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Hfq chaperone brings speed dating to bacterial sRNA

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Abstract

Hfq is a ubiquitous, Sm-like RNA binding protein found in most bacteria and some archaea. Hfq binds small regulatory RNAs (sRNAs), facilitates base pairing between sRNAs and their mRNA targets, and directly binds and regulates translation of certain mRNAs. Because sRNAs regulate many stress response pathways in bacteria, Hfq is essential for adaptation to different environments and growth conditions. The chaperone activities of Hfq arise from multi-pronged RNA binding by three different surfaces of the Hfq hexamer. The manner in which the structured Sm core of Hfq binds RNA has been well studied, but recent work shows that the intrinsically disordered C-terminal domain of Hfq modulates sRNA binding, creating a kinetic hierarchy of RNA competition for Hfq and ensuring the release of double-stranded sRNA-mRNA complexes. A combination of structural, biophysical and genetic experiments reveals how Hfq recognizes its RNA substrates and plays matchmaker for sRNAs and mRNAs in the cell. The interplay between structured and disordered domains of Hfq optimizes sRNA-mediated post-transcriptional regulation, and is a common theme in RNA chaperones.

Graphical/Visual Abstract

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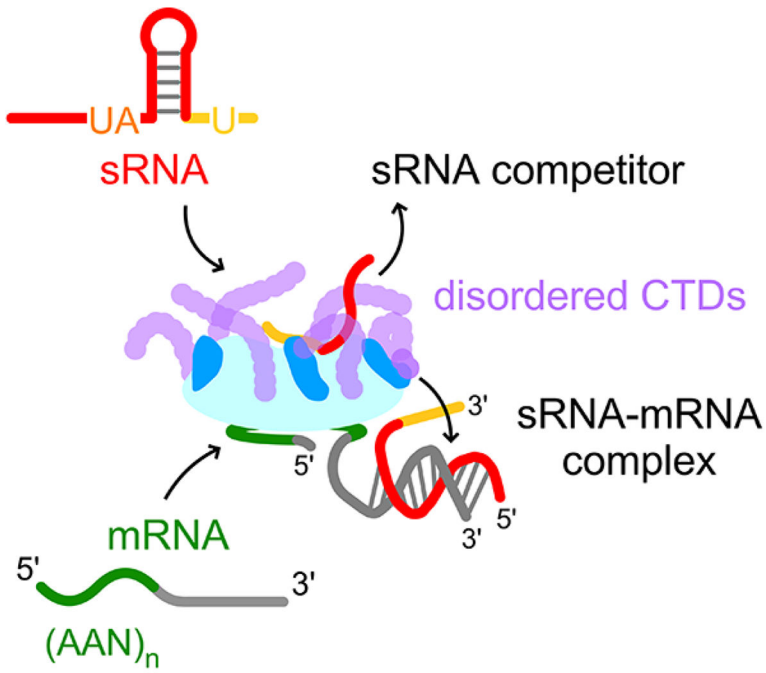

The chaperone Hfq accelerates match-making between small regulatory RNA and mRNA in bacteria. The Hfq core (cyan) binds U and A rich RNA motifs, recruiting complementary strands to arginine patches (blue). Disordered C-terminal domains (violet) sweep RNA from the core, quickly cycling prospective sRNA-mRNA pairs.

Introduction

Originally identified as a Host Factor required for the replication of bacteriophage Qβ in *Escherichia coli* (1), Hfq is a member of the Sm/Lsm family of RNA binding proteins present in the majority of sequenced bacteria (2–4). Like eukaryotic and archaeal Sm/Lsm proteins, Hfq plays several roles in bacterial RNA metabolism. These roles include destabilization of mRNA by promoting their polyadenylation (5, 6), stabilization of small regulatory RNA (sRNA) against degradation (7–9), facilitating sRNA-mRNA base pairing (8, 10) in an ATP-independent manner (11), and stimulation of sRNA and mRNA turnover by RNase E cleavage (9, 12, 13). Hfq can also directly bind certain mRNAs and regulate their translation (14–17). Hfq accomplishes these varied functions by using different surfaces to simultaneously recognize the sequence motifs and structural features of its target RNAs. This multi-faceted RNA recognition creates an adaptable and dynamic chaperone for RNA-dependent regulation.

