Mycobacterial HflX is a ribosome splitting factor that mediates antibiotic resistance

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Antibiotic resistance in bacteria is typically conferred by proteins that function as efflux pumps or enzymes that modify either the drug or the antibiotic target. Here we report an unusual mechanism of resistance to macrolide-lincosamide antibiotics mediated by mycobacterial HflX, a conserved ribosome-associated GTPase. We show that deletion of the hflX gene in the pathogenic Mycobacterium abscessus, as well as the nonpathogenic Mycobacterium smegmatis, results in hypersensitivity to the macrolide-lincosamide class of antibiotics. Importantly, the level of resistance provided by Mab_hflX is equivalent to that conferred by erm41, implying that hflX constitutes a significant resistance determinant in M. abscessus. We demonstrate that mycobacterial HflX associates with the 50S ribosomal subunits in vivo and can dissociate purified 70S ribosomes in vitro, independent of GTP hydrolysis. The absence of HflX in a ΔMs hflX strain also results in a significant accumulation of 70S ribosomes upon erythromycin exposure. Finally, a deletion of either the N-terminal or the C-terminal domain of HflX abrogates ribosome splitting and concomitantly abolishes the ability of mutant proteins to mediate antibiotic tolerance. Together, our results suggest a mechanism of macrolide-lincosamide resistance in which the mycobacterial HflX dissociates antibiotic-stalled ribosomes and rescues the bound mRNA. Given the widespread presence of hflX genes, we anticipate this as a generalized mechanism of macrolide resistance used by several bacteria.

HflX | macrolides | Mycobacterium abscessus | erm41 | ribosome

Mycobacterium abscessus has emerged as an important human pathogen during the last 10 y, causing superficial and deep-tissue infections after traumatic injury and/or surgery, as well as bronchopulmonary infections in patients with chronic lung damage, such as prior tuberculosis and cystic fibrosis, resulting in a persistent decline in pulmonary functions or acute respiratory failure (1). The major threat posed by this organism is its extremely low sensitivity to most FDA-approved antibiotics, making its infections incredibly difficult to treat (2, 3). The current treatment regimen against M. abscessus recommends a combination of an oral macrolide in conjunction with amikacin and 1 or more of the injectables (cefotaxim, imipenem, or tigecycline) for a period of several months (2, 4). The majority of these antibiotics target the ribosome, a 2.5-MDa ribonucleoprotein enzyme composed of a 50S and 30S subunit. The binding sites for most ribosome-targeting antibiotics are primarily concentrated at 3 locations within the ribosome: the decoding site on the 30S subunit, the peptidyl transferase center (PTC), and/or the nascent peptide exit tunnel (NPET) on the 50S subunit (5). Macrolide, lincosamide, and streptogramin B antibiotics are structurally distinct but are often considered together (MLS<sub>B</sub> antibiotics), as they have overlapping binding sites on the 50S subunit around the 23S rRNA nucleotide, A2058 (6). Macrolides are 14- to 16-member macrolactones and bind in the upper portion of the NPET between the PTC and the constriction formed by the proteins L4 and L22 (7, 8). Macrolide binding does not interfere with peptide bond formation per se, but hinders the passage of newly synthesized polypeptides, thereby interrupting translation elongation (9, 10). Lincosamides are smaller molecules that occupy the region between A2058 and the PTC in a way that overlaps with the aminocycl moiety of the A-site tRNA, thereby preventing peptide bond formation (11).

Intrinsic resistance to macrolides is commonly attributed to 3 primary mechanisms: target modification, active efflux by ABC transporters and the Major Facilitator superfamily, and drug inactivation by esterases, lyases, and phosphorylases (12). Target modification at A2058 of the 23S rRNA by methylases confers cross-resistance to macrolide, lincosamide, and streptogramin B, commonly referred to as the MLS<sub>B</sub> phenotype, and is the most widespread mechanism of macrolide resistance (12, 13). More recently, the Antibiotic Resistance ATP binding cassette family F (ARE ABC-F) proteins have been shown to confer macrolide resistance by ribosome protection in several Gram-positive bacteria (14, 15). While some macrolide resistance genes are constitutively expressed, the majority are inducible by low doses of antibiotics through transcriptional or translational attenuation (16, 17). In mycobacteria, the most common mechanism of macrolide resistance involves mutations in the macrolide binding site on the 23S rRNA, as well as methylation of these residues by erm-encoded RNA methyltransferases (18, 19). The expression of macrolide erm genes is under the control of a transcriptional activator, WhiB7, which is in turn controlled by translational attenuation in the presence of subinhibitory concentrations of structurally unrelated antibiotics (20, 21). Deletion of whiB7 in Mycobacterium smegmatis, Mycobacterium tuberculosis, and M. abscessus results in multidrug sensitivity to MLS<sub>B</sub> and other ribosome-targeting antibiotics (22, 23).

