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A *Legionella pneumophila* kinase phosphorylates the Hsp70 chaperone family to inhibit eukaryotic protein synthesis

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Summary

Legionella pneumophila (*L.p.*), the microbe responsible for Legionnaires' disease, secretes ~300 bacterial proteins into the host cell cytosol. A subset of these proteins affect a wide range of post-translational modifications (PTMs) to disrupt host cellular pathways. *L.p.* has 5 conserved eukaryotic-like Ser/Thr effector kinases, LegK1–4 and LegK7, that are translocated during infection. Using a chemical genetic screen, we identified the Hsp70 chaperone family as a direct host target of LegK4. Phosphorylation of Hsp70s at T495 in the substrate-binding domain disrupted the Hsp70's ATPase activity and greatly inhibited its protein folding capacity. Phosphorylation of cytosolic Hsp70 by LegK4 resulted in global translation inhibition and an increase in the amount of Hsp70 on highly translating polysomes. LegK4's ability to inhibit host

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Author Contributions S.M.M., K.M.S., and S.M. designed the experiments, analyzed the data, and wrote the manuscript. S.M.M. performed all experiments excluding those done by I.R.T. I.R.T designed and performed the *in vitro* Hsp70 activity assays and assisted with purification of the Hsp70s. J.E.G helped design all *in vitro* Hsp70 experiments. D.R. helped design all polysome experiments. All authors commented on the manuscript.

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Declaration of Interests

The authors declare no competing interests

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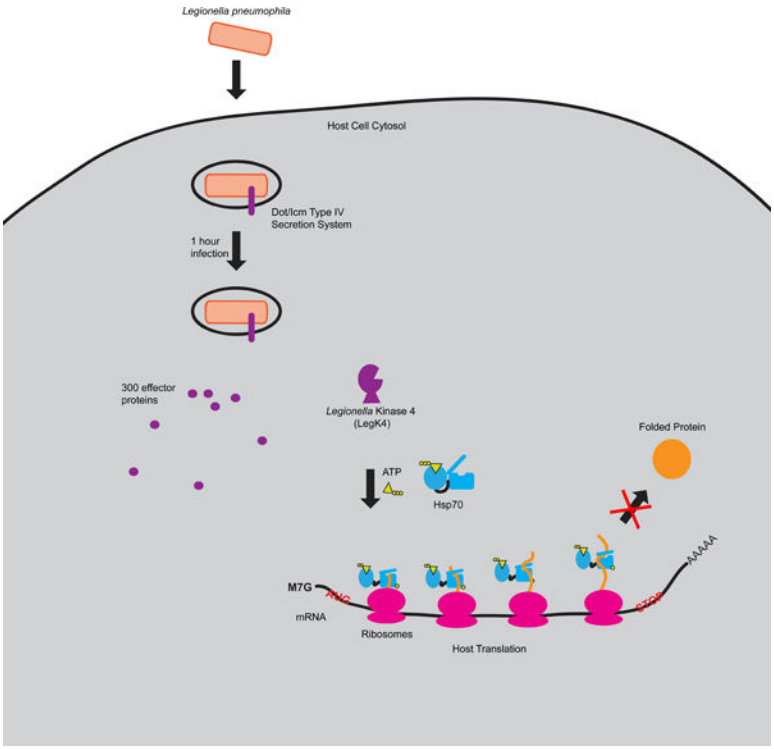
translation via a single PTM uncovers a role for Hsp70 in protein synthesis and directly links it to the cellular translational machinery.

Graphical Abstract



eTOC Blurb

Moss et. al. describe an example of a bacterial pathogen effector modifying host Hsp70 to achieve translational inhibition. The *Legionella pneumophila* kinase LegK4, phosphorylates a conserved threonine on Hsp70 to reduce the chaperone's refolding capacity and inhibit cellular protein translation.

Introduction

Intracellular pathogens are successful because they manipulate multiple host processes to escape immune detection and enable their own survival; thus, studying these pathogens has facilitated our understanding of the intricate regulation of fundamental host pathways (Mohr and Sonenberg, 2012; Salomon and Orth, 2013). Targeting eukaryotic mRNA translation is one such pathway, and is a common mechanism by which pathogens regulate their hosts. Work on Diphtheria (Collier, 1975) and Shiga toxin (Brown et al., 1980) have identified fascinating mechanisms for blocking host protein synthesis (Mohr and Sonenberg, 2012). Almost all cases of translation inhibition are a result of targeting the translation machinery itself such as inhibiting elongation factors (e.g. Diphtheria toxin) (Collier, 1975) or inhibiting the 28S rRNA (Shiga toxin) (Tumer and Li, 2012).

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The bacterial pathogen *Legionella pneumophila* (*L.p.*) has emerged as a model organism for studying host-pathogen interactions given its expert manipulation of many key regulatory pathways including host translation and eukaryotic vesicle transport. To control these host processes, *L.p.* uses a type IV secretion system called Dot/Icm that functions to translocate an astonishing ~300 bacterial effector proteins directly into infected host cells (Finsel and Hilbi, 2015). Studying the mechanisms of *L.p.* effectors has not only uncovered fascinating aspects of the host-pathogen arms race, it has also led to the identification of important regulators of host cell processes (Cornejo et al., 2017; Mohr and Sonenberg, 2012). A subset of these effectors work to inhibit protein synthesis by manipulating the host translational machinery (Belyi et al., 2006; 2008; Shen et al., 2009). Three of these proteins are a set of glucosyltransferases (Lgt1–3) that inhibit eukaryotic elongation factor1A (eEF1A), using glucose as a post-translational modification (PTM) (Belyi et al., 2006; 2008). A fourth effector protein SidI binds to eEF1A and another elongation factor eEF1Bγ to block mRNA translation (Shen et al., 2009). In addition to these four proteins, there are several other *L.p.* effectors that have been suggested to block host translation, but lack mechanistic detail or extensive characterization (Barry et al., 2013).

With the important role that PTM's play in *L.p.*'s survival and replication, its effector eukaryotic-like Ser/Thr protein kinases (eSTPKs) are emerging as important drivers of pathogenicity. *L.p.* has 5 conserved eSTPKs designated as *Legionella* kinase 1–4 and 7 (LegK1–4 and LegK7) (Hervet et al., 2011; Lee and Machner, 2018), that are translocated during infection. Many of these kinases were evolved to target important domains of host proteins. Characterization of LegK1 revealed its role in altering innate immunity by phosphorylating Ser32, on IκBα (Ge et al., 2009). Ser32 is typically phosphorylated by the mammalian kinases IKKα or IKKβ, an event that activates the canonical NF-κB pathway. In another example, an isogenic Δ*legK2* strain of *L.p.* exhibits delayed intracellular replication in amoebae due to the kinase's ability to disrupt actin polymerization (Hervet et al., 2011; Michard et al., 2015). Recently, LegK7 was shown to mimic Hippo kinase activity and phosphorylate MOB1 (Lee and Machner, 2018).

By studying the cellular phenotypes of known eSTPKs in *L.p.*, we identified a mechanism of pathogenic translation inhibition in which LegK4 phosphorylates the Hsp70 chaperone family. The effector LegK4 phosphorylates cytosolic Hsp70s at a highly conserved Thr residue. This phosphorylation reduced the chaperone's ATPase activity and subsequently decreased its overall protein refolding capacity. LegK4's phosphorylation of Hsp70 blocked protein synthesis and caused an increase in the Hsp70 load on highly translating polysomes. Thus, this work directly links Hsp70 to the translational machinery via a bacterially programmed PTM.

Results

Expression of LegK4 causes Golgi fragmentation and yeast lethality

L.p. is known to interact with the Golgi during infection (Bärlocher et al., 2017). To look at any effects the eSTPKs from *L.p.* might have on Golgi morphology, we transiently transfected these effectors into HeLa cells for 24 hours. LegK4 overexpression, but none of the other kinases, caused Golgi fragmentation in 90% of transfected cells (Fig. 1A & 1B).

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The LegK4-Δ1–58 construct in this data represents the recombinantly purified version of LegK4 that was used in later *in vitro* phosphorylation assays. In many *L.p.* strains, including the *Corby* strain used to obtain the LegK4 crystal structure (Flayhan et al., 2015), the *legK4* gene sequence begins at the conserved M59 of LegK4 from the *Philadelphia* strain. Based on this analysis, we modified the *legk4* gene from the *Philadelphia* strain of *L.p.* by truncating the first 58 amino acids (LegK4-Δ1–58). This LegK4 truncation was successfully expressed and purified from *E. coli* while the full length LegK4-wt protein was not. Both full length LegK4 and LegK4-Δ1–58 showed a similar Golgi fragmentation phenotype (Fig. 1B). A kinase dead construct of LegK4 (Fig. S1A), in which the Mg^{2+} coordinating aspartate of the DFG motif is mutated to an alanine (D271A, "LegK4-DA" for remainder of manuscript), did not show Golgi disruption (Fig. 1A & 1B). This indicates that the kinase activity of LegK4 is responsible for the Golgi fragmentation phenotype.

