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Christopher D. A. Rodrigues, Xavier Henry, Emmanuelle Neumann, Vilius Kurauskas, Laure Bellard, et al.. A ring-shaped conduit connects the mother cell and forespore during sporulation in *Bacillus subtilis*. *Proceedings of the National Academy of Sciences of the United States of America*, 2016, 113 (41), pp.11585-11590. 10.1073/pnas.1609604113 . hal-01378398

HAL Id: hal-01378398

<https://hal.univ-grenoble-alpes.fr/hal-01378398>

Submitted on 27 May 2020

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A ring-shaped conduit connects the mother cell and forespore during sporulation in *Bacillus subtilis*

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Edited by Thomas J. Silhavy, Princeton University, Princeton, NJ, and approved August 26, 2016 (received for review June 14, 2016)

During spore formation in *Bacillus subtilis* a transenvelope complex is assembled across the double membrane that separates the mother cell and forespore. This complex (called the “A–Q complex”) is required to maintain forespore development and is composed of proteins with remote homology to components of type II, III, and IV secretion systems found in Gram-negative bacteria. Here, we show that one of these proteins, SpoIIAG, which has remote homology to ring-forming proteins found in type III secretion systems, assembles into an oligomeric ring in the periplasmic-like space between the two membranes. Three-dimensional reconstruction of images generated by cryo-electron microscopy indicates that the SpoIIAG ring has a cup-and-saucer architecture with a 6-nm central pore. Structural modeling of SpoIIAG generated a 24-member ring with dimensions similar to those of the EM-derived saucer. Point mutations in the predicted oligomeric interface disrupted ring formation in vitro and impaired forespore gene expression and efficient spore formation in vivo. Taken together, our data provide strong support for the model in which the A–Q transenvelope complex contains a conduit that connects the mother cell and forespore. We propose that a set of stacked rings spans the intermembrane space, as has been found for type III secretion systems.

sporulation | SpoIIAG | type III secretion system | EscJ/PrgK/FlhF | SigG

Transport of proteins across the outer membrane of Gram-negative bacteria requires specialized secretion systems (1, 2). These transenvelope complexes span the inner and outer membranes and use ATP hydrolysis in the cytoplasm to power secretion across the outer membrane. Gram-positive bacteria lack an outer member and in most cases lack these specialized secretion systems. Endospore formation in bacteria such as *Bacillus subtilis* provides an unusual and noteworthy example of a Gram-positive, double-membrane envelope. As a result of the phagocytic-like process of engulfment, the developing endospore (called the “forespore”) is released into its sister cell (referred to as the “mother cell”), surrounded by an inner membrane derived from the forespore and an outer membrane derived from the mother cell (Fig. 1B) (3). Intriguingly, the mother cell and forespore assemble a multimeric complex spanning these two membranes that bears similarity to specialized secretion systems and is required to maintain forespore development (4–9). It has been proposed that these proteins constitute a hybrid specialized secretion system with a channel connecting mother cell and forespore. Here we provide direct evidence that this complex contains a ring-like conduit in the space between the two membranes.

B. subtilis differentiates into a stress-resistant spore in response to nutrient limitation (3). The first morphological event in this process is the formation of an asymmetrically placed septum generating a small forespore and larger mother cell. Shortly afterwards, the mother cell membranes migrate around the forespore, generating a cell within a cell. Eight mother cell proteins encoded in the *spoIIA* operon (AA, AB, AC, AD, AE, AF, AG, and AH) and one forespore protein, SpoIIQ (Q), are required during and/or shortly after engulfment to maintain forespore development. Cells lacking any of these nine proteins have the same phenotype: The engulfed

forespores fail to grow to their full size and frequently develop membrane invaginations and appear to collapse (7, 10). In addition, these forespores are unable to maintain transcriptional potential including gene expression under the late-acting forespore transcription factor SigG (5, 7).

Previous work indicates that most of the A–Q proteins reside in a multimeric complex that spans the two membranes surrounding the forespore; for simplicity, we refer to this complex as a “transenvelope complex,” although whether it spans the nascent spore coat remains unclear (7). Q produced in the forespore localizes to the inner forespore membrane and is required for the localization of AH in the outer forespore membrane (11, 12). This localization is mediated by direct protein–protein interaction between the extracellular domains of AH and Q in the intermembrane space (Fig. 1B) (11, 12). Furthermore, coimmunoprecipitation experiments have shown that AB, AD, AE, AF, and AG reside in a multimeric membrane complex (7). Finally, AG has been found to localize in the membranes surrounding the forespore (Fig. 14), and this localization depends on AH and Q (7).

