The secreted protein kinase CstK from *Coxiella burnetii* influences vacuole development and interacts with the GTPase-activating host protein TBC1D5

The intracellular bacterial pathogen *Coxiella burnetii* is the etiological agent of the emerging zoonosis Q fever. Crucial to its pathogenesis is type 4b secretion system–mediated secretion of bacterial effectors into host cells that subvert host cell membrane trafficking, leading to the biogenesis of a parasitophorous vacuole for intracellular replication. The characterization of prokaryotic serine/threonine protein kinases in bacterial pathogens is emerging as an important strategy to better understand host–pathogen interactions. In this study, we investigated CstK (for *Coxiella* Ser/Thr kinase), a protein kinase identified in *C. burnetii* by *in silico* analysis. We demonstrate that this putative protein kinase undergoes autophosphorylation on Thr and Tyr residues and phosphorylates a classical eukaryotic protein kinase substrate *in vitro*. This dual Thr-Tyr kinase activity is also observed for a eukaryotic dual-specificity Tyr phosphorylation-regulated kinase. We found that CstK is translocated during infections and localizes to *Coxiella*-containing vacuoles (CCVs). Moreover, a CstK-overexpressing *C. burnetii* strain displayed a severe CCV development phenotype, suggesting that CstK fine-tunes CCV biogenesis during the infection. Protein–protein interaction experiments identified the Rab7 GTPase-activating protein TBC1D5 as a candidate CstK-specific target, suggesting a role for this host GTPase-activating protein in *Coxiella* infections. Indeed, CstK co-localized with TBC1D5 in non-infected cells, and TBC1D5 was recruited to CCVs in infected cells. Accordingly, TBC1D5 depletion from infected cells significantly affected CCV development. Our results indicate that CstK functions as a bacterial effector protein that interacts with the host protein TBC1D5 during vacuole biogenesis and intracellular replication.

Signal transduction is an essential and universal function that allows all cells, from prokaryotes to eukaryotes, to translate environmental signals to adaptive changes. By this mechanism, extracellular inputs propagate through complex signaling networks whose activity is often regulated by reversible protein phosphorylation. Signaling mediated by serine/threonine/tyrosine protein phosphorylation has been extensively studied in eukaryotes; however, its relevance in prokaryotes has only begun to be appreciated. The recent discovery that bacteria also use Ser/Thr/Tyr kinase-based signaling pathways has opened new perspectives to study environmental adaptation, especially in the case of bacterial pathogens, with respect to host infection (1). Thus, advances in genetic strategies and genome sequencing have revealed the existence of “eukaryotic-like” serine/threonine protein kinases (STPKs) and phosphatases in a number of prokaryotic organisms (2), including pathogens such as *Streptococcus* spp. (3–6), *Mycobacteria* (7–12), *Yersinia* spp. (13, 14), *Listeria monocytogenes* (15, 16), *Pseudomonas aeruginosa* (17), *Enterococcus faecalis* (18), or *Staphylococcus aureus* (19, 20). Consequently, the study of STPKs in human bacterial pathogens is emerging as an important strategy to better understand host–pathogen interactions and develop new, targeted antimicrobial therapies. However, if on one hand it is clear that STPKs and phosphatases regulate important functions in bacterial pathogens, their signal transduction mechanism remains ill-defined and restricted to a limited number of microbes.

Importantly, STPKs expressed by pathogenic bacteria can either act as key regulators of important microbial processes or be translocated by secretion systems to interact with host substrates, thereby subverting essential host functions including the immune response, cell shape, and integrity (21). Phosphorylation of host substrates has been demonstrated for some bacterial STPKs, whereas others seem to require their kinase activity, but their phosphorylated substrates remain to be identified (21). Therefore, biochemical mechanisms of these pathogen-directed targeted perturbations in the host cell–signaling network are being actively investigated, and STPKs are proving to be translocated by secretion systems to interact with host substrates, thereby subverting essential host functions including the immune response, cell shape, and integrity (21). Phosphorylation of host substrates has been demonstrated for some bacterial STPKs, whereas others seem to require their kinase activity, but their phosphorylated substrates remain to be identified (21). Therefore, biochemical mechanisms of these pathogen-directed targeted perturbations in the host cell–signaling network are being actively investigated, and STPKs are proving to

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**4** The abbreviations used are: STPK, serine/threonine protein kinase; IPTG, isopropyl β-D-thiogalactopyranoside; HA, hemagglutinin; CCV, *Coxiella*-containing vacuole; BLAM, β-lactamase; GST, glutathione S-transferase; MBP, myelin basic protein; qRT-PCR, quantitative RT-PCR; ROI, region of interest; ANOVA, analysis of variance.

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This work was supported by grants from the ATIP/AVENIR Program (to V. M. and M. Bonazzi), the Region Occitanie (to S. B. and V. M.), Marie Curie Action Career Integration Grant 293731 (to E. M. and M. Bonazzi), and Agence Nationale de la Recherche Grant ANR-14-CE14-0012-01 through project AttaQ (to M. Bonazzi). The authors declare that they have no conflicts of interest with the contents of this article.

This article contains Table S1 and Figs. S1 and S2.

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be molecular switches that play key roles in host–pathogen interactions (21).