Significance

The erm41 gene is considered the primary mechanism of intrinsic resistance to macrolides in Mycobacterium abscessus. Here we demonstrate that the hflX gene plays a significant and equally important role as erm41. We further describe an unusual mechanism of resistance to macrolide-lincosamide antibiotics mediated by the mycobacterial HflX that likely involves the dissociation of antibiotic-stalled ribosomes. An understanding of the various mechanisms employed by bacteria for resistance to an antibiotic is critical in predicting an effective therapeutic regimen against a pathogenic isolate, and can also inform the development of novel drugs.

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Previously, we used genomewide transcriptomic profiling by RNAseq and identified ∼80 genes in the WhiB7 regulon of *M. abscessus* and *M. smegmatis*, one of which is MAB_3042c, a homolog of the universally conserved Translation Factor (TRAFAC) family of GTPases, HflX (22). Despite its widespread distribution, knockout strains of *hflX* are viable, and the precise biological function in most organisms is unclear (24, 25). The *Escherichia coli* HflX is known to be involved in splitting of stalled ribosomes generated during heat shock into free subunits, and the *Staphylococcus aureus* HflX was shown to disassemble hibernating 100S ribosomes (26, 27). Although binding of macrolides has been shown to interfere with the GTPase activity of *E. coli* HflX, the *E. coli* HflX has not been shown to be directly involved in antibiotic resistance (28). Recently, the hflX-r gene from *Listeria monocytogenes* was shown to confer macrolide resistance (29). We demonstrate here that Mab-HflX (MAB_3042c) and Ms-HflX (MSMEG_2736) are required for macrolide-lincosamide resistance in *M. abscessus* and *M. smegmatis*, conferring equivalent resistance as the *erm* genes, by an *erm*-independent pathway. We also demonstrate that Ms-HflX is a ribosome splitting factor, and disruption in its ability to dissociate ribosomes results in an inability to mediate antibiotic resistance. Our results suggest that a likely mechanism of mycobacterial HflX-mediated macrolide-lincosamide resistance involves dissociation of ribosomes stalled in the presence of these antibiotics.

