus surrounded by two membranes: an inner membrane that contains the forespore cytoplasm and an outer membrane derived from the mother cell membrane. The molecular mechanism underlying this membrane remodeling event is the focus of our work.

We describe the identification and characterization of a membrane protein, FisB, that is required for membrane fission at the last stage of engulfment. FisB is a bitopic membrane protein that is produced in the mother cell after polar division. In its absence, the migration of the mother cell membranes around the forespore proceeds normally, but engulfment stalls when the membranes reach the cell pole. In support of the idea that FisB functions in membrane remodeling, a functional GFP-FisB fusion localizes as a focus at the cell pole at the time of membrane remodeling. Consistent with a direct role in membrane remodeling, we show that recombinant FisB catalyzes lipid mixing *in vitro*. Interestingly, lipid-mixing activity required FisB in only one of the two interacting membranes, suggesting that FisB–lipid interactions drive membrane remodeling. In support of this idea, we show that the extracellular domain of FisB directly interacts with liposomes. Moreover, FisB–liposome interaction required the presence of the anionic phospholipid cardiolipin (CL). Altogether, our data support the idea that FisB

directly catalyzes the membrane fission event that marks the end of engulfment. Given the topology of FisB and that of the membrane tube to be severed, we suggest that FisB promotes fission from within or near one of the openings of the tube by interacting with CL that is enriched in the engulfment membranes and in regions of high negative curvature.

Results

FisB is required for membrane fission at the last stage of engulfment

Previous studies on membrane fission during sporulation implicated the polytopic membrane protein SpoIII_E (Sharp and Pogliano 1999, 2003). SpoIII_E is a DNA transporter that is required to translocate the forespore chromosome into the forespore compartment (Wu and Errington 1994). In the absence of SpoIII_E, sporulating cells are unable to pump the forespore chromosome and fail to make viable spores. Using an innovative membrane fission assay (described below), Sharp and Pogliano (1999) discovered that cells lacking SpoIII_E are also defective in membrane fission at a late stage of engulfment. In support of the idea that SpoIII_E functions directly in catalyzing fission, a SpoIII_E-GFP fusion protein localized to the forespore pole around the time of membrane fission (Sharp and Pogliano 1999). In the course of our studies on chromosome organization and segregation during sporulation (Burton et al. 2007; Marquis et al. 2008; Sullivan et al. 2009), we observed that sporulating cells lacking SpoIII_E exhibited defects in engulfment at stages prior to membrane fission. Under our assay conditions, 80%–90% of the SpoIII_E mutant cells had bulged septal membranes and/or invaginations that appeared to prevent complete membrane migration around the forespore (Supplemental Fig. S1). These observations led us to consider the possibility that SpoIII_E might only be indirectly involved in membrane fission and that other proteins catalyze this reaction.

We began our studies by investigating two candidate factors for a role in catalyzing fission: the *B. subtilis* dynamin homolog DynA (Bürmann et al. 2011) and the peptidoglycan synthetic machinery (Meyer et al. 2010). *B. subtilis* DynA has been shown to catalyze membrane fusion in vitro; however, cells lacking DynA have virtually no phenotype during vegetative growth (Bürmann et al. 2011). To investigate whether the DynA mutant was impaired in membrane fission during sporulation, we took advantage of the fission assay developed by Sharp and Pogliano (1999). In this assay, cells are induced to sporulate synchronously by nutrient downshift. At time points after polar division, the cells are stained with the membrane dye FM4-64 and visualized by fluorescence microscopy. Since this dye is unable to cross the lipid bilayer, the membranes surrounding the forespore are only labeled in cells that have not yet undergone fission (Fig. 1). Once fission occurs and the forespore is released into the mother cell cytosol, the membrane-impermeant dye only has access to the

peripheral mother cell membranes. To assess whether these cells have an engulfed forespore inside them, we used a forespore fluorescent reporter in which the gene encoding the cyan fluorescent protein (CFP) was placed under the control of the forespore-specific transcription factor σ^F (*P_{spoIIQ}-cfp*), as described previously (Fig. 1; Meyer et al. 2010). Using this assay, we found that engulfment and membrane fission were indistinguishable in wild-type cells and cells lacking DynA (Fig. 2A). Thus, dynamin does not appear to function in membrane fission during sporulation.

Next, we investigated whether cell wall synthesis between the forespore and mother cell membranes is involved in membrane fission. Dworkin and colleagues (Meyer et al. 2010) recently proposed that the action of cell wall synthetic complexes anchored in the mother cell membrane could promote membrane migration during engulfment and membrane fission. Using a membrane fission assay similar to the one described above, these researchers reported that the addition of antibiotics that inhibit cell wall synthesis during the engulfment process resulted in a fourfold reduction in fission. To investigate whether the force generated from cell wall synthesis was the principal driver of membrane fission, we performed similar antibiotic addition experiments (see the Materials and Methods). Under our assay conditions, we observed a defect in membrane fission, although the effect was more modest than observed previously. Membrane fission was reduced from 86% to 51% when the antibiotic fosfomycin was added at hour 1.5 of sporulation (Fig. 2A). The reduction in fission was even less pronounced when the drug was added at later time points (data not shown). These results suggest that peptidoglycan synthesis and the force generated by it could help bring the membranes into close proximity but indicate that the protein (or proteins) that catalyze membrane fission remain to be discovered.