Several *L.p.* proteins are known to cause yeast lethality when exogenously expressed. We tested our LegK4 constructs for yeast lethality and showed that they prevented yeast growth when expressed under a Galactose inducible promoter (Fig. 1C). There was no difference in yeast growth between the full length and truncated versions of LegK4. The D271A mutant again exhibited no growth phenotype.

LegK4 phosphorylates the Hsp70 chaperone family

We conducted a chemical genetic screen to identify substrates phosphorylated by LegK4. We used an ATP analog that has a benzyl (Bn) group at the N6 position of the adenine ring. These ATP analogs possess a γ-thio-phosphate group that can be differentiated from endogenous kinase phosphorylation sites (Allen et al., 2007; Blethrow et al., 2008; Hertz et al., 2010). Chemical genetic substrate identification typically requires a space-creating mutation at the gatekeeper position of the kinase of interest in order to accommodate the bulky N6-substituted ATP analogs. Surprisingly, purified LegK4-Δ1–58-wt was able to use N6-substituted analogs of ATP to thio-phosphorylate substrate proteins in HEK-293T cell lysate without the need for a gatekeeper mutation (Fig. 2A). Several other kinases are known to accept N6 substituted ATP analogs without a gatekeeper mutation, including CDPK1 from the pathogen *Toxoplasma gondii* (Lourido et al., 2013).

When HEK-293T cell lysate was incubated with purified LegK4-Δ1–58 and N6-Bn-ATP-γ-thio-phos, a striking banding pattern was observed. A minor population of LegK4-Δ1–58-wt autophosphorylation was observed at 110kDa, but there were also two or more strong bands between 70 and 80 kDa (Fig. 2A). It is unusual for a purified kinase incubated with cell lysate to phosphorylate a small number of proteins so specifically and robustly (Blethrow et al., 2008). We immunoprecipitated all thio-phosphorylated proteins followed by LC MS/MS identification and found 52 unique peptides from a cytosolic Hsp70 (HSPA8). This result, and the relative molecular weight and abundance of the phosphorylated proteins made Hsp70s stand out as the likely substrates of LegK4. Hsp70s are a family of abundant molecular chaperones whose members include Hsc70 (HSPA8) and Hsp72 (HSPA1A) of the cytosol and BiP (HSPA5) of the ER. Using purified human Hsc70, we confirmed that this protein is phosphorylated by LegK4 *in vitro* (Fig. 2B). We determined LegK4's specificity by testing other chaperones. LegK4 showed robust phosphorylation of Hsc70, Hsp72, and

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BiP, but not the closely related Hsp70 from *Escherichia coli*, DnaK, or 90 kDa heat shock proteins (Fig. S1B).

We determined the sites of phosphorylation using LC MS/MS. There was only a single observed phosphorylation at a conserved Thr in the substrate-binding domain (SBD) of the Hsp70s (T495 in Hsc70 and Hsp72, and T518 in BiP) (Fig. 2C, Fig. S2A, & Fig. S2B). Removal of the T495 phosphorylation site in Hsc70 abrogated LegK4-mediated phosphorylation, suggesting high specificity for a single site of phosphorylation (Fig. 2B). An additional Hsc70-T495S mutant showed a noticeable reduction in LegK4 phosphorylation revealing modest selectivity for Thr over Ser phosphorylation (Fig. 2B and Fig. S1).

LegK4 phosphorylates cytosolic Hsp70s during *L.p.* infection

We generated phospho-specific antibodies to Hsc70/Hsp72-pT495 and BiP-pT518 (Fig. S2C) to determine which Hsp70 isoforms were phosphorylated during infection. We also generated an isogenic *ΔlegK4* strain of *L.p* and complemented the strain with a plasmid encoding either a WT 3XFLAG-tagged LegK4-Δ1–58 or a kinase dead LegK4-Δ1–58-DA mutant. Cells stably expressing the FCγ receptor (to allow opsonization of *L.p.* with a *L.p.* specific antibody) were infected for one hour. The infection produced a strong phosphorylation signal of Hsc70/Hsp72 in the *L.p.*-WT but not in the *L.p.-ΔlegK4* or *L.p.-ΔdotA* strains (Fig. 2D). There was no noticeable phosphorylation of ER-resident BiP upon infection (Fig. 2D), consistent with the cytosolic localization of *L.p.* effectors. The complemented *L.p.-Δlegk4* strain containing 3X-FLAG-LegK4-Δ1–58-WT showed a recovery of Hsc70/Hsp72 phosphorylation, while the kinase dead strain did not (Fig. 2D).

Hsc70 phosphorylation reduces its J-protein stimulated ATPase activity

Hsp70s are ATPases known to play key roles in protein folding and homeostasis. The identified LegK4 phosphorylation site on Hsp70's substrate-binding domain was previously shown to be an important phosphoregulon in yeast Hsp70s (Beltrao et al., 2012). Additionally, T518 in the ER resident Hsp70, BiP, is adenylylated by the human Fic protein HYPE (Preissler et al., 2015; Sanyal et al., 2015). This modification reduced BiP's ATPase activity with no noticeable effect on protein folding capacity (Preissler et al., 2015). To test if LegK4-mediated phosphorylation might likewise impact ATPase activity or refolding functions, we used LegK4-Δ1–58-wt to phosphorylate recombinant Hsc70. We compared the ATPase activity of this modified chaperone to Hsc70-wt using a malachite green-based *in vitro* ATPase assay. Because the rate of ATP hydrolysis by Hsc70 is normally low, a stimulatory co-chaperone, DnaJA2 (DJA2), was added to improve signal intensity (Rauch and Gestwicki, 2014). Using this assay format, we found that the DJA2-stimulates ATPase activity of phosphorylated Hsc70 (+LegK4) was decreased (V_{max} of 24.2 ± 0.9 pmol/min) when compared to Hsc70-wt (-LegK4), (V_{max} of 33.4 ± 0.8 pmol/min (Fig. 3A).

To understand the functional consequences of this reduced DJA2-stimulated ATPase activity, we compared the ability of phospho-Hsc70 to refold the model substrate firefly luciferase *in vitro*. Denatured luciferase was incubated with Hsc70, ATP, and DJA2 and luminescence was used to measure the chaperone's ability to restore native folding. As the concentration

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of DJA2 was increased in these reactions, luminescence signal was first increased, followed by a characteristic decrease after reaching an optimal ratio of Hsc70:DJA2 (Fig. 3B). In contrast, we found that phosphorylated Hsc70 had reduced refolding capacity as compared to the Hsc70-wt (Fig. 3B). Interestingly, this finding suggests that the effects of phosphorylation are different than what was reported for adenylylation of BiP (Preissler et al., 2015). Specifically, LegK4 phosphorylation of Hsc70 decreased both its DJA2-stimulated ATPase activity and its protein refolding function.

Using a phos-tag™ gel, we quantified the amount of Hsc70 that had been phosphorylated by LegK4. We observed two distinct species, of which 53.4 ± 1.5% was phosphorylated (Fig. 3C). The sub-stoichiometric phosphorylation of Hsc70 may explain the modest reduction in ATPase activity.

LegK4 suppresses the unfolded protein response

Hsp70 family members are critical effectors of the unfolded protein response. Our previous research showed that *L.p.* has the ability to suppress certain arms of the UPR (Treacy-Abarca and Mukherjee, 2015). Following *L.p.* infection and UPR induction with Thapsigargin, there was no translational upregulation of the canonical UPR targets BiP and CHOP (Treacy-Abarca and Mukherjee, 2015).