Among emerging human pathogens, Coxiella burnetii is a highly infectious bacterium, responsible for the zoonosis Q fever, a debilitating flu-like disease leading to large outbreaks with a severe health and economic burden (22–24). The efficiency of infections by C. burnetii is likely associated with the remarkable capacity of this bacterium to adapt to environmental as well as intracellular stress. Indeed, outside the host, C. burnetii generates pseudospores that facilitate its airborne dissemination. C. burnetii has developed a unique adaptation to the host, being the only bacterium that thrives in an acidic compartment containing active lysosomal enzymes. Upon host cell invasion, bacteria reside within membrane-bound compartments that passively traffic through the endocytic maturation pathway, progressively acquiring early and late endocytic markers such as Rab5 and Rab7, respectively (25). Fusion of Coxiella-containing vacuoles (CCVs) with late endosomes and lysosomes is accompanied by the acidification of the endosomal environment, which is required to activate the translocation of bacterial effector proteins by a Dot/Icm type 4b secretion system (26). Some of these effectors modulate important signaling pathways of infected cells, including apoptosis and inflammasome activation (27–29), whereas others are essential for the development of the intracellular replicative niche. Among these, CvpB and CvpF have been recently implicated in the development of the intracellular replicative niche (30–33). C. burnetii genome analysis revealed a close homology to the facultative intracellular pathogen Legionella pneumophila, in particular at the level of Dot/Icm core genes (34). In silico analysis identified over 100 candidate effector proteins encoded in the C. burnetii genome, some of which have been validated for secretion using either C. burnetii or L. pneumophila as a surrogate model (26, 35, 36).

In this study, we investigated the candidate effector CBU_0175, which encodes a unique putative Coxiella Ser/Thr kinase (CstK). We demonstrated CstK translocation by C. burnetii during infection, and we reported its localization at CCVs. In vitro kinase assays revealed that CstK undergoes autophosphorylation on Thr and Tyr residues and displays a bona fide kinase activity toward a test substrate of eukaryotic protein kinases. Furthermore, the identification of the Rab7 GTP-activating protein TBC1D5 as a CstK interactor suggests that this protein might be involved during infection to facilitate CCVs biogenesis. Indeed, TBC1D5 is actively recruited at CCVs during Coxiella infections, and TBC1D5-targeting siRNAs significantly affect CCVs development. Our data provide the first evidence that a C. burnetii secreted kinase might control host cell infection.

Results

C. burnetii genome encodes a single putative protein kinase

In silico analysis of the virulent C. burnetii strain RSA493 NMI genome revealed only one gene encoding a putative STPK. To date, no STPKs have been characterized in this organism. This gene was named cstK for C. burnetii gerine threonine kinase and encodes a 246-amino acid protein with an estimated molecular mass of 31 kDa. The gene coding for cstK is flanked by genes CBU_0174 (which encodes an hypothetical protein) and CBU_0176, a gene coding for the serine protease domain-containing protein degP1. Of note, these genes are not part of an operon (Fig. 1A). InterProScan analysis of CstK revealed the presence of most of the essential amino acids and sequence subdomains characterizing the Hanks family of eukaryotic-like protein kinases (37). CstK shares a common eukaryotic protein kinase superfamily fold with two lobes and a Gly-rich loop. These protein kinases include the central core of the catalytic domain and the invariant lysine residue in the consensus motif within subdomain II, which is usually involved in the phosphate transfer reaction and required for the autophosphorylating activity of eukaryotic STPKs (Fig. 1A) (37–39). The activation loop in the catalytic domain is particularly short in CstK, and the DFG motif is substituted by a GLS motif. Interestingly, the transmembrane domain usually present in classical prokaryotic STPKs is lacking in CstK; thus, it is a so-called cytoplasmic STPK.

CstK is a Dot/Icm effector protein

Bioinformatics analysis using the prediction software S4TE 2.0 (40) indicated that CstK harbors features corresponding to secreted effector proteins, including a promoter motif typically found in effector proteins from intravacuolar bacterial pathogens, suggesting that CstK is indeed a Coxiella effector protein (Fig. 1A). Consistently, previous studies by Chen et al. (36) have shown that CstK is secreted in a type 4b secretion system–dependent manner by the surrogate host L. pneumophila, albeit with low efficiency. To validate CstK secretion in C. burnetii, we engineered plasmids encoding, either CstK or CvpB (a known C. burnetii effector protein) (30), fused to β-lactamase (BLAM) and expressed in WT Coxiella Tn1832 (a C. burnetii transposon mutant expressing GFP and that phenocopies WT C. burnetii) or the Dot/Icm-defective dotA::Tn mutant, also expressing GFP. By means of a BLAM secretion assay, we could observe that BLAM-CstK was secreted by WT Coxiella at 48 and 72 h, but not at 24 h postinfection (Fig. 1B). Secretion of BLAM-CvpB or BLAM-CstK was not detectable in cells infected with the dotA::Tn strain, indicating that both CvpB and CstK are C. burnetii Dot/Icm substrates (Fig. 1B). Next, the intracellular localization of CstK was investigated by ectopically expressing HA-tagged CstK either in noninfected or WT C. burnetii–infected U2OS cells. In noninfected cells, CstK localized at intracellular compartments that were negative for the lysosomal marker LAMP1, whereas it was recruited at CCVs (as revealed by the co-localization with LAMP1) in infected cells (Fig. 1C).

CstK displays autokinase and protein kinase activities

To determine whether CstK is a functional protein kinase, this protein was overproduced in Escherichia coli and purified as a recombinant protein fused to glutathione S-transferase (GST) tag. The purified tagged CstK protein (Fig. 2A, upper panel) was then assayed for autokinase activity in the presence of the phosphate donor [γ-32P]ATP. As shown in Fig. 2A (lower panel), CstK incorporated radioactive phosphate from [γ-32P]ATP, generating a radioactive signal corresponding to
the expected size of the protein isoform, strongly suggesting that this kinase undergoes autophosphorylation. To confirm CstK autophosphorylation and exclude the possibility that contaminant kinase activities from *E. coli* extracts might phosphor-
ylase CstK, we mutated the conserved Lys55 residue present in subdomain II into CstK by site-directed mutagenesis. Indeed, protein sequence analysis revealed that Lys55 in CstK is similar to a conserved Lys residue usually involved in the phosphotransfer reaction and also required for the autophosphorylating activity of eukaryotic-like STPKs (37, 38). Thus, Lys55 was substituted by a Met residue, the mutated form of CstK, CstK_K55M, was purified as described above (Fig. 2A, upper panel), and it was then tested for autophosphorylation in the presence of [γ-32P]ATP. As expected, no radioactive signal could be detected (Fig. 2A, lower panel), thus establishing that CstK displayed autophosphorylation activity. A kinetic analysis of CstK phosphorylation was next performed to determine the initial CstK phosphorylation rate (Fig. 2B). Incorporation of γ-phosphate occurred rapidly, reaching ~50% of its maximum rate within 5 min of reaction. This autokinase activity depended on bivalent cations such as Mg2+ and Mn2+ in the range of 5 mM, thus in correlation with concentrations required for canonical STPK activity, as shown in Fig. 2C, and abolished by addition of 20 mM EDTA chelating all the divalent cations available (data not shown).