DynA was not required for membrane fission, and we were unable to identify homologs of the ESCRT-III fission machinery in the *B. subtilis* genome. Accordingly, we turned to the hypothesis that fission is mediated by a fusion-like mechanism. We reasoned that if such a fission protein existed, it would likely be synthesized in the mother cell at an early stage of sporulation prior to the fission reaction and would have at least one transmembrane segment, and cells lacking this protein would have reduced sporulation efficiency. The first mother cell-specific transcription factor that is induced during sporulation is the alternative σ factor σ^E (Stragier and Losick 1996; Piggot and Losick 2002). The σ^E regulon has been defined by transcriptional profiling (Eichenberger et al. 2003; Feucht et al. 2003; Steil et al. 2005), and strains harboring deletions in most of the genes in the regulon have been generated (Eichenberger et al. 2003). Using the criteria described above, we examined a subset of this ordered library of mutants using the fluorescent membrane fission assay of Sharp and Pogliano (1999). Our analysis identified one mutant (in the *yunB* gene) that was severely impaired in membrane fission (Fig. 2A). Based on its mutant phenotype and the characterization

Figure 2. Cells lacking FisB are impaired in membrane fission. (A) Histogram shows fission efficiency (the percentage of sporulating cells that have undergone fission compared with all sporulating cells; $n > 1000$), 3 h after the onset of sporulation from a representative experiment. Wild type (BKM015), a DynA mutant (BAM027), wild type after the addition of 5 mM fosfomycin (Fos) that inhibits peptidoglycan synthesis at hour 1.5, and a FisB mutant (BTD3200) are shown. (B) Fission efficiency as a function of time from a representative experiment in a wild-type strain (wt, BKM15) and a FisB mutant (BTD3200). (C) Membrane fission was assessed by fluorescent microscopy during a sporulation time course in wild type (BKM015) and a FisB mutant (BTD3200). Both strains contained a fluorescent forespore reporter (P_{spoIIQ}-Cfp) to label all forespores. The membranes (false-colored red) were visualized with the fluorescent dye FM4-64 and merged with forespore CFP (false-colored green). Examples of sporulating cells in which fission has occurred are highlighted (yellow arrowheads). Time (in hours) after the initiation of sporulation is indicated. Bar, 1 μ m.

of the *yunB* gene product described below, we renamed this gene *fisB* for fission protein B.

To quantitatively assess the requirement for FisB in membrane fission, we compared wild type and the FisB mutant in a sporulation time course (Fig. 2B; Supplemental

Fig. S2). Two hours after the initiation of sporulation, 13% of wild-type cells ($n > 1000$) had completed membrane fission, releasing the forespore into the mother cell cytoplasm. At this time point, there was no detectable fission in the mutant. Thirty minutes later, at hour 2.5, 51% of wild-type sporulating cells ($n > 1000$) had undergone membrane fission compared with $<1\%$ in the FisB mutant ($n > 1000$). By hour 3, 79% of wild-type sporulating cells had completed membrane fission, but only 2% had completed membrane fission in the mutant. Importantly, in cells lacking FisB, the migration of the mother cell membranes around the forespore appeared indistinguishable from wild type (Fig. 2B; Supplemental Fig. S2). Moreover, similar to wild type, membrane migration appeared complete in most cells lacking FisB by hour 2.5. These results indicate that the absence of FisB does not impact the early steps of engulfment and thus suggest that FisB plays a specific role in the membrane fission process.

Interestingly, membrane fission was not completely abolished in cells lacking FisB. By hour 4.5, $\sim 21\%$ of the FisB mutant sporulating cells appeared to have undergone fission. This percentage is likely to be an overestimate because sporulating cells lacking FisB begin to lyse at this time point, reducing the total number of sporulating cells in the culture (Supplemental Fig. S2). FisB mutants have a sporulation efficiency of 12%–15%, suggesting that most of the cells that successfully undergo membrane fission are able to produce mature spores.

Altogether, these results suggest that FisB plays a specific role in membrane fission. In its absence, membrane migration around the forespore proceeds normally, but most sporulating cells fail to undergo membrane remodeling and release of the forespore into the mother cell cytoplasm. Cells that stall at the fission step ultimately lyse. Those that do complete fission do so after an ~ 2 -h delay.

FisB forms dynamic foci that localize to the pole at the time of membrane fission

FisB is predicted to be a bitopic membrane protein with a small N-terminal domain that resides in the mother cell cytoplasm and a larger (23-kDa) extracellular domain. To determine the subcellular localization of FisB, we generated an N-terminal monomeric GFP fusion to FisB expressed under its native promoter- and ribosome-binding site. The fusion protein complemented the FisB-null mutant for membrane fission and sporulation (data not shown). We visualized the GFP fusion by epifluorescence microscopy in a sporulation time course. For this experiment, we visualized engulfment and membrane fission using the membrane dye TMA-DPH. TMA-DPH inefficiently crosses the lipid bilayer and thus weakly labels the mother cell membrane that surrounds the forespore after fission is complete. Accordingly, using TMA-DPH, it is possible to distinguish pre- and post-fission stages without a forespore reporter. Supplemental Figure S3 shows a direct comparison of the two membrane dyes FM4-64 and TMA-DPH.