We wondered whether the phosphorylation of Hsc70 might be involved in this process. After transient transfection of EGFP-LegK4-Δ1–58-wt, but not EGFP or EGFP-LegK4-Δ1–58-DA, into HEK-293T cells, we observed robust levels of Hsc70 phosphorylation (Fig. S2D). We examined the UPR using fluorescence-activated cell sorting (FACS) to distinguish between EGFP-expressing and untransfected cells and then measured levels of the UPR biomarker BiP in both cell populations. There was a noticeable increase in the production of BiP after treatment with Thapsigargin in cells transfected with the EGFP control plasmid (Fig. 3D). In contrast, cells expressing the EGFP-LegK4-Δ1–58-wt construct did not show increased BiP expression in response to Thapsigargin treatment (Fig. 3D). The population of cells in the EGFP-LegK4-Δ1–58-wt experimental sample that were not transfected had a normal response to Thapsigargin treatment (Fig 3D). Interestingly, cells that were not treated with Thapsigargin but were transfected with EGFP-LegK4-Δ1–58-wt also showed a modest decrease in basal levels of BiP as compared to untransfected cells in the same experiment (Fig. 3D). The kinase activity of LegK4 is critical for this result, as the kinase dead mutant showed a normal upregulation of BiP after UPR induction (Fig. 3D). Transfected HEK-293T cells were pretreated with the proteasome inhibitor MG-132 before Thapsigargin treatment to assess whether the lack of BiP observed in LegK4 expressing cells was due to proteasomal degradation of BiP. BiP suppression by EGFP-LegK4-Δ1–58-wt was unchanged in the presence of the proteasome inhibitor (Fig. S2E).

LegK4 expression reduces host global protein synthesis

One role that Hsc70 plays in the cell is to assist in folding of nascent polypeptides during translation (Nelson et al., 1992). Hsp70's dissociation from the ribosome during extreme heat shock leads to an inhibition of global protein synthesis (Shalgi et al., 2013). LegK4's phosphorylation and subsequent inactivation of Hsc70 led us to test whether this

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modification was causing inhibition of global protein synthesis. We used a homopropargylglycine (HPG) assay in which HPG is incorporated into newly synthesized proteins and can act as a reporter of translation (Beatty et al., 2005). Transiently transfected cells were sorted into EGFP expressing and EGFP negative (untransfected) populations. We observed a high translation and low translation population of cells in all conditions (Fig. 3E). However, the cells that were transfected with EGFP-LegK4-Δ1–58-wt fell exclusively into the low translation population indicating suppression of global translation (Fig. 3E). The cells transfected with EGFP as well as the kinase dead EGFP-LegK4-Δ1–58-DA were observed in both the high and low translating populations (Fig. 3E). A previous screen for *L.p.* effectors that block host translation identified Lpg0208 (Pkn5) as a hit (Barry et al., 2013). We have now confirmed that LegK4 and Pkn5 are the same effectors.

We pharmacologically tested whether the disruption of Hsp70 is responsible for the observed reduction in translation, by inhibiting Hsp70's ATPase activity with the chemical inhibitor JG-98 (Li et al., 2013). Cells pretreated with JG-98 showed a modest suppression of UPR induction by Thapsigargin, as measured by reduced upregulation of BiP and CHOP (Fig. S3A). Treatment with JG-98 also showed a reduction in global protein synthesis using the HPG assay (Fig. S3B). We then used JG-98 to test whether the LegK4-mediated Golgi fragmentation phenotype was also produced by inhibition of Hsp70 activity. While the Golgi appeared healthy and perinuclear in the DMSO treated samples, it was difficult to observe any Golgi staining in the JG-98 treated cells due to the robust Golgi fragmentation (Fig. S3C). These results corroborated the genetic and biochemical studies, suggesting that Hsc70 may be an important biological target of LegK4.

LegK4 knockout in *L.p.* releases translational suppression of BiP

The functional redundancy between the ~300 effectors of *L.p.* causes very few single effectors to show any growth defect in macrophages. Instead of looking at growth, we decided to test translation inhibition during infection. First, we tracked levels of phosphorylated Hsp70 during an infection time course. Phosphorylation of Hsp70 appears to peak at 4 hours, but some phosphorylation is maintained throughout an 8 hour infection time course (Fig. S4).

We then focused on LegK4 in the context of other *L.p.* effectors that were also shown to block translation. We made use of the *Δ5* strain of *L.p.* that lacked the 5 previously identified effectors known to inhibit protein synthesis (Fontana et al., 2011), and tested expression of the UPR marker BiP. The *Δlegk4* and *Δ5* strains both showed similar suppression of BiP after UPR induction with Thapsigargin as compared to WT (Fig. 3F). However, the *Δ5 + Δlegk4* strain showed an upregulation in BiP after Thapsigargin induction (Fig. 3F & 3G). Interestingly, there was a small but noticeable increase in the amount of BiP expressed in the *L.p.-Δ5* (Fig. 3F & 3G), but it was not significant when compared to the Thapsigargin-induced increase in BiP from *L.p.-WT*. There was no noticeable increase in CHOP with the *Δ5 + Δlegk4* strain. We believe this is because BiP is rapidly and robustly upregulated in response to the UPR, making it easier to observe small changes in the amount of newly synthesized protein. This is in contrast to CHOP, which is a low abundance transcription factor.

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Transient transfection of LegK4 increases the Hsp70 load on ribosomes

We transiently transfected LegK4 into HEK-293T cells to see if the observed reduction in global translation could be directly linked to Hsp70's association with the ribosome. The cell lysates of LegK4-Δ1–58-wt and LegK4-Δ1–58-DA transfected cells were fractionated with a sucrose gradient. The UV traces of the gradient showed that LegK4-Δ1–58-wt transfected cells had an increased 80S monosome peak while the heavier polysome peaks were decreased as compared to the kinase dead LegK4-Δ1–58-DA transfected cells (Fig. 4A). This result confirms our previous finding that LegK4-Δ1–58-wt decreases global translation, as there are less of the highly translating polysomes present in the UV trace. To validate the possibility that the observed changes in the ratio between polysomes and monosomes was due to Hsp70, we fractionated the lysate of HEK-293T cells treated with JG-98 for 3 hours. An increase in the 80S monosome peak and subsequent decrease in the heavy polysomes was also observed in these samples, as compared to the DMSO treated sample (Fig. 4B).

To explore the interaction between phosphorylated Hsp70 and the translating ribosome, we precipitated Hsp70 from each fraction of a sucrose gradient in LegK4-Δ1–58-wt and LegK4-Δ1–58-DA transfected cells. Surprisingly, we noticed that wt LegK4 caused a higher load of Hsp70 in the fractions containing ribosomes compared to the catalytic dead mutant (Fig. 4C & 4D). As heavier polysome fractions started to emerge, the difference in the amount of Hsp70 between LegK4-Δ1–58-wt and LegK4-Δ1–58-DA transfected cells, became more pronounced.

Discussion:

Several potent bacterial toxins have been previously shown to target host protein synthesis. Almost all cases of translation inhibition are a result of targeting the translation machinery itself such as inhibiting elongation or initiation factors (e.g. Diphtheria toxin or Lgt effectors from *L.p.*) (Belyi et al., 2006; 2008; Collier, 1975). We have identified a method of translation suppression and have characterized a highly specific and functionally important target of the *L.p.* eSTPK LegK4. The most prominent previous research done on LegK4 was limited to a crystal structure of the kinase domain showing that it does indeed adopt the fold of a eukaryotic-like kinase, and contains a novel dimeric interface not observed in any eukaryotic protein kinases (Flayhan et al., 2015). Here, we provide a description of a bacteria using a PTM to directly target Hsp70 during infection. Our results show that one of the primary reasons *L.p.* targets Hsp70 is to reduce host translation. We have not excluded the fact that Hsp70 phosphorylation could serve to help *L.p.* infections in other ways.

L.p. tightly controls host translation and the UPR. While many pathogens control host protein synthesis, *L.p.* is thought to use this mechanism to increase the available amino acid pool, which the pathogen then uses for its own survival (De Leon et al., 2017). The complimentary inhibition of the UPR is a common mechanism by which many bacteria promote their own survival inside of the host cell (Celli and Tsolis, 2014). Even in cases where multiple *L.p.* effectors that control protein synthesis are removed, such as *L.p.-Δ5*, there are mixed results in recovering host translation (Barry et al., 2013). Monitoring BiP, which is produced rapidly and robustly following induction of the UPR, provides a more

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sensitive method for observing the recovery of host translation. The *L.p.-Δ5+Δlegk4* showed a recovery of BiP expression that has not previously been seen in *L.p.* infection. This further implicates LegK4's role in suppressing translation, and subsequently UPR signaling (Treacy-Abarca and Mukherjee, 2015).

We also describe a PTM of Hsp70 that is directly linked to protein synthesis and ribosomal association. There are many possible explanations as to why an increase in Hsp70 on ribosomes can cause a decrease in protein synthesis. Taken together, our data suggests that a LegK4 mediated block in protein synthesis raises the possibility that phosphorylated Hsp70 is unable to fold nascent polypeptides correctly and thus remains associated with the polysomes longer than usual. Future work will look to address the details of the mechanism by which phosphorylated Hsp70 remains on the ribosome.