The recombinant CstK protein was further characterized by studying its ability to phosphorylate exogenous proteins and was thus assayed for *in vitro* phosphorylation of the general eukaryotic protein kinase substrate, myelin basic protein (MBP). MBP is a commonly used substrate for both Ser/Thr and Tyr kinases. A radiolabeled signal at the expected 18-kDa molecular mass of MBP was detected, thus demonstrating that CstK phosphorylates protein substrates such as MBP (Fig. 2A). As expected, the CstK_K55M mutant did not phosphorylate MBP. Altogether, these data indicate that *in vitro*, CstK possesses intrinsic autophosphorylation activity and displays kinase functions for exogenous substrates.

**Identification of CstK autophosphorylation sites**

To determine the specificity of this kinase, we next identified its autophosphorylation sites. A MS approach was used because this technique allows precise characterization of post-translational modifications including phosphorylation (41, 42). NanoLC/nanospray/tandem MS (LC-ESI/MS/MS) was applied for the identification of phosphorylated peptides and for localization of phosphorylation sites in CstK. This approach led to 97% of sequence coverage, whereas the remaining residues uncovered did not include Ser, Thr, or Tyr residues.
As detailed in Table 1, analysis of tryptic digests allowed the characterization of three phosphorylation sites in CstK. Surprisingly, unlike classical Ser/Thr or Tyr kinases, CstK was phosphorylated on two Tyr residues (Tyr14 and Tyr209), in addition to one Thr site (Thr232). Because protein sequence analysis did not reveal a classical activation loop in this kinase, the contri-
bution of Thr\textsuperscript{232}, Tyr\textsuperscript{14}, and Tyr\textsuperscript{209} to CstK kinase activity was individually assessed. Hence, these residues were mutated either to phenylalanine to replace tyrosine residues or alanine to replace threonine residue, generating the single mutants CstK\textsubscript{Y14F}, CstK\textsubscript{Y209F}, and CstK\textsubscript{T232A}, as well as the CstK\textsubscript{Y14F/Y209F/T232A} triple mutant (CstK\textsubscript{FFA}). Next, in vitro kinase assays with \( ^{32}P\)ATP were carried out and revealed that maximum loss in CstK autophosphorylation activity was observed in the CstK\textsubscript{Y14F} mutant (Fig. 2D), suggesting that this site is central for CstK activation. In contrast, the CstK\textsubscript{Y209F} mutant exhibited a slight hyperphosphorylation, which might indicate that Tyr\textsuperscript{209} only plays an accessory role in controlling CstK autophosphorylation (Fig. 2D). Finally, the CstK\textsubscript{T232A} mutant showed a reduced CstK phosphorylation and displayed diminished kinase activity toward the exogenous substrate MBP (Fig. 2D). Note that mutating all three autophosphorylation sites fully abrogated CstK kinase activity (Fig. 2D). These results indicate that Tyr\textsuperscript{14} and Thr\textsuperscript{232} are the major phosphorylation sites in CstK and strongly suggest that CstK might be a dual specificity (Thr/Tyr) kinase.

**CstK activity and phosphorylated state affect its intracellular localization**

Next, we ectopically expressed HA-tagged CstK, CstK\textsubscript{K55M}, and CstK\textsubscript{FFA} derivatives in noninfected and *C. burnetii*–infected U2OS cells to investigate its intracellular localization. CstK mainly localized at vesicular compartments in noninfected cells and accumulated at CCVs upon *C. burnetii* infection, suggesting an active role in the biogenesis of this compartment (Fig. 1C). Interestingly, the inactive CstK\textsubscript{K55M} mutant localized at vesicular structures positive for the lysosomal marker LAMP1 but not at CCVs, whereas the nonphosphorylated CstK\textsubscript{FFA} displayed a diffuse localization in the cytosol of transfected cells (Fig. 2E). Overall, these data suggest that the kinase activity and phosphorylated state of CstK play an important role in its localization in cells.

**CstK regulates vacuole development and *C. burnetii* replication within infected cells**

As a first step toward the understanding of CstK functions in the course of infection and to appreciate the extent to which this kinase is required for growth and viability of *C. burnetii*, we attempted to inactivate the corresponding chromosomal gene. Unfortunately, after several attempts we were unable to generate a null mutant, suggesting that *cstK* might be essential. However, we had previously isolated a *C. burnetii* mutant (Tn2496) carrying a transposon insertion allowing GFP expression at position 156,783, 32 bp upstream of the starting codon of *cstK* (43) (Fig. 1A). To determine the effect of this transposon insertion on *cstK* gene expression, we assessed the expression level of *cstK* mRNA from WT *C. burnetii* and Tn2496 strains. Surprisingly, *cstK* expression was significantly up-regulated in the mutant strain, suggesting that the transposon insertion may have released a transcriptional negative regulation (Fig. 3A). This suggested that a putative transcriptional regulator might bind the *cstK* promoter and control its activity during host invasion.