**Results**

**Deletion of the HflX Homolog Confers Hypersensitivity to Macrolide-Lincosamide Antibiotics in *M. abscessus* and *M. smegmatis***. WhiB7, a transcriptional activator, is one of the earliest genes upregulated in response to ribosome-targeting antibiotics, and in turn activates the expression of ∼80 genes that comprise the WhiB7 regulon (22). A few genes in this regulon have known functions such as efflux pumps, the *erm* and *cis2* genes; the roles of most genes are, however, unknown. One such gene induced in response to ribosome-targeting antibiotics as part of the WhiB7 regulon is MAB_3042c, a homolog of the universally conserved ribosome binding protein, HflX (*SI Appendix, Fig. S1*). A comparison of the WhiB7 regulon of *M. abscessus* and *M. smegmatis* showed that MAB_3042c is also one of the few genes that is shared between the WhiB7 regulons of the 2 species; MSMEG_2736, the ortholog in *M. smegmatis*, displays ∼80% amino acid sequence identity to MAB_3042c (*SI Appendix, Fig. S2*) (22). Since HflX is known to bind the ribosome in several bacterial species studied, we explored whether MAB_3042c is involved in resistance to ribosome-targeting antibiotics (26, 30, 31). Isogenic deletions of MAB_3042c (Mab_hflX) in ATCC 19977 and MSMEG_2736 (∼Ms_hflX) in mc²155 were constructed using phage recombineering (22, 32). The resulting deletion strains ∆Mab_hflX and ∆Ms_hflX were hypersensitive to the macrolides erythromycin (ERT), clarithromycin (CLA), and azithromycin (AZT) and the lincosamide clindamycin (CLIND) (*Fig. L4, Table 1, and SI Appendix, Fig. S3 and Table S1*); their sensitivity to several other ribosome-targeting antibiotics remained unchanged (*SI Appendix, Fig. S4*). Constitutive expression of *hflX* driven by *phsp60* from a chromosomally integrated copy in the respective mutant strains restored sensitivity of the mutant to wild-type levels; in fact, the complementing strains displayed increased tolerance to macrolides and clindamycin compared with wild-type bacteria (*Fig. L4 and SI Appendix, Fig. S3*). Moreover, overexpression of Ms_hflX in ∆Mab_hflX
Mycobacterial HflX Dissociates 70S Ribosomes In Vitro, Independent of GTP Hydrolysis. The E. coli HflX has previously been shown to be inducible under heat stress, and an E. coli ΔhflX mutant is highly heat sensitive. However, the viability of ΔMab_hflX and ΔMs_hflX remain unchanged on exposure to elevated temperatures (SI Appendix, Fig. S5 A and B). The E. coli HflX has also been shown to be involved in splitting of stalled ribosomes generated during heat shock into free subunits (26). To determine whether mycobacterial HflX is capable of similarly dissociating 70S ribosomes in vitro, we examined the effect of adding purified Ms-HflX to 70S ribosomes, using sucrose density gradient centrifugation (SDGC). Fig. 2 A–F shows that Ms-HflX indeed promoted dissociation of 70S ribosomes in the presence of either GTP or ATP, consistent with previous studies showing that E. coli HflX can bind to both ATP and GTP (31). No ribosome dissociation was observed in the absence of added nucleotides (Fig. 2F); moreover, maximum splitting efficiency was observed in the presence of the nonhydrolysable GTP analog GMP-PNP (Fig. 2F), which implied that although nucleotide binding is necessary for ribosome splitting, GTP hydrolysis is not. The distribution of HflX in ribosomal fractions was monitored by immunoblotting using anti-his antibodies that recognized his-tagged Ms-HflX. Although HflX could be detected in the top fractions as well as 70S fractions, an enrichment was observed in the 50S subunit fractions (Fig. 2G).

In Vivo Association of HflX with Ribosomes from Antibiotic-Treated Cells. To determine whether HflX associates with ribosomal fractions in vivo, we constructed a strain in which Ms-hflX was also restored its antibiotic tolerance to wild-type levels, thereby suggesting a conserved function of Mab_hflX and Ms_hflX (Fig. 1A).

Curiously, the M. abscessus WhiB7 regulon contains additional known and putative effectors of macrolide resistance; specifically, the full-length emr41 gene and homologs of ABC-F proteins, MAB_1846 and MAB_2355, transcription of which are induced on macrolide exposure. To evaluate their relative contribution to macrolide resistance, we constructed isogenic deletions in known and putative effectors of macrolide resistance; specifically, the erm41 gene and homologs of ABC-F proteins, MAB_1846, and MAB_2355. Fig. 1 A and Table 1 show that Δerm41 and ΔhflX were both equally hypersensitive to erythromycin and clindamycin; a ΔhflX/Δerm41 double-mutant and ΔwhiB7, however, displayed significantly increased sensitivity compared with either single mutant alone. Moreover, overexpression of either hflX or emr41 restored antibiotic sensitivity of the ΔwhiB7 mutant strain, suggesting that Erm41 and HflX act via independent pathways (Fig. 1C). ΔMAB_2355 and ΔMAB_1846 strains displayed mild sensitivity to erythromycin and clindamycin, respectively (Fig. 1B).

Mycobacterial HflX Dissociates 70S Ribosomes In Vitro, Independent of GTP Hydrolysis. The E. coli hflX has previously been shown to be inducible under heat stress, and an E. coli ΔhflX mutant is highly heat sensitive. However, the viability of ΔMab_hflX and
C-terminally tagged with the 3X-FLAG epitope at its native chromosomal location. The distribution of endogenous HflX in ribosome fractions obtained from *M. smegmatis* treated with either ERT or CLIND, as well as an untreated control sample, was examined by SDGC coupled with immunoblotting. Fig. 3A shows that Ms-HflXFLAG was enriched in the 50S ribosomal fractions obtained from antibiotic-treated cells, but almost undetectable in corresponding fractions obtained from bacteria untreated with antibiotics.