The role of this complex in maintaining forespore development has been informed by the remote homologies (13) of many of the proteins in this complex (4, 6, 7). AA resembles secretion

Significance

Specialized secretion systems transport proteins across the double-membrane cell envelope of Gram-negative bacteria. Gram-positive bacteria possess a single membrane and lack many of these secretion systems. During endospore formation in Gram-positive bacteria such as *Bacillus subtilis*, a double-membrane envelope surrounds the developing spore. A transenvelope complex with similarities to Gram-negative specialized secretion systems spans the two membranes separating mother cell and endospore. This complex is essential for development and has been hypothesized to serve as a channel for molecular transport between the two cells. Here we show that it contains an oligomeric ring with architecture and dimensions similar to those found in type III secretion systems, providing direct evidence for a conduit connecting mother cell and developing spore.

Author contributions: C.D.A.R., D.Z.R., and C.M. designed research; C.D.A.R., X.H., E.N., V.K., L.B., Y.F., P.S., G.S., D.Z.R., and C.M. performed research; C.D.A.R., X.H., E.N., V.K., Y.F., P.S., G.S., D.Z.R., and C.M. analyzed data; and C.D.A.R., E.N., P.S., G.S., D.Z.R., and C.M. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

Data deposition: The cryo-EM 3D reconstruction map of the D1+D2 rings of SpoIIAG from *Bacillus subtilis* has been deposited in the EMDDataBank (EMDB ID code EMD-4072).

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1609604113/-DCSupplemental.