During engulfment at hour 2.5, GFP-FisB localized as discrete foci in both the peripheral membranes as well as the mother cell membranes that were engulfing the forespore (Fig. 3A). We were unable to quantify the number of FisB proteins in the foci, but the fluorescence intensity suggests that each focus is not a single protein and likely represents an oligomeric complex. Consistent with this idea, we found that both full-length FisB and the extracellular domain of FisB when purified from *Escherichia coli* can form oligomeric complexes in vitro (Supplemental Fig. S4). Time-lapse imaging revealed that the GFP-FisB foci were highly mobile in the membrane (Supplemental Movie M1). Importantly, at the time of membrane fission or just after it, a GFP-FisB focus was present near the cell pole adjacent to the forespore in a significant proportion of the cells. Strikingly, time-lapse imaging indicates that these polar foci remained immobile (Supplemental Movie M2). Figure 3B shows representative stills from a typical movie and an image in which each pixel is averaged over the entire time lapse. Dynamic foci are lost upon averaging, while immobile ones at the cell pole are retained.

In some cases, lowering the level of a fluorescent fusion will reveal or highlight specific subcellular localization, perhaps by reducing nonspecific or low-affinity binding (Rudner et al. 2002; Gregory et al. 2008). Accordingly, we constructed a YFP-FisB fusion expressed at levels approximately fivefold lower than the native protein (see the Materials and Methods; data not shown). The fusion protein complemented the *fisB*-null, restoring sporulation efficiency to wild-type levels. Moreover, YFP-FisB supported efficient membrane fission, albeit with a 20- to 30-min delay compared with wild type (Fig. 3C; data not shown).

During early engulfment stages, YFP-FisB localized in foci in the mother cell membranes, as observed with the

GFP-FisB fusion expressed at wild-type levels. These foci were more often found in the mother cell membranes that surrounded the forespore (Fig. 3C; Supplemental Fig. S5). Importantly, at hour 3, when sporulating cells were about to undergo fission or had just completed it, ~65% of the sporulating cells ($n > 1000$) exhibited a YFP-FisB focus at or near the cell pole adjacent to the forespore (Fig. 3C, yellow arrowheads; Supplemental Fig. S5, yellow arrowheads). In most cases, the polar focus was brighter than the foci in the peripheral and spore membranes, consistent with the idea that FisB assembles into a larger oligomeric complex at the pole. Because of the lower expression levels, we were unable to assess FisB dynamics due to rapid photobleaching of the fusion protein.

These results suggest that FisB forms mobile oligomeric complexes in the membranes of the mother cell. When the engulfing membranes reach the cell pole, a subset of these complexes localize to this site, where they participate in the fission reaction.

FisB mediates membrane remodeling in vitro

Altogether, our results show that FisB localizes to the site of fission and is required for membrane remodeling in vivo. To investigate whether FisB plays a direct role in the fission reaction and gain insight into the mechanism, we turned to in vitro experiments. Based on tomographic cryoelectron microscopy of *Caulobacter crescentus* cells undergoing cytokinesis (Judd et al. 2005), the connection between daughter cells prior to fission can be viewed as a tube 20–30 nm in length. We imagine a similarly sized membrane tube exists between the peripheral membrane of the mother cell and that surrounding the forespore. Since most of the FisB protein (amino acids 39–254) is present on the extracytoplasmic side of the membrane, if

Figure 3. GFP-FisB forms dynamic foci that become immobilized at or near the cell pole at the time of membrane fission. (A) Images show GFP-FisB (strain BAM003) at hour 3 of sporulation. The membranes were visualized with the fluorescent dye TMA-DPH. Examples of sporulating cells with a discrete GFP-FisB focus at the cell pole are highlighted (yellow arrowheads). (B) Representative images from a time-lapse movie showing the dynamic behavior of GFP-FisB foci in the mother cell membranes at hour 2.5 of sporulation. Images were acquired every 2 min. The bottom panel (AVG) shows an image in which each pixel is averaged over the entire time lapse. Yellow arrowheads highlight immobile GFP-FisB foci at the cell pole. (C) Low levels of YFP-FisB (strain BTD3124) reveal specific localization at the cell pole at the time of fission. Fluorescent images are from hour 2 (T2) and hour 3 (T3) of sporulation. Punctate YFP-FisB localization prior to fission is highlighted with white arrowheads. Yellow arrowheads show discrete YFP-FisB foci at the cell pole at the time of membrane fission. A schematic representation of FisB localization is shown below the micrographs. Bar, 1 μ m.

FisB indeed mediates membrane fission, it is unlikely to do so via a dynamin-like constriction from outside of the tube. Instead, FisB must do work on the membrane tube from within or at one of its openings, interacting with proteins and/or lipids across the tube. This mechanism is reminiscent of those employed by eukaryotic SNAREs and viral fusion proteins to force apposing bilayers into close proximity. We therefore reasoned that if FisB catalyzes fission in vivo, purified full-length FisB inserted into artificial liposomes should be able to induce lipid mixing and membrane remodeling in vitro, as has been shown for SNAREs (Weber et al. 1998). We used a standard fluorescence dequenching assay for lipid mixing (Struck et al. 1981) in which a population of liposomes bearing the fluorescent lipid 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(7-nitro-2,1,3-benzoxadiazol-4-yl) (NBD-PE) and its lipid-linked quencher, 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) (LR-PE), are mixed with unlabeled liposomes. Fusion of labeled and unlabeled liposomes increases the mean distance between the NBD-PE fluorophores and their quenchers, resulting in an increase in fluorescence (Fig. 4).