We next analyzed the polysome profile of ribosomes isolated from wild-type and ΔMs_hflX strains when exposed to ERT, as well as in the absence of the drug, by SDGC. As seen in Fig. 3C, an increased accumulation of the 70S fraction was observed in the presence of ERT and likely represents ribosomes stalled in the presence of the drug. The quantity of 70S ribosome was, however, significantly (*P < 0.05*) greater in ERT-exposed ΔMs_hflX cells than that observed in ERT-exposed wild-type bacteria and could reflect a decrease in dissociation of antibiotic-stalled ribosomes in the absence of HflX (Fig. 3B and SI Appendix, Fig. S6).

**Ribosome Splitting Function of HflX Correlates With Its Ability to Mediate Antibiotic Resistance.** Bacterial and eukaryotic HflX are 3 domain proteins composed of a unique N-terminal HflX domain (NTD), a central GTPase domain (G-domain), and a C-terminal domain (CTD) (26, 33). Interaction with the large ribosomal subunit is a conserved feature of all HflX proteins and is thought to be facilitated by the N-terminal and C-terminal domains (30, 31, 34). Moreover, the ribosome splitting function of *E. coli* HflX has previously been shown to require both the N- and C-terminal domains (26). To determine whether the ribosome splitting function of Ms-HflX influences its ability to confer macrolide resistance, we created deletions in the N- and C-terminal domains of Ms-HflX identified based on the structure of Ms-HflX modeled on ribosome-bound *E. coli* HflX (Fig. 4A and B). The Ms-HflX mutants containing deletions of either the N-terminal (HflX236–471/NTD) or the C-terminal (HflX1–402/CTD) domains were then assayed for ribosome splitting activity in vitro and antibiotic resistance in vivo. Fig. 4 C–F shows that removal of either the NTD or the CTD of Ms-HflX resulted in a defect in their ability to dissociate 70S ribosomes. Preliminary analyses show that both mutant proteins retain their ability to bind ribosomes (SI Appendix, Fig. S7A). Importantly, both HflXΔNTD and HflXΔCTD from *M. smegmatis* and *M. abscessus* were functionally defective in their ability to complement the antibiotic sensitivity of their respective ΔhflX mutant strains (Fig. 4H, Table 2, and SI Appendix, Fig. S7 B and C). Finally, a point mutant of Ms HflX (K258A/S259A) defective in ribosome splitting was also found to be defective in complementing the antibiotic sensitivity of ΔMs_hflX (Fig. 4G and H). Together, these data indicate that the ability of mycobacterial HflX to mediate antibiotic resistance correlates directly with its ribosome splitting ability.

**Macrolide Sensitivity of Mycobacterial ΔhflX Mutant Can Be Partially Restored by the Ribosome Recycling Factor.** The ribosome recycling factor (RRF) is required for dissociation of the posttermination complex during a normal translation cycle, thereby making the subunits available for a new round of translation. Although key differences exist between the requirements of HflX and RRF, both proteins are required for recycling ribosomes that are stalled under different physiological conditions (26). We therefore tested the ability of *M. smegmatis* RRF to complement the antibiotic resistance of ΔMs_hflX. As seen in Fig. 5 and SI Appendix, Table S1, constitutive expression of Ms-RRF partially restored antibiotic sensitivity of the mutant, supporting a role of ribosome splitting as a mechanism of antibiotic resistance. Partial complementation of HflX function by RRF in antibiotic resistance is also consistent with the notion that ribosomal substrates of HflX and RRF are likely to be fundamentally different (26).