We next examined the effects of CstK overexpression on *C. burnetii* infections by challenging Vero cells with WT *C. burnetii*, the Dot/Icm-defective dotA::Tn mutant, or the Tn2496 mutant. Intracellular growth of the CstK-overexpressing strain was significantly reduced over 7 days of infection with an intermediate phenotype between WT and the dotA::Tn mutant (Fig. 3B). Accordingly, multiparametric phenotypic profile analysis of the Tn2496 mutant indicated that this strain exhibited a major defect in CCV development as compared with WT *C. burnetii* (Fig. 3, C and D). To further investigate the effects of CstK overexpression on *C. burnetii* infections, GFP-expressing *C. burnetii* were transformed with plasmids expressing HA-tagged WT CstK or its corresponding mutants (CstK\textsubscript{K55M} and CstK\textsubscript{FFA}) under the control of an IPTG promoter. U2OS cells expressing cytoplasmic mCherry were challenged with the three *C. burnetii* strains in the presence or absence of IPTG. After 6 days of infection, the cells were fixed, labeled with Hoechst and anti-LAMP1 antibody to visualize host cells nuclei and CCVs, respectively, and processed for automated image analysis. In all cases, the overexpression of CstK was detrimental for CCVs biogenesis and bacterial replication (Fig. 3E). Next, U2OS cells were challenged with WT *C. burnetii*, the GFP-expressing Tn2496 mutant strain, or a combination of the two for 6 days (Fig. 3F). The cells were then fixed and labeled with an anti–C. burnetii antibody to label both bacteria strains and incubated with Hoechst to visualize host cells nuclei (Fig. 3F). Automated image analysis was then used to determine the effects of CstK overexpression on the replication of WT bacteria, in trans. Co-infections resulted in a significant increase in the size of Tn2496 colonies, indicating that WT *C. burnetii* can partially restore the growth of the CstK-overexpressing strain (Fig. 3G). However, a significant decrease in the size of bacterial colonies labeled by the anti– *C. burnetii* antibodies indicated that CstK overexpression has a detrimental effect in trans on the development of WT bacteria (Fig. 3G). Of note, vacuoles harboring WT or mutant colonies alone were never observed in co-infection experiments. Therefore, we concluded that CstK participates in the formation of the *C. burnetii* replicative vacuole and that its expression must be finely tuned for optimal intracellular replication.

**CstK specifically interacts with host cell proteins**

Because CstK is a secreted protein, we assume that this kinase would interfere with host cell signal transduction pathways to subvert host cell defenses to the benefit of the bacteria. To identify host cell proteins that could interact with CstK, we made use of the model amoeba *Dictyostelium discoideum*. *D. discoideum* is a eukaryotic professional phagocyte amenable to genetic and biochemical studies. Lysate from cells overex-

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**Table 1**

| Phosphorylated tryptic peptide sequence of CstK | Number of detected phosphate groups LC/MS/MS | Phosphorylated residue |
|------------------------------------------------|---------------------------------------------|-----------------------|
| [8LSVADFpYDLKK\textsubscript{14}]\textsuperscript{pY} | 1                                           | Tyr\textsuperscript{14} |
| [2[220]EQNpTAGHLR\textsubscript{237}]               |                                             | Thr\textsuperscript{232} |
| [209pYCNPHIK\textsubscript{115}]                   | 1                                           | Tyr\textsuperscript{209} |
pressing CstK tagged with a C-terminal FLAG epitope (CstK-FLAG) was incubated with beads coupled to an anti-FLAG antibody. The beads were extensively washed, and bound proteins were separated by SDS-PAGE before MS analysis. Among the putative interactants of CstK identified by this approach, some were discarded on the basis of their
intracellular localization, whereas other retained candidates were mostly involved in the endocytic pathway (Table S1). Among these, the Rab GTPase-activating protein/TBC domain-containing protein, DDB_G0280253 (UniProtKB Q54VM3), is a 136.4-kDa protein homologous to mammalian TBC1D5 (dictyBase), a GTPase-activating protein for Rab7a and Rab7b (44–46) that acts as a molecular switch between the endosomal and the autophagy pathway (47).

Given the recently reported implication of TBC1D5 in the biogenesis of L. pneumophila-containing vacuoles (48) and the role of autophagy in the biogenesis of CCVs (25, 31, 49), we aimed at validating the interaction between human TBC1D5 (Hs-TBC1D5) and CstK in HEK-293T cells. The cells co-expressing Hs-TBC1D5-GFP and HA-CstK or CstK mutants were used for immunoprecipitation using anti-HA beads. WT and CstK derivatives were detected as co-immunoprecipitated in the presence of Hs-TBC1D5-GFP, thus confirming that Hs-TBC1D5 is a bona fide CstK interactant (Fig. 4A). Significantly higher levels of TBC1D5 were co-immunoprecipitated by the CstK mutants, suggesting that the interaction might be increased in the absence of phosphorylation turnover of the kinase (Fig. 4A, bottom panel). Interestingly, the interaction is not dependent on the phosphorylation status of CstK because the K55M mutant and the triple FFA mutant are still able to interact. Other candidates identified by MS are currently being investigated.

TBC1D5 is recruited at CCVs and regulates their biogenesis

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cells of TBC1D5 prior to C. burnetii infection, to investigate a possible role in CCVs development and intracellular bacterial replication. Indeed, vacuole development was significantly reduced in cells exposed to Hs-TBC1D5–targeted siRNAs as opposed to cells treated with nontargeting siRNA oligonucleotides (Fig. 4C).

TBC1D5 is not phosphorylated in vitro by recombinant CstK

We assessed whether CstK might phosphorylate the recombinant Hs-TBC1D5. Despite the in silico prediction of several Ser/Thr and Tyr phosphorylatable residues in Hs-TBC1D5, we failed to detect Hs-TBC1D5 phosphorylation using several in vitro kinases assays (Fig. S2). In addition, Hs-TBC1D5 phosphorylation status was also investigated upon transfection with CstK or its inactive derivative (K55M) followed by Hs-TBC1D5 immunoprecipitation. No phosphorylation could be detected in our experimental conditions.