Full-length his-tagged FisB was expressed in *E. coli* and purified from detergent-solubilized membranes using the

Figure 4. FisB catalyzes membrane remodeling in vitro. The lipid-mixing assay based on fluorescence dequenching is shown schematically above the graph. Fusion of labeled and unlabeled liposomes increases the mean distance between the NBD-PE fluorophores and their quenchers, resulting in an increase in fluorescence. See the Materials and Methods for details. Lipid mixing was monitored as an increase in NBD fluorescence as a function of time after incubation of labeled and unlabeled liposomes. Liposomes contained FisB or mock-purified proteins (mock) or were protein-free (pf). Fluorescence is expressed as the fraction of the maximum dequenching obtained after addition of detergent at the end of the experiment. The graph shows the average of four independent fusion experiments from three different reconstitutions (error bars correspond to the standard error).

lipid-like detergent Fos-choline-12 (see the Materials and Methods). The purified protein was then reconstituted into labeled or unlabeled liposomes composed of a mixture of phospholipids (phosphatidylcholine [PC], phosphatidylglycerol [PG], phosphatidylethanolamine [PE], and CL) found in sporulating *B. subtilis* cells (Griffiths and Setlow 2009) or a mixture of the minimal lipids (1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine [POPC] and dioleoyl phosphatidyl serine [DOPS]) commonly used for SNARE-mediated fusion (Weber et al. 1998; Karatekin et al. 2010). To control for contaminating membrane proteins in the His-FisB-purified fraction, we performed a mock purification from *E. coli* cells harboring the empty expression plasmid (Supplemental Fig. S6). Labeled liposomes reconstituted with FisB or with mock-purified proteins (mock) or that were protein-free were mixed with unlabeled protein-free, FisB, or mock liposomes, and lipid mixing was monitored as an increase in NBD fluorescence as a function of time.

Using liposomes composed of lipids found in sporulating *B. subtilis*, efficient lipid mixing was observed when FisB liposomes were mixed with either FisB or protein-free liposomes (Fig. 4). Labeled FisB liposomes and unlabeled FisB liposomes resulted in slightly less efficient fusion than between FisB and protein-free liposomes, perhaps due to surface coverage by FisB reducing the available area of target membranes to interact with FisB in *trans*. Importantly, all combinations involving protein-free liposomes and liposomes containing mock-purified proteins resulted in negligible lipid mixing (Fig. 4; data not shown). Interestingly, using liposomes composed of POPC/DOPS (the minimal SNARE lipid mixture), no fusion was observed between FisB-FisB or FisB-protein-free liposomes (data not shown). In contrast, neuronal SNAREs incorporated into liposomes containing POPC/DOPS resulted in efficient lipid mixing comparable with previous reports (Supplemental Fig. S7). These results show that FisB possesses an intrinsic capacity to remodel lipid membranes in vitro and strongly suggest it can also do so in vivo. The data further imply that the lipid remodeling activity of FisB critically relies on specific protein-lipid interactions.

FisB binds to CL

The results of the membrane fusion assay with the two different lipid mixtures suggest that an interaction between a particular lipid in the *B. subtilis* lipid composition was essential for FisB's membrane remodeling activity. The simplest model is that this interaction occurs in *trans* and involves the extracellular domain of FisB (ECD). To test this idea and identify the lipids to which FisB binds, we used a coflotation assay (Fig. 5A; Bigay et al. 2005). The extracellular domain of FisB lacking its transmembrane segment was fused to a His tag, and the fusion protein was expressed in *E. coli* and purified from the soluble fraction (see the Materials and Methods). The soluble protein was then mixed with protein-free liposomes consisting of either the *B. subtilis* lipid mix (PG/PE/CL/PC) or the standard minimal SNARE composition

Figure 5. The extracellular domain of FisB binds CL in vitro. (A) Schematic representation of the coflotation assay. Protein-free liposomes were incubated with the soluble extracellular domain of FisB (ovals) for 2 h at room temperature. Liposomes and bound protein were separated from unbound protein using a four-step Nycodenz gradient. (B) The interface between the 0% and 30% step was collected and analyzed by SDS-PAGE stained with Coomassie blue. FisB bound to liposomes composed of a mixture of *B. subtilis* lipids (PG/PE/CL/PC) but not to liposomes used in SNARE fusion assays (POPC/DOPS). Input protein (20% of what was used in the binding assay) was loaded for comparison. Fractions from a step gradient lacking FisB are shown (ct). Molecular weight standards (50, 37, 25, and 20 kDa) (M) are indicated. (C,D) Coflotation assay in which the lipid composition of the liposomes was varied: PC/PG (70 mol/30 mol), PC/PE (70 mol/30 mol), PC/CL30 (70 mol/30 mol), PC/CL45 (55 mol/45 mol), and POPC/DOPS (85 mol/15 mol). (C) All liposomes contained a small percentage of NBD-PE as a fluorescent marker. Visualization of liposomes by fluorescence before (pre-float) and after (post-float) separation using a three-step idoxanol gradient (see the Materials and Methods). (D) Four fractions were collected as indicated in the post-float box and analyzed by SDS-PAGE stained with SYPRO Orange. FisB bound efficiently to liposomes containing CL but not to liposomes without CL.

(POPC/DOPS). The mixture was loaded at the bottom of a density step gradient, and the free liposomes and liposomes with bound protein were separated from unbound protein by ultracentrifugation (see the Materials and Methods). Consistent with the membrane fusion results, the His-FisB extracellular domain bound to and coflotated with the liposomes composed of the *B. subtilis* lipid mixture but not of the SNARE lipid mix (Fig. 5B).

To identify the lipids that FisB binds, we repeated the coflotation assay with liposomes in which we varied the lipid compositions (Fig. 5C,D). These experiments revealed that the extracellular domain of FisB binds CL but not to any appreciable degree to PC, PS, PE, or PG. The binding of FisB to CL was confirmed using both CL extracted from *E. coli* and synthetic tetra-oleoyl CL (data not shown). These results rule out the possibility that FisB binding was due to an impurity in the CL from *E. coli* and further suggest that FisB interacts with the head group of CL.