**Discussion**

The *erm*41 gene is considered to be the primary mechanism of intrinsic macrolide resistance in *M. abscessus* (18, 35). In the present study, we show that the *M. abscessus* hflX gene constitutes a...
significant effector of macrolide-lincosamide resistance and confers equivalent levels of resistance as ermH1 via an erm-independent mechanism. We demonstrate that Ms-HflX strongly associates with ribosomal subunits in vivo in bacteria that are exposed to either ERT or CLIND, and an absence of HflX in the ΔMs_hflX deletion strain results in an increased population of 70S ribosomes on erythromycin exposure compared with wild-type bacteria. The mycobacterial HflX is also capable of dissociating 70S ribosomes in vitro independent of GTP hydrolysis, similar to that observed in *E. coli* HflX during heat shock. Nevertheless, the cryoEM structure of *E. coli* HflX reveals that the NTD binds to the 50S ribosomal subunit and protrudes into the PTC, making extensive contact with ribosomal RNA, whereas the CTD interacts with the βL12 stalk base and occupies a position distant from the PTC (26). Owing to the conservation of mycobacterial HflX with that of *E. coli*, an alternate scenario is that the mycobacterial HflX-NTD occupies a similar position within the ribosome and occludes macrolide-lincosamide binding. *SI Appendix*, Fig. S8; however, reveals that the presence of HflX neither interferes with binding of $^{3}$H-ERT to ribosomes nor dissociates $^{3}$H-ERT that is already bound to ribosomes. Instead, the evaluation of HflX mutants demonstrating that mutants that are defective in their ability to restore antibiotic tolerance of ΔMs_hflX are also defective in ribosome splitting, as well as the ability of mycobacterial RRF to restore macrolide tolerance of ΔMs_hflX, together strengthen the importance of ribosome splitting in macrolide resistance (Figs. 4 and 5). Finally, the involvement of Ms-HflX-CTD, which is distant from the PTC, making extensive contact with ribosomal RNA, whereas the CTD interacts with the βL12 stalk base and occupies a position distant from the PTC (26). Owing to the conservation of mycobacterial HflX with that of *E. coli*, an alternate scenario is that the mycobacterial HflX-NTD occupies a similar position within the ribosome and occludes macrolide-lincosamide binding. *SI Appendix*, Fig. S8; however, reveals that the presence of HflX neither interferes with binding of $^{3}$H-ERT to ribosomes nor dissociates $^{3}$H-ERT that is already bound to ribosomes. Instead, the evaluation of HflX mutants demonstrating that mutants that are defective in their ability to restore antibiotic tolerance of ΔMs_hflX are also defective in ribosome splitting, as well as the ability of mycobacterial RRF to restore macrolide tolerance of ΔMs_hflX, together strengthen the importance of ribosome splitting in macrolide resistance (Figs. 4 and 5). Finally, the involvement of Ms-HflX-CTD, which is distant from the PTC, in conferring antibiotic tolerance reinforces the conclusion that antibiotic occlusion/ejection is unlikely to be the primary mechanism of HflX-mediated resistance to macrolides/lincosamides.

We therefore envisage a scenario in which binding of macrolides/lincosamides to ribosomes in the early stages of elongation results in accumulation of stalled, nonproductive ribosomes and a concomitant inhibition of translation. Association of HflX with antibiotic-stalled ribosomes presumably causes dissociation of the 50S and 30S subunits and rescue of the bound mRNA, which can then be translated by antibiotic-free ribosomes, thereby enabling survival in the presence of the drug. The postdissociated 50S subunit–HflX–antibiotic complex could either be sequestered from further rounds of translation due to the anti-association properties of HflX (26) or could serve as a substrate of accessory proteins that displace bound antibiotic/HflX. These possibilities are illustrated in Fig. 6. Moreover, it is likely that accessory proteins (if any) are also macrolide-lincosamide inducible and are included within the WhiB7 regulon. We speculate that MAB_1846 and MAB_2355, homologs of ABC-F proteins that displace ribosome-bound antibiotics, constitute strong candidates for accessory proteins that mediate recycling of the postdissociated 50S subunit–HflX–antibiotic complex. Although deletions in MAB_1846 or MAB_2355 alone do not significantly influence macrolide-lincosamide resistance, further investigations will be required to determine whether they act in concert with HflX, as well as to determine the identity of additional accessory proteins.

Our data in Fig. 3 shows preferential association of HflX with ribosomal fractions obtained from ERT/CLIND-treated cells; negligible levels of ribosome-bound HflX were observed in untreated cells. This difference could be attributed to a WhiB7-dependent increase in HflX expression in antibiotic-treated bacteria (*SI Appendix*, Fig. S9). However, HflX levels in lysates of ERT-treated bacteria do not show an increase proportionate to the enrichment of HflX observed in ERT-treated ribosomes (∼10-fold; *SI Appendix*, Fig. S9). We therefore speculate that recruitment of HflX could be facilitated by structural changes within antibiotic-bound ribosomes that are recognized either directly or indirectly by HflX. However, we cannot exclude a basal level of HflX interaction with antibiotic-free ribosomes, which is suggested by the lethal effect of multicopy expression of HflX from a strong promoter (*SI Appendix*, Fig. S10). Further investigations will be needed to elucidate the characteristics of ribosomes that serve as substrates for HflX binding, as well as the role of accessory proteins (if any) in facilitating HflX binding to ribosome.