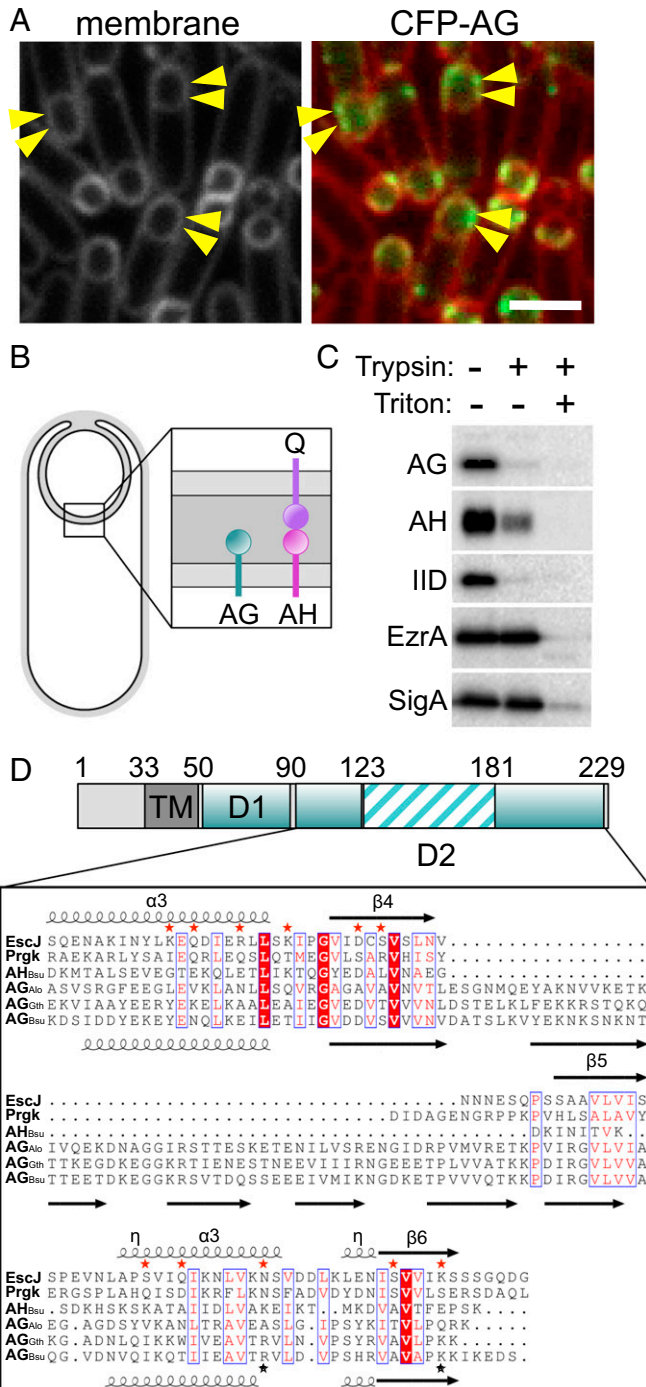


Fig. 1. The extracellular domain of AG has remote homology to PrgK/EscJ proteins in type III secretion systems. (A) CFP-SpolIIAG (CFP-AG, false-colored green) localizes as discrete foci (arrowheads) in the mother cell membranes that surround the forespore. CFP-AG displays weak localization at the second potential polar division site, but the relevance of this localization remains unclear. CFP-AG and membranes (stained with the fluorescent probe TMA-DPH) were visualized by fluorescence microscopy at hour 2 of sporulation. (Scale bar, 2 μ m.) (B) Schematic diagram showing the localization and topology of AG, SpolIIAH (AH), and SpolIIQ (Q) during sporulation. (C) The extracellular domains of AG, AH, and SpolIID (IID) are extracytoplasmic. Immunoblot of a protease susceptibility assay in which sporulating cells were protoplasted and treated with trypsin with or without Triton X-100. A soluble transcription factor (SigA) and a membrane-anchored cytoplasmic protein (EzrA) were inaccessible to trypsin. (D) Domain structure of AG showing the transmembrane segment (TM) and the extracytoplasmic D1 and D2 domains. Numbering refers to the *B. subtilis* AG sequence.

ATPases found in type IV secretion systems, whereas AB and AE both have domains with remote homology to GspF that helps tether the secretion ATPase to the membrane complex in type II secretion systems. Finally, AF, AG, and AH have remote homology to the EscJ/PrgK family of ring-forming proteins found in type III secretion systems. Importantly, cryocrystal structures of a heterodimeric complex consisting of the extracellular domains of AH and Q revealed structural similarity between AH and EscJ/PrgK family members (14, 15). Furthermore, using this structure, the extracellular domains of AH and Q could be modeled into oligomeric rings. Based on the remote sequence homologies and this structural similarity, it has been proposed that AH and Q form a channel in the intermembrane space and that the A-Q complex functions as a specialized secretion system or a feeding tube, allowing the mother cell to nurture the forespore and maintain forespore development (4-7, 14, 15). The structure of this transenvelope complex and whether it functions to transport molecules between the two cells remain important outstanding questions.

Here, we show that the extracellular domains of AG from three endospore formers, *B. subtilis*, *Geobacillus thermodenitrificans*, and *Acetone nema longum*, assemble into large oligomeric rings in vitro. 3D reconstruction of images generated by cryo-EM indicates that the *B. subtilis* AG ring has a “cup-and-saucer” architecture, similar to that of the EscJ/PrpK family member FliF, which is part of the flagellar basal body (16). Structural modeling of AG generated a ring with dimensions similar to those of the saucer, and point mutants in the predicted oligomeric interface disrupted ring formation in vitro and impaired SigG activity and spore formation in vivo. These data indicate that the A–Q complex contains a conduit that connects the mother cell and the forespore and support a model in which stacked rings similar to those found in type III secretion systems span the intermembrane space.

Results

The Extracellular Domain of AG Has Remote Homology to the EscJ/PrgK Family. The AG protein has a single N-terminal transmembrane segment and a large soluble domain (Fig. 1D). A sequence alignment of more than 20 AG orthologs using ClustalW (17) indicates that the soluble domain displays low sequence conservation over the first ~40 amino acids (designated “D1”) and higher sequence conservation over the remaining ~140 C-terminal residues (called “D2”) (Fig. 1D). The D2 domain of AG has been reported previously to have remote homology to the EscJ/PrgK family of ring-forming proteins in type III secretion systems (6, 7). Alignment of the D2 domain from AG orthologs with EscJ from *Escherichia coli* EPEC (18) and PrgK from *Salmonella typhimurium* SPI-1 (19, 20) revealed that AG proteins contain two regions (residues 90–123 and 181–229) that share weak sequence and secondary structure similarities with these ring-forming proteins. However, AG orthologs contain an insertion between these regions (residues 124–180) with no homology to known structures (Fig. 1D). This extended D2 domain was recently reported by Bergeron (21). To investigate whether the soluble portion of AG (D1+D2) resides in the cytoplasm or, like AH, in the space between the mother cell and forespore membranes (Fig. 1B), we analyzed its localization by protease susceptibility. Sporulating cells were treated with lysozyme in hypertonic buffer to generate