Discussion

The release of the engulfed forespore into the mother cell cytoplasm through membrane fission is a key step in the developmental process of sporulation. Here, we showed that the mother cell membrane protein FisB plays a central

role in this membrane fission reaction. Collectively, our data support a model in which FisB is inserted into the mother cell membrane during the early stages of engulfment, where it forms mobile complexes. As engulfment nears completion, FisB oligomers assemble at the site of membrane closure that connects the peripheral and spore membranes. FisB then severs this membrane tube, releasing the spore into the mother cell cytoplasm (Fig. 6). Virtually all endospore-forming bacteria in the low G+C Gram-positive branch possess a FisB homolog, while the protein appears to be absent in all other bacteria. Thus, FisB is likely to function exclusively in the fission reaction that occurs at the final stage of engulfment during sporulation, taking advantage of the unusual topology of the membranes at the site of fission.

Given enough time, a subset of sporulating cells lacking FisB successfully complete engulfment and membrane fission. This implies that an additional mechanism promotes fission during sporulation. Possible candidates include peptidoglycan synthesis as proposed by Dworkin and colleagues (Meyer et al. 2010) or peptidoglycan hydrolysis carried out by the membrane-anchored peptidoglycan hydrolase complex composed of SpoIID, SpoIIP, and SpoIIM (Abanes-De Mello et al. 2002; Aung et al. 2007; Morlot et al. 2010). Either of these machines could bring the engulfing membranes into close proximity

Figure 6. Model for FisB-mediated fission. Schematic representation of the membrane tube formed at the cell pole by the engulfing mother cell membranes. Due to its high negative curvature, CL (not shown) is thought to become enriched within the tube. FisB (green) becomes immobilized at this site through its interaction with CL. Oligomerization of FisB or FisB–lipid interactions are proposed to sever the tube, releasing the spore into the mother cell cytoplasm. See the Discussion for details.

and promote fission (Judd et al. 2005), although at a slower rate than FisB. Alternatively, SpoIIIE or an as yet unidentified factor could function in an independent fission pathway.

SpoIIIE and membrane fission

Our analysis of engulfment in cells lacking the SpoIIIE DNA translocase suggests that its activity is critical for membrane migration around the forespore prior to fission. These results do not exclude the possibility that it could also function in the fission reaction. However, our analysis of the FisB protein in SpoIIIE mutants provides a simpler explanation for the original observations of Sharp and Pogliano (1999). Immunoblot analysis of sporulating cells revealed that the levels of FisB were significantly reduced in the SpoIIIE-null mutant compared with wild type (Supplemental Fig. S8A). Moreover, in a SpoIIIE point mutant (SpoIIIE36) that binds DNA but is impaired in DNA transport (Wu and Errington 1994; Wu et al. 1995), FisB protein levels were unaffected, but YFP-FisB localization was disrupted (Supplemental Fig. S8B). In accordance with this finding, Sharp and Pogliano (1999) reported that the SpoIIIE36 mutant was impaired in membrane fission but had a less severe defect than the null. Based on these observations, we favor the idea that SpoIIIE is only indirectly involved in the fission reaction, impacting membrane migration and FisB abundance and/or localization. Interestingly, it has also been

proposed that SpoIIIE participates in fission during asymmetric division in sporulating cells (Liu et al. 2006; Fleming et al. 2010). This membrane remodeling event, wherein cytosol fills the gap inside the closing membrane annulus, has the opposite topology to the fission at the end of engulfment, where the extracellular medium fills the gap to be closed by the engulfing membrane (Supplemental Fig. S9). Intriguingly, SpoIIIE's architecture (large cytoplasmic domain anchored to the membrane) is opposite to that of FisB (membrane-anchored extracytoplasmic domain). Establishing a direct role for SpoIIIE in membrane remodeling awaits *in vitro* reconstitution.

Interactions between the extracellular domain of FisB and CL

We found that the extracellular domain of FisB interacts with CL, and our data suggest that this interaction is important for membrane remodeling *in vitro*. The specificity of this interaction is remarkable for a soluble domain. Although CL has high affinity for proteins with transmembrane segments involved in oxidative phosphorylation, such as the F_0F_1 ATPase (Haines 2009), many soluble proteins that bind CL, such as cytochrome *c* (Kagan et al. 2009) and tBid (Epand et al. 2002) in eukaryotes and MinD (Mileykovskaya et al. 2003) in bacteria, also interact with other, negatively charged lipids such as PS and PG to varying extents. In contrast, we could not detect significant interaction between the extracellular domain of FisB and the acidic lipids PG and PS or with the zwitterionic lipids PE and PC (or with digalactosyldiacylglycerol) (data not shown). Thus, we expect the dynamics of FisB and CL to be intimately coupled (see below). In this regard, it is noteworthy that during sporulation, the levels of CL increase, and CL is enriched in the engulfing membranes (Kawai et al. 2004). *B. subtilis* possesses three genes that encode proteins homologous to CL synthases: *clsA* (*ywnE*), which encodes the major CL synthase; *clsB* (*ywjE*); and *ywiE* (Kunst et al. 1997; Kawai et al. 2004). The expression of both *clsA* and *clsB* increases during sporulation, consistent with the idea that CL plays an important role during this process. Cells lacking all three genes are viable and have undetectable levels of CL during vegetative growth (Kawai et al. 2004). However, CL is readily detectable in the triple mutant during the early stages of sporulation, indicating that an additional sporulation-specific CL synthase exists or that CL is produced as a side reaction by a lipid synthesis/modification enzyme (Kawai et al. 2004, 2006; Tan et al. 2012). In accordance with the presence of CL during engulfment in cells lacking *ClsA*, *ClsB*, and *YwiE*, we found that the triple mutant was able to undergo fission at a frequency comparable with wild type (data not shown). This result suggests that if FisB requires CL to catalyze fission *in vivo*, then the reduced amount of CL made in the absence of the known synthases must be sufficient. Alternatively, FisB could use another phospholipid in addition to CL *in vivo*.