Two classes of ribosome-associated proteins have been implicated in antibiotic resistance: the Ribosome Protection Proteins that confer tetracycline resistance and the ARE ABC-F group of proteins that confer resistance to the macrolide-lincosamide-ketolide antibiotics. Both classes of proteins show homology to the translation factors, EF-G and EF-Tu, and actively trigger the release of the antibiotic bound to the ribosome (14, 36–39). Dissociation of ribosomes stalled in the presence of antibiotics and rescue of mRNA has, however, not been described as a mechanism of antibiotic resistance until very recently, when Duval et al. (29) demonstrated that deletion of *Listeria monocytogenes* hflX (lm0762) confers sensitivity to erythromycin and lincomycin and results in accumulation of 70S ribosomes in the mutant strain on antibiotic exposure. Interestingly, *L. monocytogenes* encodes 2 HflX paralogs, only 1 of which is involved in antibiotic resistance. Such a duplication event is observed in other firmicutes, as well as proteobacteria; the actinomycetes, however, encode a single copy of *hflX*. Curiously, a phylogenetic analysis shows that mycobacterial and *E. coli* hflX are more closely related to *lmo1296*, the hflX paralog in *L. monocytogenes* incapable of conferring resistance to macrolides/lincomycin (*SI Appendix*, Fig. S11) (29). We also note that the *E. coli* HflX is unable to complement the antibiotic sensitivity of ΔMs_hflX and ΔMab_hflX mutants, suggesting either that *E. coli* HflX is not involved in macrolide-lincosamide resistance or that it confers antibiotic resistance only in its native host (*SI Appendix*, Fig. S12). Functional metagenomics from antibiotic-rich environments that

![Fig. 5. Overexpression of the ribosome recycling factor (Ms-RRF) partially restores macrolide sensitivity of ΔMs_hflX. Complementing strains were created by integrating either Ms-RRF or Ms_hflX at the Bxb1 attB site of ΔMs_hflX. Expression of Ms-RRF in the complementing strain was verified using real-time PCR (*SI Appendix*, Table S2). Tenfold serial dilutions of wild-type *M. smegmatis*, ΔMs_hflX, and the complementing strains were grown to A600 of 0.7 and spotted on Middlebrook 7H10 ADC containing indicated concentrations of macrolides.](https://www.pnas.org/content/117/1/633/fig/5)
identify hflX homologs as putative antibiotic resistance genes belong to both the HflX-r (Enemescu spp) and HfX (Sinamka spp) clades (40, 41). A possible explanation is that while all HfX proteins retain their ability to split 70S ribosomes into their subunits, their duplication allows each paralog to recognize ribosomes that are stalled under different physiological conditions. The ability of HfX to confer antibiotic tolerance may therefore be a generalized and widespread mechanism used by several bacteria, and not restricted only to species that contain the hflX-r paralog.


**Materials and Methods**

Referenced details of the materials and methods, including plasmids, strains, and oligonucleotides are provided in the SI Appendix. Proteins were purified using Ni-NTA chromatography. Antibiotic sensitivity assays were carried out either using broth dilution method or by spotting a 10-fold dilution series on plates containing the desired antibiotic concentrations. For analysis of polypeptide profiles on antibiotic exposure, bacteria were exposed to either ERT (20 μg/mL) or CLIND (16 μg/mL). Cells were lysed using the CyoMill (Retsch), and crude ribosomes were prepared as described (42). After lysis, 50% of the cell lysate was layered on 10% to 40% sucrose gradients, and centrifuged using a Beckman SW 41 rotor followed by Brandel Teledyne fractionation. Ribosomes were isolated from wild-type McfSS, Mcr_hflX, and McfSS-Ms_hflX-FLAG strains as described (42). For in vitro translation assays, purified ribosomes (0.2 μM) were incubated with 15-fold molar excess of full-length Ms-hflX, point mutants, or truncated mutants (Ms-hflX JNTD and αCTD) in a 50-μL total volume in HMA-7 buffer in the presence of 1 mM GTP, ATP, or GMP-PNP. The reactions were incubated at 37°C for 45 min and layered on 5-μL 10% to 40% sucrose gradients.

**Data Availability.** All data have been provided in the main article and SI Appendix.

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