LegK4's phosphorylation of Hsp70 at a site that reduced global translation also indicates the possibility of an endogenous mammalian kinase that is capable of causing Hsp70 phosphorylation. Many PTMs observed during *L.p.* infection mimic mammalian signaling. There are also scenarios in which a cell reduces or alters global translation, particularly under stress (Lindquist, 1980). Future work holds promise in identifying an endogenous kinase as well as further characterizing the phosphorylation at this site in relevance to other diseased or stressed conditions.

STAR Methods

Contact For Reagent and Resource Sharing

Further information and requests for resources and reagents should be directed and will be fulfilled by the lead contact, Shaeri Mukherjee (Shaeri.Mukherjee@ucsf.edu).

Experimental Model and Subject Details

All HEK-293T (Female) and HeLa (Female) cell lines were grown at 37°C and 5% CO_2 in DMEM (Gibco) containing 10% (vol/vol) FBS (Axenia BioLogix). HeLa cells were authenticated through the UCSF Cell Culture Facility.

The LP02 thymidine auxotrophy strain of *L.p.* (LP02) was used in all cases of infection. All strains were grown on charcoal-yeast extract (CYE) plates for 48 hours. These plates were supplemented with the appropriate antibiotic when plasmids were introduced, and thymidine (100 μg/mL) for all LP02 growth. The *ΔlegK4* and *Δ5 + ΔlegK4* strains were constructed by allelic exchange using the gene replacement vector pSR47S as previously described (Merriam et al., 1997). Plasmids for *L.p.* transformation were made by cloning the specified DNA into pJB1806 (Bardill et al., 2005), a kind gift from the lab of Dr. Craig Roy, behind an introduced 3x FLAG tag. *L.p.* strains that were overexpressing 3X-Flag epitope tagged proteins were transformed with a pJB1806 plasmid containing an IPTG inducible tac promoter. The transformation was done by electroporation as previously described (Berger and Isberg, 1993). The recovery was done on CYE plates containing chloramphenicol (10ug/mL). All *L.p.* proteins were cloned from genomic DNA prepared from the Philadelphia strain of WT *L.p.* described above (LP02) and cloned into their respective vectors.

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Hsc70 proteins and vectors (pMCSG7) were gifts from J.E.G. and they were cloned from the *Homo sapien* Hsc70 and included a 6XHis tag followed by a TEV cleavage site. Protein purifications for *L.p.* proteins used a pPROEX HTb vector that included a 6XHis tag followed by a TEV cleavage site.

All yeast experiments were done with the with the BY4741 strain. For yeast overexpression experiments, proteins were cloned into a pCH043 plasmid, a kind gift from the lab of Dr. Jaime Fraser at UCSF. For mammalian transfection, all proteins were cloned into a pEGFP-C2 plasmid. All point mutants were generated by site directed

Method Details

Transfections and Western Blotting—Fugene HD (Promega) and opti-MEM (Gibco) were used for all transfections of HeLa and HEK-293T cells. Cells were transfected (conditions based on manufacturers recommendations) once the cells reached 60% confluency, for 24 hours. Cells were harvested at 75–85% confluency. Mammalian cell lysis was performed in cell lysis buffer (20 mM Tris at pH 7.6, 100 mM NaCl, 1 mM MgCl2, 1 mM DTT, 1% Triton, 1x Roche EDTA-free Complete protease inhibitor mixture, and 1x PhosStop phosphatase inhibitor (Roche)) unless otherwise specified. For western blotting, cells were washed once with ice cold PBS, and lysed in cell lysis buffer for 30 minutes on ice. Protein concentrations were measured using a Bio-Rad Protein Assay Dye (procedure based on factory recommendations).

After this, 10–20 μg of protein were separated by SDS/PAGE using pre-cast 4–12% Bis-Tris gels (Thermo-Fischer) and run in MOPS (Invitrogen) buffer at 200V for 1 hour. Following this, gels were transferred to 0.45μM nitrocellulose paper (Bio-Rad) in transfer buffer (1X TOWBIN, 10% v/v MeOH) at 90V for 35 minutes. Membranes were blocked for 1 hour, and then incubated with antibody concentrations based on the manufacturer's guidelines in all cases except the pT495-Hsc70/Hsp70 and the pT518-BiP antibodies from GenScript, which were used at 1:10,000. All blocking and primaries were done in a 5% bovine serum albumin (BSA) (Sigma-Aldrich) in TBST solution containing 0.02% (w/vol) sodium azide. Blots were imaged on a Licor system. All biological experiments were performed in triplicate and a representative blot was chosen for publication.

Immunofluorescence—HeLa cells were plated on glass coverslips and grown to 60% confluency. Cells were then transfected, grown for 24 h, and fixed. For Hsp70 drug treatment, cells were grown to 60% confluency, treated with JG-98 (Gifted from J.E.G.) for 6 hours at indicated concentrations, and then fixed. Fixing was done with 4% paraformaldehyde, followed by permeabilization with 0.1% saponin (Sigma Aldrich), and staining. Transient transfection staining included rabbit anti-GFP and mouse anti-GM130 (Invitrogen) for 1 hour, followed by washing, then anti-rabbit and anti-mouse antibodies conjugated to Alexa-488 and Alexa-568, respectively. Drug treated cells were stained with rhodamine-phalloidin (Invitrogen) and mouse anti-GM130 followed by anti-mouse Alexa-488. Coverslips were then stained with Hoeschst reagent, fixed to slides, imaged, and quantified manually.

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Quantification for transient transfection was performed by randomizing samples with numbers assigned by labmate that were hidden to experimenter. One-hundred EGFP positive cells were counted and the Golgi were observed for fragmentation. Following quantification, the identity of samples was revealed to the experimenter.

Yeast Growth Assay—Handling and transformations were done based on previously published methodologies (Lundblad and Struhl, 2001). Following transformations, each yeast strain was streaked on SD –URA. A single colony was grown overnight in SD –URA media with shaking. In the morning, a new liquid culture was started at OD_{600}=0.25. When cultures reach an OD_{600}=1.0, 5 mL was collected, washed in sterile ddH_2O, and resuspended in 500uL of ddH_2O. Five μL of each condition was plated on SD –URA and Gal/Raf –URA plates, and grown for 3 days to analyze growth. Experiment was done in biological triplicate and a representative image is shown in the figure.

Purification of LegK4 and Hsp70s—LegK4-Δ1–58-wt was purified as previously described (Flayhan et al., 2015). Hsc70, Hsp72, BiP, and all point mutations of these proteins were purified as previously described (Chang et al., 2010). Briefly, plasmids containing His-tagged proteins were transformed into *E. coli* BL21 (DE3). Following transformation, Bacteria were grown in TB broth at 37°C to an OD_{600}=0.6. Protein expression was induced by adding 1mM Isopropyl β-D-1-thiogalactopyranoside (IPTG), and the bacteria grew at 18°C for 16 hours. Selection antibiotic concentrations used for respective plasmids are as follows: ampicillin – 100 μg/mL, kanamycin – 50 μg/mL, chloramphenicol – 10 μg/mL (GoldBio). The cells were harvested by centrifugation and then mechanically lysed with a microfluidizer in lysis buffer (50 mM Tris at pH 8.0, 500 mM NaCl, 10% glycerol (vol/vol), 20 mM Imidazole, and 1x Roche Complete protease inhibitor mixture). The cleared lysate was incubated with 1mL/L Ni/NTA agarose resin (Qiagen) for 1 hour at 4°C. Bound proteins were washed with lysis buffer and eluted in buffer containing 300 mM imidazole. LegK4-Δ1–58-wt was further purified by size-exclusion chromatography on a Superdex 200 column (Amersham Biotech) and concentrated. In cases where the His tag needed to be cleaved, The purified LegK4-Δ1–58 was mixed with 6x-His tagged TEV protease at a 1:40 (w/w) ratio in the presence of 0.5 mM EDTA and 0.5 mM DTT. The mixture was dialyzed against 20 mM Tris at pH 8.0, 150 mM NaCl, and 5% glycerol (vol/vol) at 4°C. Cleaved LegK4- Δ1–58-wt was separated from uncleaved protein by reloading dialyzed protein onto Ni/NTA agarose resin. This was incubated for 1 hour at 4°C, and initial flow through was collected and con centrated. All Hsp70s were further purified by an ATP-agarose column using previously established protocols (Chang et al., 2008). Purified DnaK, Hsc70-NBD, Hsc70-SBD, Hsp72-NBD, and Hsp72-SBD were acquired from J.E.G. Purified Grp92 and Hsp90α were gifts from the lab of Dr. Jack Taunton at UCSF.