Late forespore gene expression does not require the completion of engulfment

Previous work has shown that the developmental programs of gene expression in the mother cell and forespore are not free-running clocks but are linked to each other by cell–cell signaling pathways (Stragier and Losick 1996; Rudner and Losick 2001; Rudner and Doan 2008). Furthermore, it has been hypothesized that these cell type-specific transcriptional programs are coupled to morphogenesis. Specifically, it was proposed that the activation of the forespore transcription factor σ^G is coupled to the completion of engulfment and membrane fission (Stragier and Losick 1996; Rudner and Losick 2001; Rudner and Doan 2008; Regan et al. 2012). One compelling model was that the isolation of the forespore from the external environment as a result of fission served as the trigger for σ^G . Recently, we reported evidence that argued against the coupling of σ^G activation to membrane fission (Doan et al. 2009). Specifically, we found that a subset (13%) of sporulating cells that was blocked at early stages of engulfment successfully activated σ^G in the forespore. However, the severe defect in morphogenesis in these mutants and the small number of cells that turned on σ^G raised concern that the activation might not be biologically relevant. Furthermore, recent genetic experiments using large insertions into the *B. subtilis* chromosome that delay DNA transport into the forespore suggest that σ^G activation could indeed be coupled to completion of engulfment (Regan et al. 2012). The FisB mutant allowed us to revisit the link between σ^G activity and morphogenesis in a strain in which engulfment proceeds normally and only fission is impaired.

Analysis of σ^G activity in the FisB mutant revealed that 47% ($n > 1000$) of the sporulating cells that were stalled at the membrane fission step successfully activated σ^G in the forespore compartment (Supplemental Fig. S10). This observation lends further support to the idea that σ^G activation and the fission reaction are not coupled events. Intriguingly, we also found that sporulating FisB mutants lyse at an elevated frequency, and this lysis was suppressed in cells lacking σ^G (Supplemental Fig. S11). This observation suggests that activation of σ^G prior to the completion of membrane fission is deleterious to the sporulating cell and leads to death. Thus, although a mechanism that holds σ^G inactive until fission is complete would appear to be beneficial, our data suggest that *B. subtilis* does not employ one. We suspect that under normal circumstances (in wild-type cells), σ^G activation and fission are temporally distinct enough that a coupling device is not required.

How might FisB mediate membrane scission?

Although membrane fission is a fundamental and ubiquitous biological process used by species ranging from the simplest bacteria and enveloped viruses to the most sophisticated neuronal cells, only two general fission machineries have been described. The first and best understood involves constriction of a membrane neck or tube from the outside by dynamin and its homologs

(Ferguson and De Camilli 2012). This constriction process relies on oligomerization of dynamin around the membrane neck and GTP hydrolysis to sever it. The second fission machinery is the ESCRT-III system (Hurley 2010; Henne et al. 2011), in which all components are soluble proteins that are recruited to the membrane via protein–protein and protein–lipid interactions. The only ESCRT-III component that seems to be absolutely required for fission is Snf7, whose oligomerization initiates the fission process, at least in vitro (Wollert and Hurley 2010). Other subunits likely terminate Snf7 filament assembly and recruit Vps4, an ATPase that disassembles the ESCRT-III complex and perhaps contributes to the fission reaction. The mechanism by which ESCRT-III mediates fission is still intensely debated (Hurley 2010; Henne et al. 2011); however, it is clear that the machinery works at the opening of and/or inside the membrane neck to achieve fission, rather than using constriction from outside the tube. Based on the topology of FisB with its large extracytoplasmic domain and very short (15-amino-acid) cytoplasmic domain, we suspect that FisB also catalyzes fission by doing work at one (or both) of the openings of the tube or from within it.

The activity of eukaryotic fusion and fission machines such as SNAREs and dynamin are exquisitely regulated in time and space by other proteins and intracellular signals. Potential regulators of FisB other than CL have not been identified. However, given the topology of the membranes during engulfment of the forespore, it is possible, in principle, that the dynamics of CL during sporulation, its propensity to associate with negatively curved membranes, FisB binding to CL, and homo-oligomerization of FisB could provide sufficient control over when and where fission occurs.

At the final stages of engulfment, the tube connecting the membrane engulfing the forespore to the rest of the mother cell membrane must shrink in diameter (Fig. 6). This will increase the negative curvature of the tube's inner leaflet, making it increasingly favorable for CL localization. We hypothesize that FisB becomes immobilized at the fission site as a result of its interaction with CL within the membrane tube. We found that FisB forms oligomers in vitro, and one possibility is that oligomerization leads to a filling of the tube with FisB and overflow to the tube's openings. The positive curvature of these openings would be unfavorable for CL localization. If FisB recruits CL to these positively curved regions, this could lead to the destabilization and membrane scission. In a variation of this model, FisB would localize to the highly positively curved regions at the openings of the tube by virtue of its own curvature preference and, by recruiting CL to such regions, would accumulate stresses, which could be relieved by severing the tube.