Hfq is best known for its function in post-transcriptional gene regulation by sRNA. sRNAs range in size from 37–500 nt (18), and can stimulate or inhibit mRNA translation through a variety of mechanisms (Box 1). Many sRNAs act by base pairing with a complementary sequence in the target mRNA, either sequestering the ribosome binding site (RBS) (19) or

rendering it more accessible for translation (20, 21). In *E. coli* and many other bacteria, Hfq accelerates and stabilizes sRNA-mRNA base pairing (8, 10, 21).

Box 1

Mechanisms of sRNA regulation

Positive regulation involves the annealing of an sRNA to the 5′ end of the mRNA (22), generally remodelling inhibitory RNA structures around the ribosome binding site (RBS) to expose the RBS more for translation (23, 24). Alternatively, the sRNA may anneal to and sequester ribonuclease cleavage sites on the mRNA (25). Negative regulation occurs when the sRNA anneals to or around the RBS in a manner that inhibits binding of the ribosome (26). In some cases the annealed sRNA directly targets the mRNA for degradation by RNase E (9, 12, 13). In yet other examples, an sRNA indirectly regulates gene expression as a decoy for another sRNA or a sponge for an RNA binding protein (27).

Bacterial sRNAs often regulate several different mRNAs, and an mRNA may be targeted by more than one sRNA (18). These overlapping regulatory interactions may synergistically act towards a similar physiological outcome, or could antagonize each other. Since RNAs are typically in competition for access to Hfq (28, 29), a bacterium's response to a given signal is affected by the current cellular milieu of Hfq-dependent sRNAs and mRNAs, and the ability of each RNA to compete against the others. Consequently, bacterial non-coding RNAs and Hfq variants may have co-evolved in bacterial lineages (30), so that specific regulatory pathways are prioritized over others. For example the appearance of a long Hfq C-terminal domain is coincident with the expansion of the sRNA repertoire in Enterobacteriaceae (30), and the establishment of two classes of sRNAs that compete for Hfq with different efficiencies (31, 33).

One challenge in understanding Hfq-mediated regulation is that the RNA substrates vary in size, structure, and sequence motif combinations. This diversity of RNA ligands contrasts with the defined nature of guide RNAs in other RNA silencing systems such as miRNA/Argonaute and CRISPR-Cas (34). The highly varied nature of Hfq's RNA substrates is mirrored in the broad scope of its proposed protein partners, which have a variety of enzymatic functions (35). Thus, Hfq must be able to flexibly interact with a range of protein and RNA partners, while retaining selectivity for its proper substrates.

The biological functions of Hfq, its interactions with other nucleic acids and proteins in the cell, and newly identified modes of Hfq- and sRNA-mediated posttranscriptional regulation have been recently reviewed elsewhere (36–38). Here, we provide biophysical and biochemical perspectives on how sRNA and mRNA compete for binding to Hfq, how Hfq discriminates between different RNA substrates, and how Hfq facilitates the annealing of complementary RNA strands. Although most studies have used *E. coli* Hfq, where possible we discuss the results for bacterial and archaeal homologs.

RNA recognition by the Sm core

The Sm domain of Hfq assembles into a stable, homo-hexameric ring with sequence-specific binding surfaces on either face (Figure 1). Hfq-dependent sRNAs bind Hfq by interacting with its proximal pore, via their ρ-independent terminators that end with 4–6 uridines. By contrast, the distal face of Hfq recognizes an A-rich motif (46) that is often present in mRNA targets of Hfq and sRNA regulation (23, 24), and in Class II sRNAs (see Box 2 for sRNA definitions). These motifs enable Hfq to recognize its substrates, although the sequences and secondary structures of individual sRNAs and mRNAs vary enormously. All RNA substrates are thought to make additional stabilizing contacts with basic, arginine-rich patches found on the outer rim of the Hfq hexamer (47–51). It is at these basic rim patches where the sRNA-mRNA pair is annealed (39, 52). Finally, the structured core of Hfq is flanked by disordered N-terminal and C-terminal domains (NTDs and CTDs) (53, 54) (Figure 1A), which in some cases either bind RNA (45, 55) or compete for RNA-protein interactions on the core (32, 40). As discussed below, these disordered regions modulate the RNA binding properties of *E. coli* Hfq, and convert Hfq from an RNA binding protein into a dynamic RNA chaperone.