Thiophosphorylation assays and immunoprecipitations.—Thiophosphorylation assays were performed as previously described (Levin et al., 2016). In brief, assays for cell lysates and purified Hsp70s with purified LegK4-Δ1–58-wt were performed in buffer containing 50 mM Tris at pH 7.5, 150 mM NaCl, and 10 mM $MgCl_2$. For experiments with purified substrate, the buffer was supplemented with 1 μg of purified Hsp70 per condition,

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0.1 μg of purified LegK4-Δ1–58-wt per condition, and ATP-γ-thiophosphate (Axxora) at 250 μM. Cell lysate labeling experiments were done with HEK-293T at a concentration such that there was 20 μg of protein per condition. Lysate was incubated with buffer supplemented with 250 μM of the designated ATP-γ-thiophosphate analog (6-Benzyl-ATP-γ-S (Bn) Axxora) 250 μM ATP, 3 mM GTP, and 1% (w/w) purified LegK4-Δ1–58-wt. Labeling reactions were left at room temperature for 1 h before quenching with 25 mM EDTA. Thirty μL aliquots of each reaction were alkylated with 2 uL of 100 mM p-nitro benzyl-mesylate (PNBM) for 30 minutes at room temperature. Thiophosphorylation was detected by western blot with the antithiophosphate antibody. Immunoprecipitations of thiophosphorylated substrates were performed using Bn-ATP-γ-S as previously described (Allen et al., 2007).

LC-MS/MS Phosphosite Identification—Purified Hsc70-wt was phosphorylated with LegK4 as described for the thiophosphorylation assay, with the exception that ATP-γ-thiophosphate was replace with 1 mM ATP. Mass Spectrometry was performed as previously described (Levin et al., 2016) with some modifications. After the 1 hour incubation at room temperature, ammonium bicarbonate and DTT were added to the reaction to reach final concentrations of 50 mM and 5 mM, respectively. The proteins were denatured at 55°C for 30 min. Denatured proteins were alkylated with Iodoacetamide that was added to 10 mM, and the solution was incubated at room temperature for 30 min. Samples were digested overnight at 37°C with trypsin or Asp-N (P romega) at a 1:20 (w/w) ratio. Peptides were acidified with 2% (vol/vol) formic acid, desalted with ZipTips (Millipore), and speed-vacuumed to dryness.

Desalted peptides were resuspended in 0.1% formic acid and diluted so that only 0.1 μg of peptides were analyzed per LC-MS/MS run. Peptides were loaded onto a nanoACQUITY (Waters) UPLC instrument for reversed-phase chromatography with a C18 column (BEH130, 1.7-μM bead size, 100 μm x 100 mm) in front of an LTQ Orbitrap Velos. The LC was operated at a 600-nL/min flow rate and peptides were separated over a 60-min gradient from 2 to 50% buffer B (Buffer A: water and 0.1% formic acid; buffer B: acetonitrile and 0.1% formic acid). Survey scans were recorded over a 350–1,800 *m/z* range and MS/MS fragmentation was performed using HCD on the top eight peaks. Peak lists were generated with an in-house software called PAVA and searched against the SwissProt *Homo sapien* database (downloaded June 27, 2013; 20,264 entries), as well as a manual user input of the LegK4 amino acid sequence, using Protein Prospector (v5.21.2). Data were searched with a 20-ppm tolerance for parent and fragment ions allowing for standard variable modifications and S/T/Y phosphorylation.

***L.p.* Infections.—**HEK-293 cells expressing FCγRIII (Gift from the lab of Dr. Craig Roy) cells were infected as described previously (Treacy-Abarca and Mukherjee, 2015). Cells were grown to 80% confluency and infected with the designated *L.p.* strain or isogenic mutant at an MOI of 100. Following a 48 hour heavy patch, *L.p.* was grown in ACES buffered yeast extract (AYE) supplemented with 0.33 mM $Fe(NO_3)_2$, 3.3 mM L-cysteine, and any necessary antibiotics or auxotrophy supplements. In any 3X-Flag overexpressing strains, AYE broth included 0.5 mM IPTG to induce protein production. Liquid cultures

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were grown overnight and collected at an OD_{600} of 3.0–3.5. HEK-293 cells require opsonization which uses a lab-generated anti *L.p.* antibody at 1:2000 for a 20 min incubation with *L.p.* before infection. For the 3X-Flag overexpressing strains of *L.p.*, all media used during infection included 0.5mM IPTG. Infection was initiated with a centrifugation spin at 1000xg. Treatment of cells with thapsigargin (Enzo) was done as described previously (Treacy-Abarca and Mukherjee, 2015). Briefly, cells were washed once with PBS after 1 hour of infection followed by the addition of either thapsigargin at 1uM or DMSO for 5 hours. All infected cells were harvested after infection, and snap frozen at specified time points.

Isolation of purified phosphoT495-Hsc70—The Hsc70 purification process started as described above. Following the lysis with a microfluidizer and clearing of the lysate by centrifugation, the protein concentration of the crude cellular supernatant was measured using a Bio-Rad protein assay dye. The lysate was split into two even aliquots (phosphorylated and nonphosphorylated samples). Both samples were treated with $MgCl_2$ to a final concentration of 10mM and ATP to a final concentration of 1 mM. Purified LegK4-Δ1–58-wt without a 6x-His tag, was added to one of the samples (phosphorylated) at 1% (w/w) of total protein concentration. The samples were both incubated at room temperature for 2 hours with gentle rotation. Following this, the samples were purified in the same way as other Hsp70s starting with Ni/NTA-agarose resin.

ATPase assays with Malachite Green—The ATPase activity of phosphorylated and non-phosphorylated Hsc70 was done with malachite green (MG) (Sigma Aldrich) as described previously (Chang et al., 2008). Briefly, in a clear 96-well plate, phosphorylated or non-phosphorylated Hsc70 were incubated with human DnaJA2 (DJA2) in 25 μL total volume. The assay buffer was 100 mM Tris at pH 7.4, 20 mM KCl, 6 mM $MgCl_2$, and 0.01% Triton. The reaction was initiated by the addition of ATP at a final concentration of 1 mM and incubated at 37 °C for 1 hour. After incubation, 80 μL of MG reagent was added, followed by 10 μL of saturated sodium citrate to quench the reaction. Absorbance was measured at 620 nm on a SpectraMax M5 plate reader (Molecular Devices). ATP hydrolysis rates were calculated by comparison to a phosphate standard. Displayed curves are a combination of 6 replicates.

Luciferase Refolding—Luciferase refolding assays were preformed as previously described (Wisén and Gestwicki, 2008). Briefly, native firefly luciferase (Promega) was denatured in 6 M guanidinium hydrochloride for 1 h at room temperature and then diluted into assay buffer (28 mM HEPES at pH 7.6, 120 mM potassium acetate, 12 mM magnesium acetate, 2.2 mM DTT, 8.8 mM creatine phosphate, and 35 U/mL creatine kinase). Solutions were prepared of phosphorylated and nonphosphorylated Hsc70, denatured luciferase (at 0.1 μM), DnaJA2 (DJA2) and 1 mM ATP. Total volume was 25 μL and incubation time was 1 hour at 37 °C. Steady Glo rea gent was prepared fresh and added to the plate immediately prior to reading luminescence. ATPase activity was determined using a nonlinear fit and Michaelis-Menten graphical analysis. Displayed curves are a combination of 6 replicates.

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SDS-PAGE Phos-Tag Gels—Hsc70 that was phosphorylated by LegK4 during the purification process was separated into phosphorylated and nonphosphorylated species using 8% SDS-PAGE gels with 100μM Phos-tag™(Wako Chemicals) acrylamide and 200μM $MnCl_2$. Gels were run under standard electrophoresis conditions. Gels were then incubated in transfer buffer containing 1 mM EDTA for 20 minutes, followed by incubation in transfer buffer for another 20 minutes. Gels were transferred and treated as described above. This experiment was repeated in triplicate and then quantified to obtain the ratio of phosphorylated vs. nonphosphorylated Hsc70 in the sample.

Flow Cytometry Experimentation and Analysis—All flow cytometry experiments were performed on a FACSCantoII (BD Biosciences) using 405 nm, 488 nm, and 635 nm lasers, and the software analysis was done using FlowJo (v. 10.3.0). Cells were first gated for singlets by graphing forward scatter height vs. forward scatter area and excluding any outliers. These cells were then gated for viability by removing any cells above background control of the Zombie Aqua dye with 405 nm laser. Any further analysis was done only on this population of cells.