Discussion

Bacterial Ser/Thr/Tyr kinases expressed by pathogenic bacteria can either act as key regulators of important microbial processes or be translocated by secretion systems to interact with host substrates; thereby our results provide the first biochemical analysis of the secreted C. burnetii kinase CstK and its involvement in the process of infection and CCVs development. Importantly, CstK presents important differences as compared with classical Ser/Thr kinases. In particular, we provided evidence that CstK is a dual kinase able to autoprophosphorylate on Thr and Tyr residues. Moreover, the observation that a transposon insertion 32 bp upstream of the cstK starting codon leads to an increase in the levels of cstK mRNAs was indicative of the presence of a negative transcriptional regulation of gene expression, suggesting a fine-tuning of the levels of CstK. Indeed, the Tn2496 mutant displays a severe CCV biogenesis defect when used to challenge U2OS cells, highlighting the importance of regulating cstK expression during CstK infections. Accordingly, inducing the expression of WT CstK in WT C. burnetii severely impairs CCVs development and bacterial replication. Co-infection experiments demonstrated that CstK overexpression can also act in trans, by perturbing the intracellular replication of WT C. burnetii. The identification of candidate eukaryotic interactors of CstK further corroborated a role of the bacterial kinase in subverting host functions during infection. Here we confirmed that CstK interacts with TBC1D5, but we failed to detect phosphorylation of the eukaryotic target by CstK. However, we cannot exclude that TBC1D5 is a genuine CstK substrate in vivo because the lack of phosphorylation of host interactors of bacterial STPKs is not uncommon. Interaction between STPKs and host proteins might well perturb protein interaction networks at play in host cells (21). Indeed, the induced overexpression of CstK mutants lacking kinase activity in WT C. burnetii impaired CCVs development to the same extent as the overexpression of WT CstK. The biochemical mechanisms of these pathogen-directed targeted perturbations of host cell–signaling networks are being actively investigated. Regardless, siRNA depletion of TBC1D5 in C. burnetii–infected cells points at a role of the eukaryotic protein in CCVs development. In mammals, TBC1D5 was suggested to function as a molecular switch between endosomal and autophagy pathways. Indeed TBC1D5 associates the retromer VPS29 subunit involved in endosomal trafficking, and upon autophagy induction, the autophagy ubiquitin-like protein LC3 can displace VPS29, thus orienting TBC1D5 functions toward autophagy instead of endosomal functions (47). It is thus tempting to propose that CstK might interfere with this tight regulation between TBC1D5, LC3, and VPS29 and redirect TBC1D5 functions to support efficient C. burnetii intracellular replication. Further work will need to be carried out to decipher how CstK recognizes these host substrates and how they participate in the establishment of C. burnetii parasitophorous vacuoles. Another perspective of this work is the opening of a new field of investigation for future drug development to fight this pathogen. Because CstK seems to be essential, specific inhibitors of this kinase preventing C. burnetii growth would be extremely useful for the development of new therapies.

Experimental procedures

Bacterial strains and growth conditions

Bacterial strains and plasmids are described in Table 2. Strains used for cloning and expression of recombinant proteins were E. coli TOP10 (Invitrogen) and E. coli BL21 (DE3)Star (Stratagene), respectively. E. coli cells were grown and maintained at 25 °C in LB medium supplemented with 100 μg/ml ampicillin when required. C. burnetii RSA439 NMII and transposon mutants Tn1832, Tn2496, and dotA_:Tr were grown in ACCM-2 (45) supplemented with chloramphenicol (3 mg/ml) in a humidified atmosphere of 5% CO₂ and 2.5% O₂ at 37 °C.

Cloning, expression, and purification of CstK derivatives

The cstK (CBLU_0175) gene was amplified by PCR using C. burnetii RSA439 NMII chromosomal DNA as a template with the primers listed in Table 3 containing a BamHI and HindIII restriction site, respectively. The corresponding amplified product was digested with BamHI and HindIII and ligated into the bacterial pGEX(M) plasmid, which includes a N-terminal GST tag, thus generating pGEX(M)_cstK. pGEX(M)_cstK derivatives harboring different mutations were generated by using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) and resulted in the construction of plasmids detailed in Table 3. For overexpression assays, cstK and its derivatives were cloned in the pJ-A-LACO-4xHA vector (30) using KpnI and BamHI restriction sites. All constructs were verified by DNA sequencing. Transformed E. coli BL21 Star cells with pGEX(M)_cstK derivatives were grown at 16 °C in LB medium containing 1 g/liter of glucose and 100 μg/ml of ampicillin and protein synthesis induced with 0.5 mm IPTG overnight. Bacteria were disrupted by sonication (Branson, digital sonifier) and centrifuged at 14,000 rpm for 25 min. Purifications of the GST-tagged recombinants were performed as described by the manufacturer (GE Healthcare). cstK coding sequence was also optimized for mammalian cell expression (GenScript), amplified by PCR, and cloned into pDXA-3C (50) containing a FLAG tag for C-terminal fusion. After sequencing, the plasmid was linearized by the restriction
enzyme Scal and transfected in *D. discoideum* as described (51). Clone selection was made with 10 mg/ml of G418, and protein expression was assayed by Western blotting analysis of *D. discoideum* crude extract with an anti-FLAG rabbit polyclonal antibody (GenScript). For ectopic expression assays, *cstK, cstK_K55M*, and *cstK_FFA* with optimized codons (IDT) were cloned in pRK5_HA using the primer pair *cstK, cstK_K55M* assays, of *D. discoideum* clones ready for transfer to Gateway-compatible expression vectors). HsTBC1D5 coding sequence has been recombined into pEGFP-N1 RFC Destination vector by Gateway reaction (MGC Platform Montpellier), thus generating pEGFP-N1_HsTBC1D5 coding for HsTBC1D5 with a C-terminal GFP-tag (MGC Platform Montpellier), thus generating pEGFP-N1_HsTBC1D5 for mammalian expression (AmpR).