Other alternatives exist that are not mutually exclusive. For example, FisB oligomerization with concomitant conformational changes could be coupled to the destabilization of the tube, perhaps via twisting or compression. Alternatively, FisB could induce lateral segregation of CL (de Kruijff and Cullis 1980), with the resulting line tension and/or the discontinuity in elastic

properties between the CL-rich and CL-poor phases leading spontaneously to fission. Such fission occurs at domain boundaries between cholesterol-rich and cholesterol-poor tubules under certain conditions (Allain et al. 2004; Roux et al. 2005). Finally, FisB might act through a mechanism reminiscent of viral fusion proteins. Although fusion and fission are topologically opposite processes, microscopically, both require cutting, bending, and rejoining of membranes. From this point of view, it is perhaps not surprising that some homologs of dynamin-1 have been described to mediate fusion, not fission (Kozlov et al. 2010; Ferguson and De Camilli 2012). In this model, FisB anchored inside the membrane tube would interact with CL across the tube via its extracytoplasmic domain. CL binding could trigger a conformational change in FisB, as in the case of cytochrome c (Kagan et al. 2009), ultimately leading to membrane fission.

Further biochemical analysis of FisB *in vitro*, along with the identification of other proteins that function with or regulate FisB and the missing CL synthase, will help distinguish among these models for FisB function. However, our characterization of FisB raises the intriguing possibility that it is part of a novel fission mechanism.

Materials and methods

General methods

All *B. subtilis* strains were derived from the prototrophic strain PY79 (Youngman et al. 1983). Sporulation was induced by resuspension at 37°C according to the method of Sterlini-Mandelstam (Harwood and Cutting 1990) or by exhaustion in supplemented DS medium (Schaeffer et al. 1965). Sporulation efficiency was determined in 36-h cultures as the total number of heat-resistant (20 min at 80°C) colony-forming units (CFUs) compared with wild-type heat-resistant CFUs. Analysis of membrane fission after inhibition of cell wall synthesis was performed as described previously (Meyer et al. 2010) except cells were sporulated at 37°C and fosfomycin (5 mM final) was added at hour 1.5. Membrane fission was assessed at hours 2, 2.5, and 3. Insertion-deletion mutations were generated by isothermal assembly (Gibson et al. 2009) of PCR products followed by direct transformation into *B. subtilis*. Tables of strains, plasmids, and oligonucleotide primers and a description of plasmid construction can be found online in the Supplemental Material (Supplemental Tables S1–S3; Supplemental Material).

Protein purification and antibody production

His₆-FisB^{FL} and His₆-FisB^{ECD} fusion proteins were expressed in *E. coli* BL21 DE3 pLysS at 30°C and 16°C, respectively, and purified by affinity chromatography on Ni²⁺-NTA agarose (Qiagen). MBP-FisB^{ECD} fusion protein was expressed in *E. coli* NB42 and purified by affinity chromatography on amylose resin (New England Biolabs). A complete description of the purifications can be found in the Supplemental Material. MBP-FisB^{ECD} peak fractions were pooled and used to generate rabbit polyclonal antibodies (Covance). Crude serum was affinity-purified as described (Campo and Rudner 2006) using His₆-FisB^{ECD}.

Immunoblot analysis

Whole-cell lysates from sporulating cells induced by resuspension were prepared as described (Doan and Rudner 2007).

Samples were heated for 10 min at 50°C prior to loading. Equivalent loading was based on OD₆₀₀ at the time of harvest. Proteins were separated by SDS-PAGE on 12.5% polyacrylamide gels, electroblotted onto Immobilon-P membranes (Millipore), and blocked in 5% nonfat milk in phosphate-buffered saline (PBS) and 0.5% Tween-20. The blocked membranes were probed with affinity-purified anti-His (1:1000; Santa Cruz Biotechnology), anti-FisB (1:10,000), and anti-σ^F (15,000) (Carniol et al. 2004) diluted into 3% BSA in 1× PBS-0.05% Tween-20. Primary antibodies were detected using horseradish peroxidase-conjugated goat anti-rabbit G (Bio-Rad) and the Western Lightning reagent kit as described by the manufacturer (PerkinElmer).

In vitro lipid mixing assay

The protocol described in Weber et al. (1998) was followed with minor modifications. All lipids were purchased from Avanti Polar Lipids. For fluorescent donor liposomes, 100 μL of 3 mM lipids (L-α-PG [*E. coli*; sodium salt], CL [*E. coli*; sodium salt], L-α-phosphatidylethanolamine [*E. coli*, PE], L-α-PC [egg, chicken], NBD-PE [ammonium salt; 18:1], LR-PE [ammonium salt; 18:1]) in a molar ratio of 30:45:13:10:1:1 dissolved in chloroform were dried under nitrogen gas, placed under a vacuum desiccator for 1 h, and then dissolved in reconstitution buffer (RB; 25 mM Hepes at pH 7.4, 100 mM KCl, 10% glycerol, 1 mM DTT) supplemented with 1% (w/v) octyl-β-D-glucopyranoside (OG) and either FisB protein, the mock purification, or nothing to obtain protein-free liposomes. Unlabeled acceptor liposomes were prepared using 100 μL of 15 mM lipids as above in a molar ratio of 30:45:15:10. FisB protein was reconstituted into the lipids at a 1:1000 (protein:lipid) molar ratio, and then the OG was removed by overnight dialysis (6000- to 8000-Da molecular weight cutoff). Unincorporated protein was removed by flotation of the liposomes through a Nycodenz (Accurate Chemicals) step gradient. Labeled liposomes were recovered in a 150-μL volume (2 mM lipid). Unlabeled liposomes were collected in 400 μL (3.75 mM lipid).