BiP Expression Assay for FACS—HEK-293T cells were grown and transiently transfected in 6-well plates, as described above. For experiments with MG-132, cells were pretreated with 10 μM MG-132 (Selleckchem) or DMSO for 1 hour. All cells were treated with 1 μM Thapsigargin or DMSO for 6 hours. Cells were removed from the plate, washed 2x with phosphate buffered saline (PBS), and stained with Zombie Aqua (BioLegend) at 1:1000 in PBS to test for viability. Cells were washed 2x with PBS, fixed with 4% paraformaldehyde, washed once with PBS, washed once with permeabilization buffer (3% (w/vol) BSA, 0.5% saponin (w/vol), and PBS), and incubated in permeabilization buffer for 30 min at room temperature. Cells were spun down and resuspended in permeabilization buffer containing BiP antibody at 1:100 (manufacturer recommended conditions for fixing, permeabilization, and antibody concentrations) for 30 min at room temperature. Cells were washed 3 times with permeabilization buffer and resuspended in permeabilization buffer, with anti-rabbit Alexa-647 (Invitrogen) at 1:1000, for 30 min at room temperature. Cells were washed twice with permeabilization buffer and twice with FACS buffer (1% BSA (w/vol) in PBS) and then resuspended in FACS buffer for analysis by flow cytometry.

Measuring Translation with Homopropargylglycine—Homopropargylglycine (HPG) labeling was performed as described previously (Beatty and Tirrell, 2008), with some modifications. HEK-293T cells were grown on 6-well plates and were either transiently transfected as described above, or grown to 80% confluency and treated with drug for 1 hour (JG-98 at 20 μM and cyclohexamide (Cell Signaling Technologies) at 100 μg/mL). Media was removed and cells were washed with PBS. HPG was pulsed for one hour at 37°C and 5% CO_2, by adding cys/met-free DMEM (Thermo Fisher - 21013024) supplemented with 10% dialyzed FBS (Thermo Fisher), 200 μM cys (Sigma Aldrich), and 1 mM HPG (ClickChemistryTools) or 1 mM Methionine (Sigma Aldrich) as a negative control. Cells were washed once with PBS, then removed from plates with 250 μL of 0.25% Trypsin-EDTA solution (LifeTechnologies). Cells were washed twice with PBS and stained with Zombie Aqua at 1:1000 in PBS. Cells were washed 2x with PBS, then fixed with 4%

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paraformaldehyde. Fixed cells were washed twice with PBS, and twice with permeabilization buffer. Cells were resuspended in 25 μL of permeabilization buffer and 100 μL of azide click mixture (50 mM HEPES at pH 7.5, 150 mM NaCl, 400 μM Tris(2-carboxyethyl)phosphine hydrochloride (TCEP) (Pierce), 250 μM Tris[(1-benzyl-1*H*-1,2,3-triazol-4-yl)methyl]amine (TBTA) (ClickChemistryTools), 5μM AlexaFluor-647 azide (Thermo Fisher), and 200 μM $CuSO_4$ (Sigma Aldrich)) was added. This was gently mixed and incubated overnight in the dark. The cells were washed three times with permeabilization buffer, twice with FACS buffer, and then analyzed via flow cytometry.

Polysome Fractionation and Protein Precipitation for Western Blotting— Polysome profiling was performed as previously described (Truitt et al., 2015). In brief, 5 X 10 cm plates of transiently transfected or drug treated (JG-98 or DMSO at 20μM for 3 hours) cells were treated with 100 μg/mL cyclohexamide for 5 min at 37°C and 5% CO_2. Cells were collected, washed with PBS containing 100 μg/mL cyclohexamide, then lysed in polysome buffer (10 mM HEPES at pH 7.4, 100 mM KCl, 5 mM $MgCl_2$, 100 μg/mL cyclohexamide, and 2 mM DTT) supplemented with 1% Triton, 1x Roche EDTA-free Complete protease inhibitor mixture, 1x PhosStop phosphatase inhibitor (Roche), and 100 U/mL of RNaseOUT (Invitrogen). Lysates were cleared by centrifugation for 10 min at 9300xg and supernatants were loaded onto a 10–50% sucrose gradient. Sucrose gradients were made by diluting 60% Sucrose solution (60% w/v sucrose in polysome buffer) to 10% and 50% sucrose solutions with polysome buffer. Each centrifuge tube was filled half-way with 10% and then 50% sucrose solutions, and a Gradient Master 108 (BIOCOMP) was used to make the gradients.

Samples were spun at 37,000rpm for 2.5 h at 4°C in a Beckman L8–70M ultracentrifuge using a Beckman SW-40 rotor, and then separated on an ISCO gradient fractionation system to evaluate polysome profiles and collect polysome fractions. Protein was precipitated from each individual fraction using a trichloroacetic acid (TCA) (Fisher) - acetone precipitation. A 6M stock solution of TCA was added to each fraction to reach a final concentration of 20% (vol/vol). This was incubated on ice for 30 min, followed by centrifugation at 20,000xg for 30 min. The pellet that formed was washed twice with ice-cold acetone and then air dried for 2 min. The protein pellet is dissolved in Laemmli buffer at pH 8.8 to neutralize any remaining TCA. Samples were denatured at 95°C for 10 min and then used in western blotting.

Antibodies used were as follows, with product number in parenthesis: Abcam: Antithiophosphate ester (Ab92570); proteintech: BiP (11587–1-AP), CHOP (15204–1-AP); Enzo: HSC70/HSP70 (ADI-SPA-820); NovusBio: RPL10A (NBP2–47298); Cell Signaling Technology: GAPDH (2118); Invitrogen: FLAG (MA1–91878-D680); BD: GM-130 (610823). Both the pT495 HSC70/HSP72 rabbit polyclonal antibody and the pT518 Grp78/BiP mouse monoclonal antibody were raised by GenScript and are available upon request.

Quantification and Statistical Analysis—Quantification of the transient transfection immunofluorescence images was graphed using Prism 6.0. error bars in Fig. 1B represent

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s.e.m. and the data is pooled from 3 biological replicates that included the counting of 100 EGFP positive cells for all conditions.

The *In vitro* Hsc70 assays in Fig. 3A & 3B were analyzed using Prism 6.0. Each line represents the averages of 6 replicates. The data in Fig. 3A was fit to a Michaelis-Menten curve and the error bars for both Figures represent s.e.m. for each data point. The acquired V_{max} was determined with the same analysis and represents $V_{max} \pm$ s.e.m.

Quantification of western blots was performed using ImageJ64. Raw quantifications were used for analysis and standardization as specified in the figure legends for Fig. 3F & 4D. Error bars in these graphs represent s.e.m. Significance indicated by "*" in the text is designated at $P<0.05$ using a Student's t-test. Each experiment was done in biological triplicate.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- *Legionella* kinase 4 (LegK4) phosphorylates host cytosolic Hsp70s during infection.
- Phosphorylated Hsp70 has reduced ATPase and refolding activity.
- LegK4 inhibits host translation via Hsp70 phosphorylation.
- LegK4 increases the amount of Hsp70 associated with translating polysomes.

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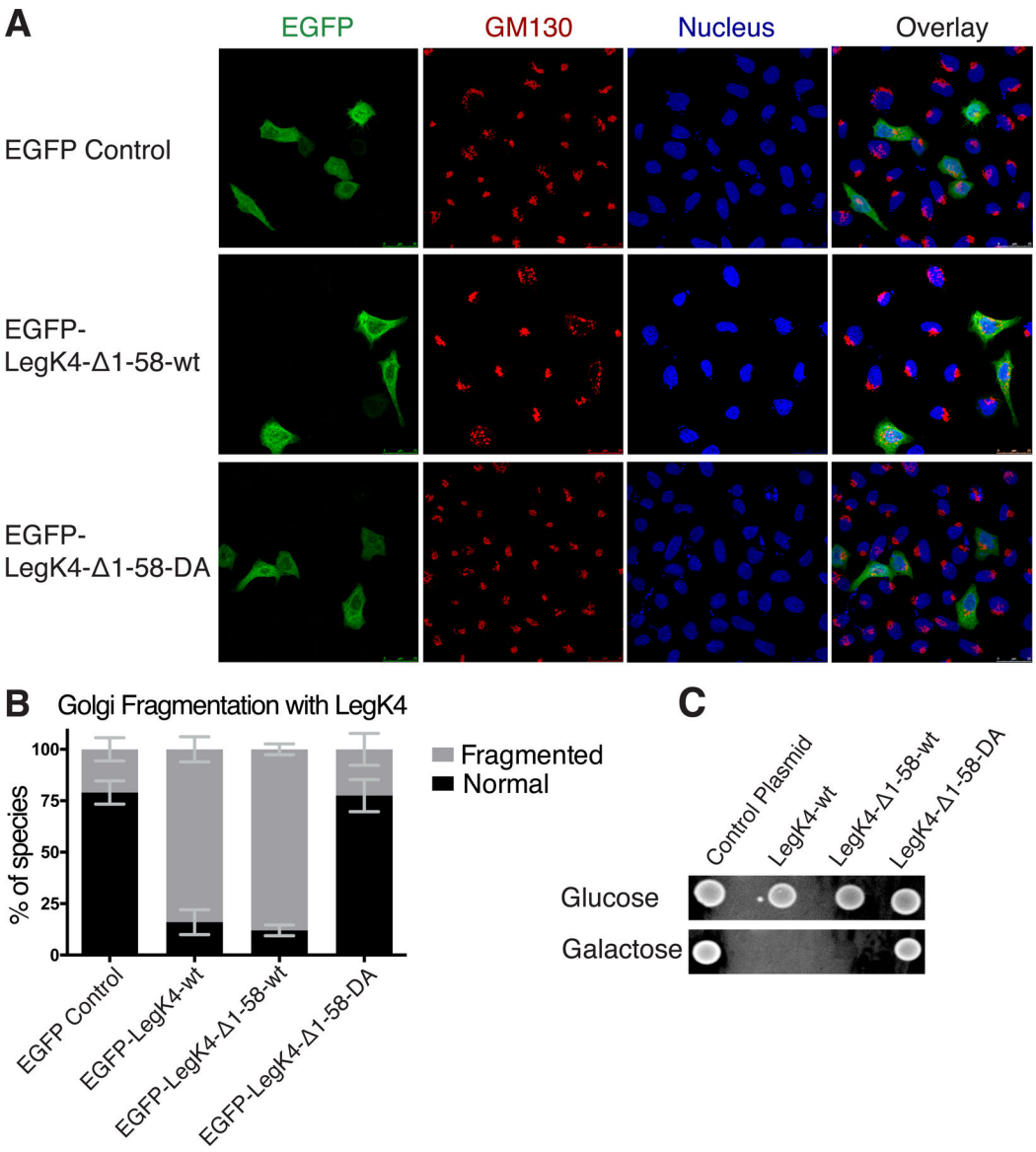