Cloning, expression, and purification of TBC1D5 derivatives

The *D. discoideum* GFP-tagged TBC1D5 was previously generated (48). Cells were grown at 22 °C in HL5 medium as previously described (51). Human TBC1D5 coding sequence was obtained from the hORFeome v8.1 (ORF 2659, Q92609, fully sequenced cloned human ORFs in Gateway Entry vectors). HsTBC1D5 coding sequence has been recombined into pEGFP-N1 RFC Destination vector by Gateway reaction (MGC Platform Montpellier), thus generating pEGFP-N1_HsTBC1D5 coding for HsTBC1D5 with a C-terminal GFP tag. pmCH_Hs-TBC1D5-mCherry has been generated by the same method (MGC Platform Montpellier).

**RNA extraction and quantitative RT-PCR (qRT-PCR)**

50 ml of *C. burnetii* culture was harvested, resuspended in 600 µl of RNA protect reagent (Qiagen) and incubated for 5 min at room temperature. Bacteria were centrifuged and resuspended in 200 µl of TE (10 mM Tris-HCl, 1 mM EDTA, pH 8) containing 1 mg/ml lysozyme. Bacterial suspension was incubated at room temperature for 5 min, and bacteria were disrupted by vigorous vortexing for 10 s every 2 min. 700 µl of lysis buffer from RNA easy kit (Qiagen) were added to the bacterial

**Table 2**

| Strain or plasmid | Genotype or description | Source or reference |
|-------------------|-------------------------|---------------------|
| **E. coli strains** |                         |                     |
| *E. coli TOP10*   | *E. coli* derivative ultra–competent cells used for general cloning; F<sup>-</sup>mcRA D(mrr-hsdRMS-mlcB) B0dlacZAM15 D lacX74 endA1 recA1 araL araD139 D (ara, leuB7697 galU galK rpsL nspG–tonA) | Invitrogen          |
| *E. coli BL21(DE3)Star* | F2 ompT hsdSB (rB2 mB2) gal dcm (DE3); used to express recombinant proteins in *E. coli* | Stratagene           |
| **C. burnetii strains** |                         |                     |
| *C. burnetii RSA439 NMII* | WT *C. burnetii* RSA439 NMII nonfluorescent | Ref. 55 |
| *C. burnetii Tn2496* | *C. burnetii* RSA439 NMII carrying an Himar1–CAT–GFP cassette 32 bp upstream of CBU_0175, expressing GFP | This study           |
| *C. burnetii Tn1832* | *C. burnetii* RSA439 NMII carrying an Himar1–CAT–GFP cassette in the intergenic region between CBU_1847b and CBU_1849, expressing GFP | Ref. 43 |
| *C. burnetii Tn292* | *C. burnetii* RSA439 NMII carrying an Himar1–CAT–GFP cassette in CBU_1648 (dotA), expressing GFP | Ref. 43 |
| **Plasmids** |                         |                     |
| pGEX(M) | pGEX with a 321-bp EcoRI/BamHI fragment from pET19b introducing a HindIII site in the pGEX polylinker | Ref. 56 |
| pGEX(M)_cstK | pGEX(M) derivative used to express GST-tagged fusion of CstK (Amp<sup>+</sup>) | This study |
| pGEX(M)_cstK_K55M | pGEX(M) derivative used to express GST-tagged fusion of CstK_K55 M (Amp<sup>+</sup>) | This study |
| pGEX(M)_cstK_Y14F | pGEX(M) derivative used to express GST-tagged fusion of CstK_Y14F (Amp<sup>+</sup>) | This study |
| pGEX(M)_cstK_Y209F | pGEX(M) derivative used to express GST-tagged fusion of CstK_Y209F (Amp<sup>+</sup>) | This study |
| pGEX(M)_cstK_T232A | pGEX(M) derivative used to express GST-tagged fusion of CstK_T232A (Amp<sup>+</sup>) | This study |
| pGEX(M)_cstK_Y14F_Y209F_T232A | pGEX(M) derivative used to express GST-tagged fusion of CstK_Y14F_Y209F_T232A (Amp<sup>+</sup>) | This study |
| pGFPN1 | Vector used for mammalian expression of C-terminal GFP-tagged fusion proteins | Addgene |
| peGFPN1_Hs-TBC1D5-GFP | peGFP-N1 derivative used to express C-terminal GFP-tagged fusion of Hs_TBC1D5 for mammalian expression | This study |
| pmCH_Hs-TBC1D5-mCherry | pmCH derivative used to express C-terminal mCherry-tagged fusion of Hs_TBC1D5 for mammalian expression | This study |
| pRK5_HA_cstK | pRK5_HA derivative used to express N-terminal HA-tagged fusion of CstK codon-optimized for mammalian expression (AmpR) | This study |
| pRK5_HA_cstK_K55M | pRK5_HA derivative used to express N-terminal HA-tagged fusion of *cstK_K55M* codon-optimized for mammalian expression (AmpR) | This study |
| pRK5_HA_cstK_FFA | pRK5_HA derivative used to express N-terminal HA-tagged fusion of *cstK_FFA* codon-optimized for mammalian expression (AmpR) | This study |
| pXDC61K-Blam | Vector with IPTG-inducible expression of β-lactamase | Ref. 57 |
| pXDC61K-Blam-cvpB | pXDC61K-Blam derivative used to express Blam-tagged fusion of CvpB | This study |
| pXDC61K-Blam-cstK | pXDC61K-Blam derivative used to express Blam-tagged fusion of CstK | This study |
| pJA-LACO-4/H-A | Vector with IPTG-inducible expression of 4 × HA-tagged proteins | Ref. 30 |
| pJA-LACO-4/H-cstK | Vector with IPTG-inducible expression of 4 × HA-tagged cstK | This study |
| pJA-LACO-4/H-cstK_K55M | Vector with IPTG-inducible expression of 4 × HA-tagged cstK_K55M | This study |
| pJA-LACO-4/H-cstK_FFA | Vector with IPTG-inducible expression of 4 × HA-tagged cstK_FFA | This study |
| **D. discoideum plasmids** |                         |                     |
| pDXA-3C_cstK-FLAG | Vector for overexpression of CstK with a C-terminal FLAG tag in *D. discoideum* | This study |
| pDXA-3C_DdTBC1D5-myc | Vector for expression of myc-DdTBC1D5 in *D. discoideum* | This study |
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Table 3
Primers used in this study
Restriction sites are underlined and specified in parentheses. Mutagenized codons are shown in bold type.