The sequences of AG from *B. subtilis* (AG_{Bsu}), *G. thermodenitrificans* NG-80 (AG_{Gth}), and *A. longum* (AG_{Ala}) were aligned with *S. typhimurium* SPI-1 and PrgK and *E. coli* EPEC LEE EsC. Conserved residues are in red boxes; similar residues are shown by red letters boxed in blue. The secondary structures of EsC and the ones predicted for AG_{Bsu} are indicated above and below the sequence alignment, respectively. Arrows indicate β -strands; α , α -helix; η , 3_{10} -helices. Residues at the oligomeric interface of EsC protomers are indicated by red stars. Predicted AG_{Bsu} interface residues important for ring formation and sporulation are indicated by with black stars.

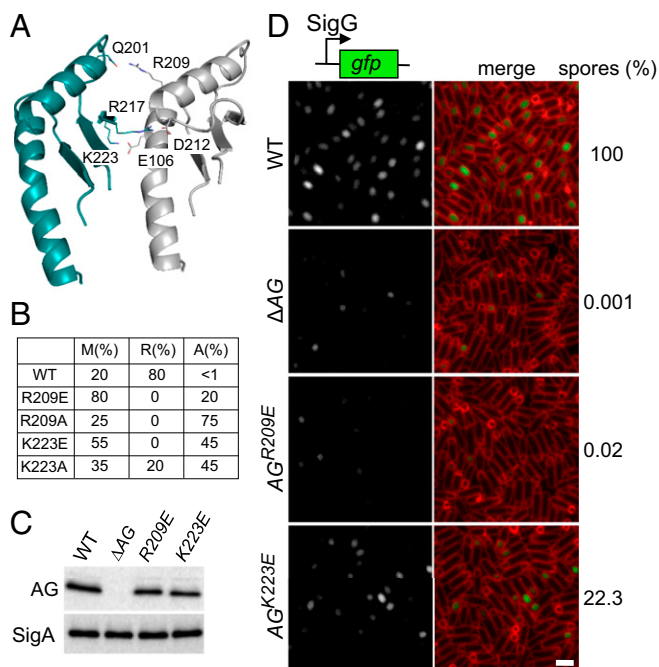


Fig. 4. In vitro and in vivo test of the AG ring model. (A) Close-up view of the interface between two adjacent subunits in the saucer region of the AG_{Bsu} D2 ring model. Residues predicted to make up the oligomeric interface are labeled. (B) Table showing the proportion of monomers (M), rings (R), and aggregates (A) assessed by gel filtration and negative-stain EM for the AG_{Bsu} D1+D2 variants. The primary data can be found in Fig. S5. (C) Immunoblot analysis of whole-cell lysates from sporulating wild-type (BCR1434), ΔAG mutant (BCR776), AG(R209E) (BCR1435), and AG(K223E) (BCR1436) with anti-AG antibodies. SigA levels were monitored to control for loading. (D) SigG activity (Left) and sporulation efficiency (Right) of *B. subtilis* cells with AG mutants. Wild-type (BCR1438), ΔAG mutant (BCR1437), and cells expressing AG(R209E) (BCR1439) or AG(K223E) (BCR1440) were visualized 4 h after the initiation of sporulation. All strains harbor a SigG-responsive promoter (P_{sigG}) fused to GFP. Images of P_{sigG} -GFP fluorescence (Left) and of P_{sigG} -GFP fluorescence images merged with TMA-DPH-stained membranes (Right) are shown. The images were scaled identically. (Scale bar, 2 μ m.)

presented above, suggest that AG and AH both contribute to the conduit that connects the mother cell and the forespore.

Discussion

Here, we have shown that AG homologs from *B. subtilis* and two distantly related species that form endospores assemble into large rings with a cup-and-saucer architecture. Mutations in predicted interface residues in the AG_{Bsu} ring result in impaired ring assembly in vitro and reduced SigG activity and sporulation in vivo. Collectively, these results provide evidence for a conduit between the mother cell and forespore and support the idea that the A–Q complex could function as a channel or secretion complex.