Fig. 1. LegK4 causes Golgi fragmentation and inhibits yeast growth.

(**A**) HeLa cells were transiently transfected with an EGFP tagged LegK4-Δ1–58-wt, as well as EGFP control, and the D271A kinase dead construct of LegK4. Golgi was stained with GM130 in red, and nuclei were stained with blue. (**B**) Amount of Golgi fragmentation was quantified by counting 100 EGFP positive cells per experimental condition. Each condition was done in triplicate. Error bars represent S.E.M. (**C**) LegK4-wt as well as LegK4-Δ1–58-wt were transformed into yeast behind a galactose promoter. Yeast growth is shown on both glucose and galactose conditions. Shown is a representative of the experiment which was confirmed in 3 separate biological replicates.

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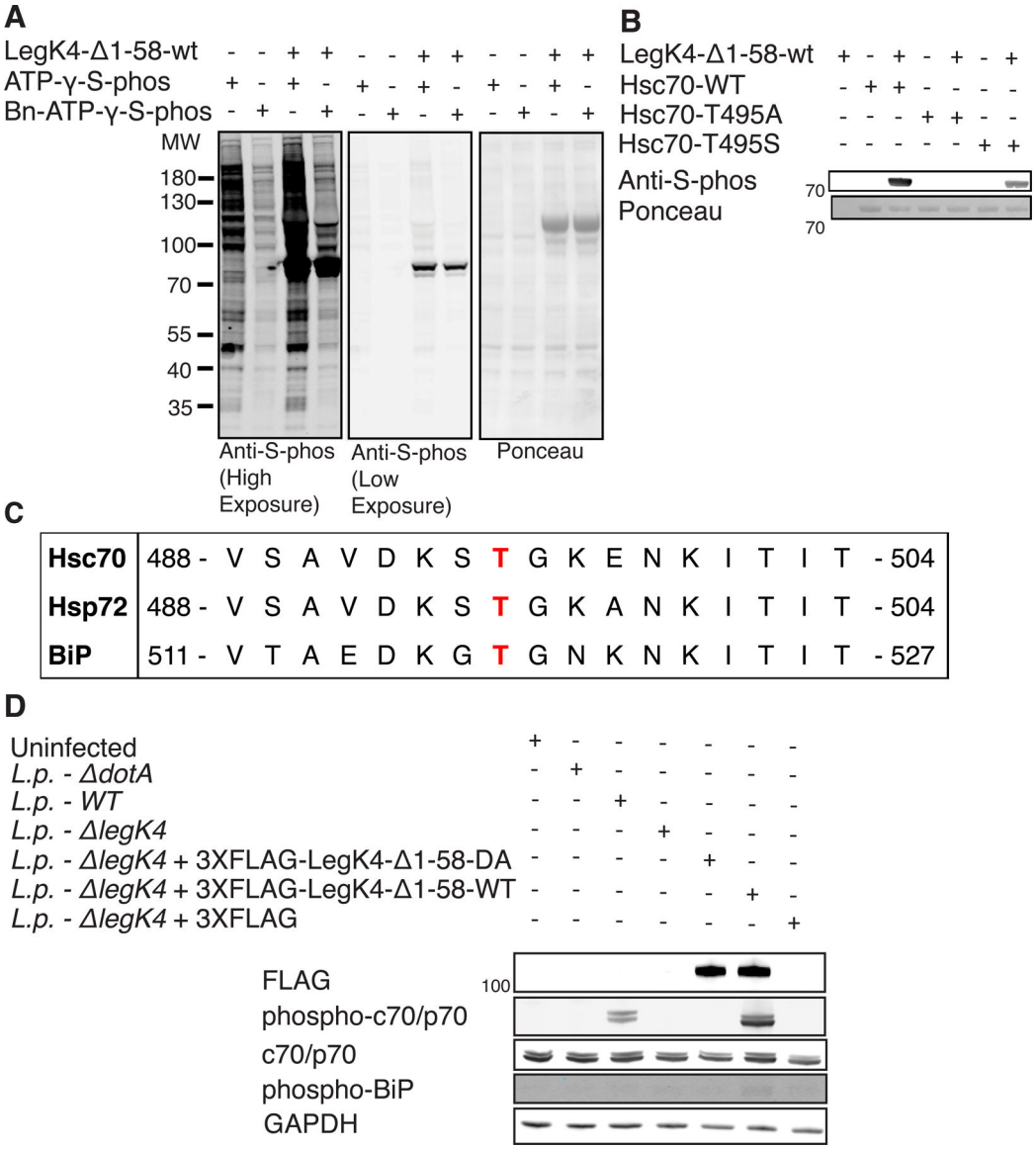

Fig. 2. Identification of Hsp70 as the substrate of LegK4.

(**A**) Purified LegK4-Δ1–58-wt was added to HEK-293T cell lysate along with ATP-γ-thiophosphate (ATP-γ-S-phos) or the corresponding Benzyl-N6-substitued ATP derivative (Bn-ATP-γ-S-phos). (**B**) Following identification of the phosphorylated residue in Hsp70, Hsc70-T495A and T495S constructs were purified and the same assay was performed. (**C**) Amino acid sequence of the identified phosphosites. The targeted threonine is in bold red. (**D**) HEK-293T-FCγRIII cells were infected for 4 hours with various strains of L.p. including the Dot-Icm secretion system knockout (*ΔdotA*) as well as the LegK4 knockout (*ΔlegK4*). Cells were additionally infected with recovery strains transformed with 3XFLAG-*legK4* variants. Phospho-Hsc70/Hsp72 as well as phospho-BiP, represent custom antibodies. See also Fig. S1 & Fig. S2.