| Primer        | 5’ to 3’ sequence |
|---------------|-------------------|
| NtermCstK     | TATGATCCCTTAAAGGCCTTTAATATAGGGCTTAGT (BamHI) |
| CtermCstK     | ATGAAGGCGAGCGGCGCTTAAACCATTCATTTTTC (HindIII) |
| RwCstK K55M   | ATGAAGGCGAGCGGCGCTTAAACCATTCATTTTTC (HindIII) |
| FwCstK KY14F  | CTATGATCCCTTAAAGGCCTTTAATATAGGGCTTAGT (BamHI) |
| FwCstK KY209F | ATGAAGGCGAGCGGCGCTTAAACCATTCATTTTTC (HindIII) |
| FwCstK KT232A | ATGAAGGCGAGCGGCGCTTAAACCATTCATTTTTC (HindIII) |
| CstKopt BamHI-Fw | GATGGTACCAGCAGACAGCCATCATTG |
| CstKopt EcoRI-Rv | GATGGTACCAGCAGACAGCCATCATTG |
| CstK-KpnI-Fw  | AGGGGATCCTAATTAATCCCATTCAATATTTTCTAA |
| CstK-KpnI-Rv  | AGGGGATCCTAATTAATCCCATTCAATATTTTCTAA |
| CvpB-KpnI-Fw  | CTTGAATTCTTAGTCCCACTCGATGTTTTCCAGATG |
| CvpB-KpnI-Rv  | CTTGAATTCTTAGTCCCACTCGATGTTTTCCAGATG |
| RT-PCR primers | GCTCCAGCATTGACATCAG |
| DotA-F        | GCGATTGATATATGAGGCTTAGT |
| DotA-R        | GCGATTGATATATGAGGCTTAGT |
| CstK-F        | GCGAAGTATTAGGCGGGAA |
| CstK-R        | GCGAAGTATTAGGCGGGAA |
| CtermCstK     | GATGTATACCCAGACAGGCTATGTCATG |
| NtermCstK     | GATGTATACCCAGACAGGCTATGTCATG |

In vitro kinase assays

In vitro phosphorylation was performed with 4 μg of WT CstK or CstK derivatives in 20 μl of buffer P (25 mM Tris-HCl, pH 7.0, 1 mM DTT, 5 mM MnCl2, 1 mM EDTA, 50 μM ATP) with 200 μCi/ml (65 nM; γ32P]ATP; PerkinElmer, ref: NEG 602H250UC, 3000 Ci mmol−1) for 30 min at 37 °C. For substrate phosphorylation, 4 μg of MBP (Sigma) and 4 μg of CstK were used. Each reaction mixture was stoped by addition of an equal volume of Laemmli buffer, and the mixture was heated at 100 °C for 5 min. After electrophoresis, the gels were soaked in 16% TCA for 10 min at 90 °C and dried. Radioactive proteins were visualized by autoradiography using direct exposure to films.

Mass spectrometry analysis

For MS analysis, CstK was phosphorylated as described above, except that [γ32P]ATP was replaced with 5 mM cold ATP. Subsequent MS analyses were previously reported (52, 53). Briefly, the samples were submitted to trypsin digestion and analyzed using an Ultimate 3000 nano-RSLC (Thermo Scientific, San Jose, CA) coupled on line with a quadrupole Orbitrap Q Exactive HF mass spectrometer via a nano-electrospray ionization source (Thermo Scientific). The samples were injected and loaded on a C18 Acclaim PepMap100 trap-column (Thermo Scientific) and separated on a C18 Acclaim Pepmap100 nano-column (Thermo Scientific). MS data were acquired in a data-dependent strategy selecting the fragmentation events based on the 20 most abundant precursor ions in the survey scan (350–1600 Th). The resolution of the survey scan was 60,000 at m/z 200 Th, and for MS/MS scan the resolution was set to 15,000 at m/z 200 Th. Peptides selected for MS/MS acquisition were then placed on an exclusion list for 30 s using the dynamic exclusion mode to limit duplicate spectra. Data files were then analyzed with Proteome Discover 2.2 using the SEQUEST HT algorithm against the Uniprot D. discoideum, which included the sequence of CstK.

C. burnetii infections

U2OS epithelial cells were challenged with C. burnetii RSA439 NMII, the transposon mutants Tn1832, dotA::Tn, or Tn2496 as previously described (38, 49). For co-infection experiments, the cells were challenged with a 1:1 ratio of C. burnetii RSA439 NMII and Tn2496 transposon mutant. For gene silencing, U2OS cells were seeded at 2,000 cells/well in black, clear-bottomed, 96-well plates in triplicate and transfected with siRNA oligonucleotides 24 h later by using the RNAiMAX transfection reagent (Thermo Fisher Scientific) according to the manufacturer’s recommendations. At 24 h post-transfection, the cells were challenged with C. burnetii (MOI of 100) and further incubated for 5 days. The cells were then fixed and processed for immunofluorescence. Where appropriate, anti-LAMP1 antibodies were used to label lysosomes and CCVs as previously described (54). Samples were imaged with a Zeiss Axio Imager Z1 epifluorescence microscope (Carl Zeiss) connected to a CoolSNAP HQ2 CCD camera (Teledyne Photometrics, Tucson, AZ). Images were acquired with 40× oil immersion objectives and processed with Metamorph (Molecular Devices, San Jose, CA). For phenotypic screening, the samples were imaged with an ArrayScan VTI Live epifluorescence automated microscope (Cellomics) equipped with an ORCA-ER CCD camera (Hamamatsu). 25 fields/well were acquired for image analysis. ImageJ and ICY software were used for image analysis and quantifications. Phenotypic profiles (expressed as z scores) were calculated using CellProfiler, from triplicate experiments as previously described (54) following median based normalization of 96-well plates. Plates effects were cor-
rected by the median value across wells that are annotated as control.