Our modeling suggests that the AG cup results from the oligomerization of a region inserted within the ring-building motif (RBM) that is predicted to contain two long antiparallel β -strands. Intriguingly, the third RBM of FliF in the flagellar basal body also has an insertion region predicted to be rich in β secondary structures (21). Accordingly, we hypothesize that the FliF cup (called the “R region”) may be similarly composed of this insertion and form a large β -barrel. In the case of FliF, the cup faces the outer membrane where it engages the flagellar rod complex (16, 24). Although the orientation of the AG cup is not known, we favor a model in which it also faces toward the forespore (away from the mother cell membrane), because our data suggest that the D1 domain is part of the base of the saucer and this domain is directly preceded by AG’s transmembrane segment (Fig. 1B). In addition, this orientation of the ring is similar to that of the PrgK and EscJ rings in type III secretion systems (18, 20).

The structural similarities between the extracellular domain of AH and EscJ/PrgK proteins and in silico modeling suggested that AH together with Q oligomerize into a pair of rings (14, 15). Our finding that three distinct AG homologs assemble into rings strengthens and extends this model. Because Q is synthesized in the forespore and AH in the mother cell, the Q ring likely resides in close apposition to the forespore membrane followed by AH (Fig. 5). We have previously shown that the localization of AG to the membranes surrounding the forespore requires AH and Q, suggesting that the AG ring could stack against AH by analogy to the stacked rings found in type III secretion systems (2, 18). If, as our data suggest, the AG cup faces toward the forespore, then in the context of this model the cup region would contact the AH ring (Fig. 5). How it does so is unknown at present. To accommodate steric clashes between AH protomers (and, separately, Q protomers), AH–Q ring models containing 12, 15, and 18 subunits have been proposed (14, 15). A 15-member AH–Q ring has a pore size of ~ 7 nm and could not accommodate the AG cup with an outer diameter of ~ 11 nm. Accordingly, in this model, the “lip” of the cup would contact the AH ring. If instead the cup is inserted into the pore of the AH ring, then the latter ring must contain at least 18 protomers. An alternative possibility is that AH assembles into a 24-member ring, which would allow 1:1 interactions between AG and AH subunits, as is the case with PrgK and PrgH (20). It is noteworthy that, in addition to the mother cell-specific (SigE) promoter upstream of the *spoIIIA* operon, there is a second SigE-responsive promoter within the *spoIIIAF* (AF) gene (25). This promoter results in increased expression of AG and AH, suggesting that the stoichiometry of these two components is higher than that of the other SpoIIIA proteins in the complex and is consistent with a stacked ring model. Our attempts to generate heteromeric complexes with purified extracellular domains of AG, AH, and Q have been unsuccessful thus far (SI Materials and Methods). Addressing the precise stoichiometry and organization of the rings in the A–Q complex will ultimately require a high-resolution structure of the intact complex, ideally purified directly from sporulating cells.

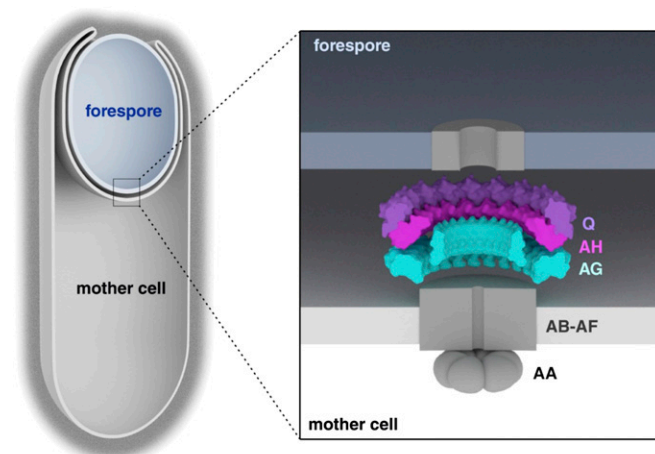


Fig. 5. AG, AH, and Q form stacked rings in the intermembrane space. Schematic diagram showing the A–Q complex in the two membranes that surround the forespore. AH (in magenta) and Q (in purple) are shown as a double ring containing 18 protomers of each, as proposed by Meisner et al. (15). The AG D2 ring model fitted in the experimental EM map is shown in cyan. The other membrane proteins encoded in the *spoIIIA* operon (AB–AF) are shown schematically as a single complex (gray) with a predicted membrane pore. AA is shown as a hexamer by analogy to other secretion ATPases. Evidence suggests the existence of a pore in the forespore membrane (6) (shown schematically in gray); the identity of this protein is unknown.