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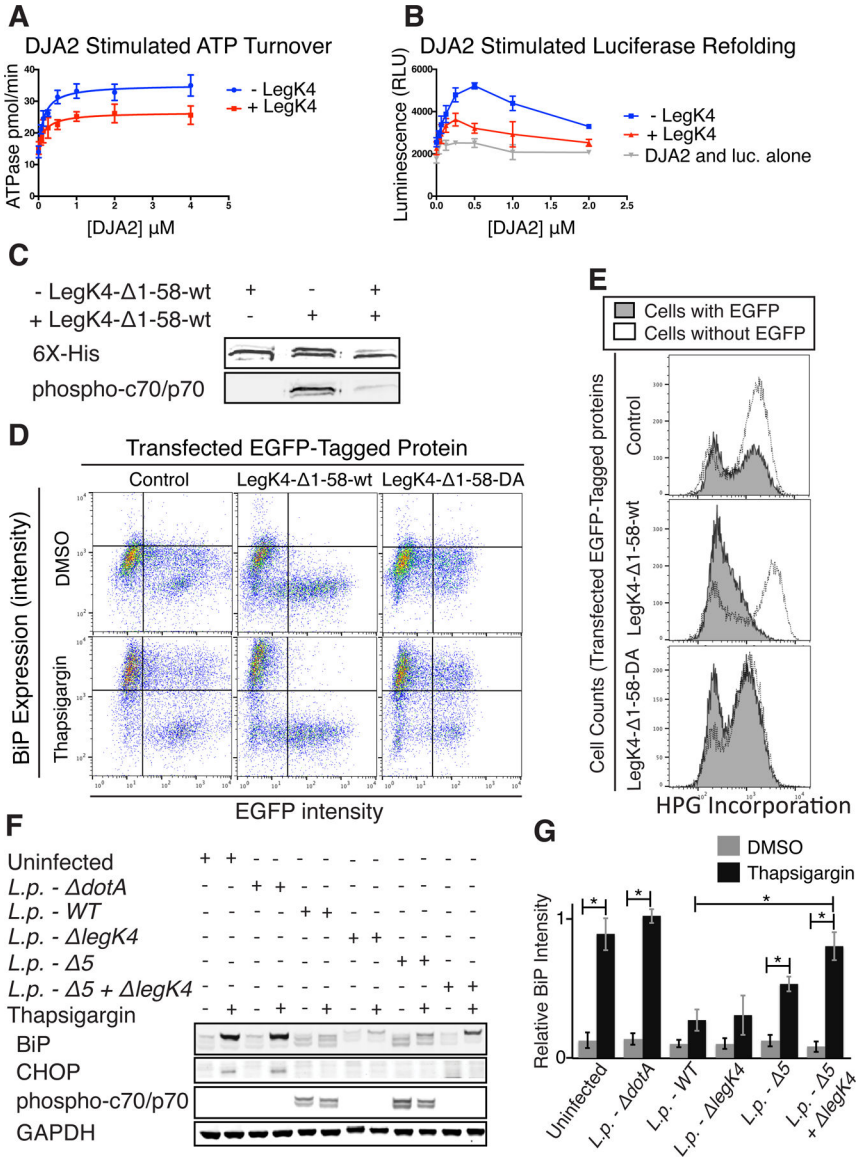

Fig. 3. Phosphorylation of Hsp70 by LegK4 decreases activity and causes a reduction in global protein translation.

(**A** & **B**) *In vitro* analysis of phosphorylated Hsc70 using purified protein. (**A**) ATPase activity was tested using a malachite green assay with the J-protein DNAJ2 to stimulate ATPase activity. (**B**) The refolding activity of Hsc70 was determined using a luciferase assay. Luminescence signal was read upon luciferase refolding by Hsc70 in the presence of DNAJ2 to stimulate ATPase activity and refolding. Each graph represents 6 experimental replicates. (**C**) The amount of phosphorylated Hsc70 after incubation with LegK4 was quantified using Phos-tag™ SDS-Page gels. The Hsc70 species were quantified in triplicate to show that 53.4 ± 1.5% of the Hsc70 was phosphorylated. All recombinant Hsc70 was purified with a 6XHis tag, as represented by the western blot. For **A**, **B**, & **C**, +LegK4 and −LegK4 indicate purified Hsc70 samples that were or were not incubated with LetK4, respectfully. (**D**) BiP levels were tested following transient transfection with EGFP tagged LegK4-Δ1–58-wt, LegK4-Δ1–58-DA, or EGFP control plasmid. The transfected HEK-293T

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cells were treated with DMSO or 1uM Thapsigargin for 6 hours. Cells were then analyzed by FACS based on expression of transfected protein (EGFP) and levels of BiP (AF-647). (**E**) Global translation was tested using a HPG assay. EGFP expressing and nonexpressing cells were gated and are shown in grey and white respectively. (**F**) HEK-293T-FCγRIII cells were infected with L.p. for 1 hour followed by 5 hour treatment with 1uM thapsigargin. Blotting was done for the UPR stress markers BiP and CHOP. (**G**) BiP levels were quantified from 3 biological replicates using the same experimental conditions. Individual data points were normalized to GAPDH, and all the data was then normalized to the uninfected condition. The values in graphs are mean ± s.e.m. *P<0.05, Student's t-test. See also Fig. S2.

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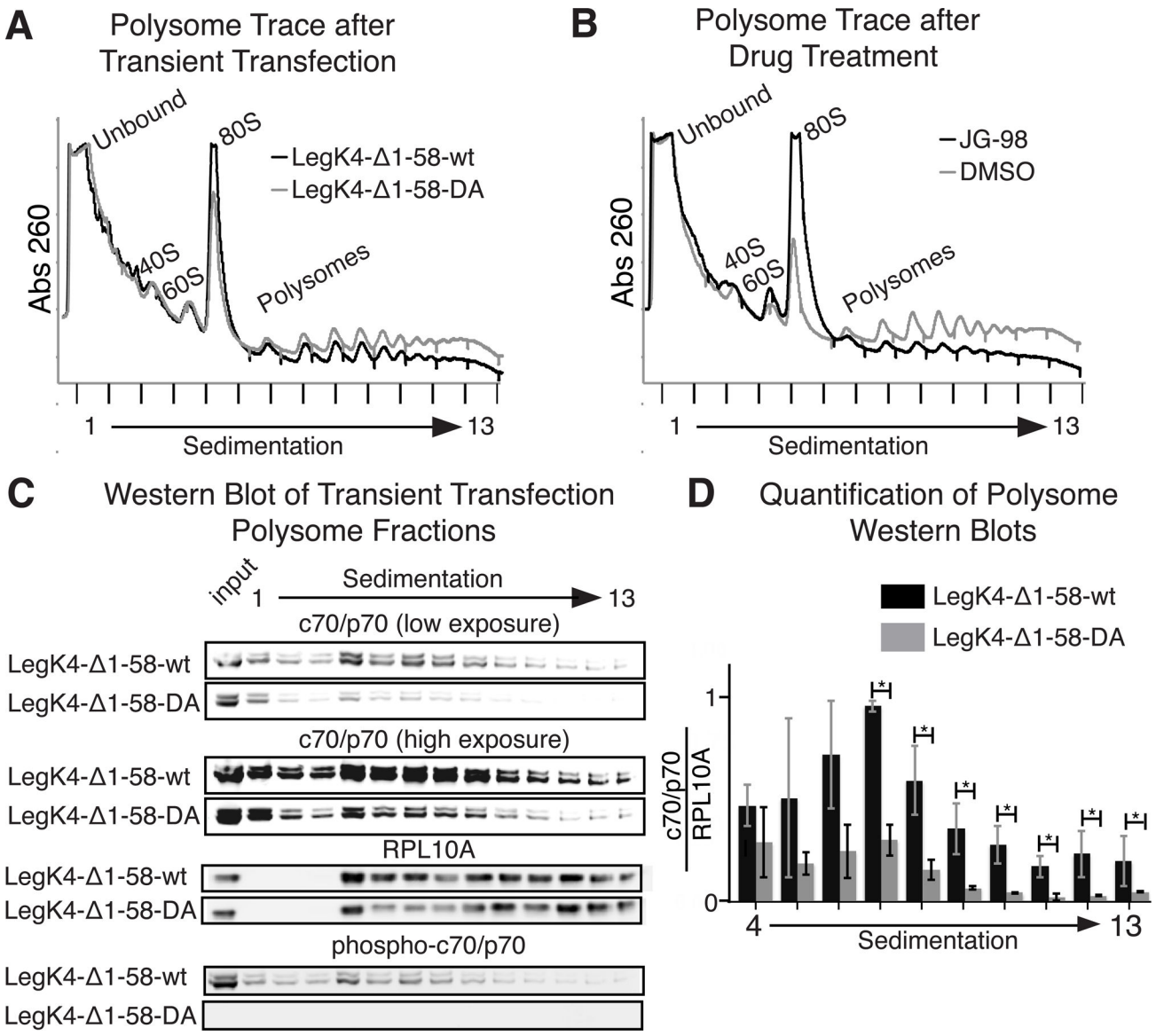

Figure 4. Interactions of phosphorylated Hsc70 with the ribosome.

(**A & B**) HEK-293T cells were transiently transfected with LegK4-Δ1–58-wt or LegK4-Δ1–58-DA (**A**), or treated with DMSO or JG-98 for 3 hours (**B**), and the lysates were fractionated into polysomes. The graph is a representative of the polysome UV trace which was done in triplicate. (**C**) The protein was precipitated and western blots were run on all fractions from the transient transfection experiment in **A** to determine amount of cytosolic Hsp70 (c70/p70) associated with the polysomes. (**D**) c70/p70 levels of each fraction containing RPL10A were quantified by dividing the raw quantitation of c70/p70 by the amount of RPL10A. The data was then normalized to the input. The values in graphs are mean ± s.e.m (n=3). *P<0.05, Student's t-test.