**β-Lactamase translocation assay**

Effectors proteins translocation assays were performed as previously described (30). Briefly, C. burnetii Tn1832 (WT) and dotA::Tn were transformed with pXDC-Blam (negative control), pXDC-Blam-CvpB (positive control), or pXDC-Blam-CstK. Each strain was used to infect U2OS epithelial cells. After 24, 48, or 72 h of infection, the cells were loaded with the fluorescent substrate CCF4/AM (LiveBLAzer-FRET B/G loading kit; Invitrogen) in Hank’s balanced salt solution (20 mM HEPES, pH 7.3) containing 15 mM probenecid (Sigma). The cells were incubated in the dark for 1 h at room temperature and imaged using an EVOS inverted fluorescence microscope. Images were acquired using 4,6-diamino-2-phenylindole and GFP filter cubes. The image analysis software CellProfiler was used to segment and identify all cells in the sample (GFP) and positive cells (4,6-diamino-2-phenylindole) and to calculate the intensity of fluorescence in each channel. The percentage of positive cells *versus* the total number of infected cells was then calculated and used to evaluate effector translocation.

**Immunoprecipitation from D. discoideum lysates**

For immunoprecipitation assays, 2 × 10^7^ cells were lysed in lysis buffer (50 mM Tris-HCl, pH 7.5, 300 mM NaCl, 0.5% Nonidet P-40, protease inhibitors (Roche)) and cleared by centrifugation for 15 min at 14,000 rpm in a microfuge. Lysate supernatants were incubated overnight at 4°C with anti-FLAG mAb coated on agarose beads (GenScript). The beads were then washed five times in wash buffer (50 mM Tris-HCl, pH 7.5, 300 mM NaCl, 0.1% Nonidet P-40) and once in PBS. Bound proteins were migrated on SDS-PAGE and analyzed by LC-MS/MS.

**Cell culture, heterologous expression, and anti-HA immunoprecipitation**

HEK-293T cells were grown in DMEM (Gibco) containing 10% (v/v) FBS, 1% GlutaMAX (Gibco, 200 mM stock), 0.5% penicillin/streptomycin (Gibco, 10,000 units/ml stock) and maintained under standard conditions at 37°C in a humidified atmosphere containing 5% CO2. The cells were transiently transfected using the jetPEI transfection reagent (Polyplus-Transfection Inc.) to express either Hs-TBC1D5-GFP, CstK_HA derivatives, or each CstK derivatives with TBC1D5_GFP protein. The cells were used 24 h after transfection for immunoprecipitation assay. Transfected cells were washed two times in cold PBS and lysed in lysis buffer (50 mM Tris, 150 mM NaCl, 0.5 mM EDTA, 0.5% Nonidet P-40, protease, and phosphatases inhibitors (Roche)). Cleared lysate (950 μl, ~1 mg of total proteins) were incubated with anti HA magnetic Beads (Pierce) for 30 min at room temperature under gentle rotation. The beads were washed three times in lysis buffer, boiled in 2× Laemmli sample buffer and loaded on ExpressPlus™ PAGE gels (GenScript). The eluted proteins were visualized by Western blotting with the following antibodies: anti-HA from Chromotek, anti-GFP from Torrey Pines, donkey anti-rat, or anti-rabbit from Jackson ImmunoResearch.

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**Densitometry**

Regions of Interest (ROIs) were obtained from each band of interest, and the intensity was measured using ImageLab (From Bio-Rad). For each band, the same ROI was used for background calculation and removal from areas adjacent to each band.

**Author contributions**—E. M., S. H.-B., S. B., J. A., M. Burette, M. M., F. L., M. Bonazzi, and V. M. conceptualization; E. M., S. H.-B., S. B., J. A., F. C., M. Burette, M. M., F. L., M. Bonazzi, and V. M. data curation; E. M., S. H.-B., S. B., J. A., F. C., L. G.-Z., M. Burette, M. M., F. L., M. Bonazzi, and V. M. formal analysis; E. M., S. H.-B., S. B., J. A., L. G.-Z., M. Burette, M. M., F. L., M. Bonazzi, and V. M. investigation; E. M., S. H.-B., S. B., J. A., F. C., M. Burette, M. M., F. L., M. Bonazzi, and V. M. methodology; E. M., M. Burette, M. M., F. L., M. Bonazzi, and V. M. project administration; E. M., M. Burette, M. M., F. L., M. Bonazzi, and V. M. writing and editing; S. H.-B., S. B., M. Burette, M. M., F. L., M. Bonazzi, and V. M. writing—original draft; F. L., M. Bonazzi, and V. M. funding acquisition.

**Acknowledgments**—We thank the Montpellier RIO imaging facility at the University of Montpellier, a member of the national infrastructure France-BioImaging, which is supported by the French National Research Agency through Grant ANR-10-INBS-04 (“Investments for the Future”). We acknowledge the contribution of the Protein Science Facility of the Structure Fédérative de Recherche Biosciences through Grant UMS3444 (to CNRS, I2B/INSERM, ENS de Lyon, UCBL), especially Frédéric Delolme and Adeline Page, who performed the MS analysis.

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