The lipid-mixing assay was performed in white 96-well Maxisorp plates (Nunc). Liposomes (10 μL of labeled and 45 μL of unlabeled) were mixed on ice and then placed in a room temperature SpectraMax M5 Microplate Reader (Molecular Devices), and NBD fluorescence was recorded with filters set at 460 nm (excitation) and 538 nm (emission) at 30-sec intervals for 45 min. To determine the maximum NBD fluorescence, 10 μL of 2.5% (w/v) *n*-dodecyl-β-D-maltoside (DM) (Thermo Scientific) was added at the end of the experiment, and fluorescences were monitored for 15 min.

Flotation assay

Natural (PG, PE, and CL, all from *E. coli*, or egg PC) lipids or synthetic lipids (POPC), 1,2-dioleoyl-*sn*-glycero-3-phospho-L-serine (sodium salt), 1',3'-bis(1,2-dioleoyl-*sn*-glycero-3-phospho)-*sn*-glycerol (sodium salt; 18:1 CL), and 1,2-dioleoyl-*sn*-glycero-3-phospho-(1'-*rac*-glycerol) (sodium salt) from Avanti Polar Lipids were used. All liposomes included 0.2 mole percent NBD-PE (ammonium salt; 18:1) as a fluorescent marker. The lipids were dissolved in a 2:1 (v/v) mixture of chloroform and methanol. Lipids (1 μmol total) were mixed at desired ratios, and the mixtures were dried under nitrogen flow. Residual organic solvent was removed under vacuum for at least 2 h in a vacuum desiccator. Lipids were hydrated in 1 mL of RB-EDTA buffer [25 mM Hepes at pH 7.4, 140 mM KCl, 1 mM EDTA, 0.2 mM tris(2-carboxyethyl)phosphine] by shaking for >30 min. The lipid suspension was then immersed in liquid nitrogen and then allowed to thaw in a water bath at 37°C. This freeze-thaw cycle

was repeated five additional times to form large unilamellar vesicles (LUVs) (Pick 1981). The LUVs were either flash-frozen in liquid nitrogen and stored at -80°C or used fresh for extrusion. The LUV stock was extruded 19 times through 50-nm pore size polycarbonate filters (Avanti) at 40°C using a miniextruder.

The extruded liposomes (40 nmol total lipid) were incubated with 40 pmol of FisB extracytoplasmic domain in a total volume of 100 μL for 1 h at room temperature. Two-hundred milliliters of 60% iodoxanol density gradient medium (Optiprep, Sigma-Aldrich) was added to make a 40% iodoxanol solution (density, 1.215 g/mL) that was layered at the bottom of a 5-mm $\times$ 41-mm Beckman Ultra-Clear ultracentrifuge tube (Beckman Coulter, Inc.). This was overlaid with 200 μL of 20% iodoxanol solution (diluted in RB-EDTA; density, 1.110 g/mL), followed by a top layer of 200 μL of RB-EDTA. To assess liposome redistribution after floatation, NBD-PE fluorescence was measured before and after centrifugation as follows: Step gradients were prepared in parallel, and the tubes were placed in a rack in a gel-imaging instrument (ImageQuant LAS 4000, GE Healthcare). A mirror was placed at a 45° angle next to the rack, reflecting the image of the tubes to the camera placed above (Fig. 5C, pre-float; Bigay and Antonny 2005; Bigay et al. 2005). Blue epi-illumination was used, and fluorescence was collected through a green long-pass filter (Y515 Di). After imaging, the tubes were spun at 48,000 rpm (269,000g) for 1.5 h at 20°C . After floatation, the tubes were imaged again to assess the position of the liposomes in the gradient, and then fractions were collected from the bottom of each tube as indicated in Figure 5C, post-float. Because FisB could have varying affinities for the different lipids, we quantified both lipid and protein in all fractions. The amount of lipid in each fraction was quantified using NBD-PE fluorescence, and the amount of protein was quantified by PAGE (Novex mini Nu-PAGE Bis-tris, 12%, 1 mm thick; Invitrogen), stained with SYPRO Orange (Invitrogen). The sum of the protein from each fraction was usually less than the total initially mixed with liposomes (Fig. 5D). This is probably because some of the protein sticks to the walls of the ultracentrifuge tube. However, this was not an issue when comparing relative amounts of protein in different fractions (Bigay and Antonny 2005; Bigay et al. 2005).

Fluorescence microscopy

Fluorescence microscopy was performed on an Olympus BX61 microscope as previously described (Doan et al. 2005). Fluorescent signals were visualized with a phase-contrast objective UplanF1 100 $\times$ and captured with a monochrome CoolSnapHQ digital camera (Photometrics) using Metamorph software (Molecular Device). Exposure times were typically 500 msec for CFP and GFP and 2000 msec for YFP-FisB. Membranes were stained with either TMA-DPH or FM4-64 (Molecular Probes) at a final concentration of 0.01 mM and 3 $\mu\text{g}/\text{mL}$, respectively. Exposure times were typically 200 msec. Images were analyzed, adjusted, and cropped using Metamorph software. Cells were concentrated by centrifugation (8000 rpm for 30 sec) prior to visualization. This step had no impact on the localization of the fusion proteins.

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