Extending the analogy between the A–Q complex and type III secretion systems and the flagellar basal body, a needle or inner rod complex might be expected to reside in the pore generated by AG and AH. No proteins currently known to be required for SigG activity are similar to those that make up these tube-like structures. Accordingly, whether such a structure exists in the A–Q complex remains an outstanding question. The absence of any candidate protein for an inner rod or needle raises the possibility that the A–Q complex uses its type III-like proteins to generate a channel (4, 5). In line with this idea, AH, AG, and AF are the only proteins in the A–Q complex that share sequence similarity with proteins found in type III secretion systems. Indeed, AA most closely resembles secretion ATPases found in type IV secretion systems, and the polytopic membrane proteins AB and AE both have domains with remote homology to GspF from type II secretion systems. One popular model that accounts for the absence of an inner rod or needle is that the complex functions as a feeding tube (5) in which the ATPase component and associated integral membrane proteins act as a gate or energy source for transport of undefined molecules across the outer forespore membrane and into the AG/AH/Q channel.

Interestingly, recent work in the endospore former *Clostridium difficile* suggests that the A–Q complex may be dispensable for late forespore gene expression under SigG control and therefore may not play a direct role in maintaining transcriptional potential in the *C. difficile* forespore (8, 9). Intriguingly, both studies identified morphological defects in spore differentiation in the absence of the A–Q complex, suggesting that the complex could play a structural role in maintaining forespore development. These findings raise the possibility that this highly conserved transenvelope complex might not be a secretion complex or a feeding tube. One possibility that is compatible with the remote homologies and structural similarities described here is that the complex is a noncanonical piliation system

and, for example, functions to adhere the two membranes together. In its absence, the forespore develops morphological defects, fails to mature, and, in the case of *B. subtilis*, loses transcriptional potential. Although currently there is no candidate pilin or pseudopilin protein that would connect the two cells, it is possible that, as in the case of the SpoIIIA proteins, this factor has remote or even undetectable homology to counterparts in canonical piliation and type II secretion systems. Future genetic and biochemical studies will be required to distinguish among these models, while structure characterization will help unravel the global architecture of the A–Q complex.

Materials and Methods

All *B. subtilis* strains were derived from the prototrophic strain PY79 (26). Sporulation assays and fluorescence microscopy were performed as previously described (7). All recombinant proteins were overexpressed in *E. coli* Rosetta (DE3) pLysS and were affinity purified as His-SUMO fusions (27) followed by size-exclusion chromatography. For cryo-EM, purified AG_{BSU} was loaded onto a Quantifoil R2/1 holey grid and vitrified using a Mark IV Vitrobot; images were acquired on a Polara electron microscope. Detailed protocols are provided in *SI Materials and Methods*, and strains, plasmids, and oligonucleotide primers are listed in Table S1.

ACKNOWLEDGMENTS. We thank members of the Vernet, D.Z.R., Dessen, and Bernhardt laboratories for advice and encouragement; Janet Iwasa for figure preparation; Daphna Fenel for negative-stain EM imaging; Amy Camp for sharing strains; and Christine Ebel and Aline Le Roy for analytical ultracentrifugation analyses. Support for this work came from NIH Grant GM086466 (to D.Z.R.) and Agence Nationale de la Recherche (ANR) Grant ANR-11-BSV8-005-01 PILIPATH. This work used the platforms of the Grenoble Instruct Centre (Integrated Structural Biology Grenoble, UMS 3518 CNRS-Commissariat à l'Energie Atomique et aux Energies Alternatives-Université Grenoble Alpes-EMBL), with support from the French Infrastructure for Integrated Structural Biology Initiative (FRISBI) Grant ANR-10-INSB-05-02 and the Grenoble Alliance for Integrated Structural Cell Biology (GRAL) Grant ANR-10-LABX-49-01 within the Grenoble Partnership for Structural Biology (PSB).

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