Box 2

sRNA Class definitions

E. coli sRNAs have been divided into two groups based on how they bind Hfq (31). The predominant group of sRNAs, Class I, binds to the Hfq proximal pore via a single-stranded U-rich 3′ end and to the Hfq rim via a combined A/U-rich single-stranded region followed by a stem-loop (31, 48, 49). The targets of Class I sRNAs frequently contain (AAN)x motifs and bind the distal face of Hfq (23). Class II sRNAs interact with the proximal pore, but also bind to the distal face via 5′ $(AAN)_x$ motifs (31, 33). The targets of Class II sRNAs are U-rich and interact with the rim of Hfq (31). While the accumulation of Class I sRNAs depends on residues on the rim of Hfq, Class II sRNA accumulation relies on the distal face (31).

Proximal Face

The proximal face contains a conserved, sequence-specific interaction site for U-rich single-stranded RNA (ssRNA). A deep channel around the proximal inner pore of the Hfq ring binds single-stranded uridines (Figure 1B) (56), with each monomer recognizing a single uridine base (10, 56). A slight selectivity for a terminal 3′-hydroxyl group is observed in Enterobacteriaceae (57, 58), but not in some Listeriaceae (59). There is no evidence that the RNA threads through the narrow central pore of the hexamer. Specificity for this U-rich RNA motif at the sRNA 3′ end is highly conserved across all bacteria (56, 59, 60) and archaea (55), and is similar to the binding site for eukaryotic and archaeal Sm proteins (61, 62). The analogous surface of the Lsm2-8 complex recognizes a very similar site in U6 snRNA, leading to the suggestion that the proximal face of the Sm ring is an ancient protein domain that recognizes RNA terminators and ssRNA (63, 64).

Distal face

The distal face of Hfq recognizes A-rich ssRNA (46). In mRNAs this A-rich site is typically found 5′ to the sRNA-targeted region (65, 66). Hfq's from Proteobacteria typically bind a triplet $(AAN)_x$ motif, with one triplet per monomer, in which A is adenine and N is any nucleotide (44, 46, 67) (Figure 1C). The discovery of this additional binding surface explained why Hfq could bind either A or U-rich RNA (68), and why the A-rich recognition sequence was generally longer than the U-rich site (69). RNAs that bind Hfq's distal face typically possess 2–4 AAN repeats (65, 66), although 6 repeats (18 nucleotides) can be accommodated on a single hexamer. The distal face residues involved in RNA contacts are well conserved within Proteobacteria, whereas Firmicute Hfqs interact with two nucleotides per monomer, or a total of 12 nucleotides for the full hexamer. Crystallography and tryptophan quenching experiments suggest that *S. aureus*, *B. subtilis* and *L. monocytogenes* Hfq bind an $(AN)_x$ motif, in which the second nucleotide makes non-sequence-specific H-bonding and stacking interactions with the protein (59, 67, 70, 71). Finally, some archaeal Hfq's possess diverged distal faces that no longer recognize adenine (55, 72). This loss in binding is thought to be due to mutations that cause i) steric occlusion of the binding pockets, ii) an absence of necessary polar contacts and iii) an increase in the negative potential of the distal face (55, 72).

Lateral rim

The discovery of sequence-specific RNA binding sites on the proximal and distal faces revealed how a single Hfq hexamer could bring sRNA and mRNA together, but not how Hfq might position an sRNA to pair with a complementary target. It was also unclear how Hfq protects the 5′ ends of sRNAs from ribonuclease digestion, if sRNAs are only anchored to the proximal pore of Hfq by their 3′ ends (73, 74). These questions were answered by the discovery that the lateral rim of the Hfq hexamer interacts with the "bodies" of sRNAs (48, 49) (Figure 1B, D). Moreover, Hfq's RNA chaperone activity was found to crucially depend on the number of arginines in basic patches on the lateral rim formed by each subunit (39, 48) (blue in Figure 1B). *E. coli* Hfq lacking one or more rim arginines per subunit binds sRNA and mRNA through proximal and distal face contacts, but is unable to promote annealing *in vitro* and is less active in sRNA regulation *in vivo* (39). Additionally, the annealing activities of Hfqs from the Proteobacterium *Pseudomonas aeruginosa* and the Firmicutes *Listeria monocytogenes*, *Bacillus subtilis* and *Staphylococcus aureus*, correlate with the number of arginines in the basic patch (52).

In vivo, sRNA-Hfq rim interactions were particularly important for the stabilization and accumulation of Class I sRNAs (31). The proximal side of the rim of *E. coli* Hfq is important for binding single-stranded A/U-rich motifs in sRNAs (Figure 1D) (31, 48, 49, 65) and U-rich loops in mRNAs (51). Discrimination against cytosine in preference for uracil is supported by recent structural data of Hfqs from *E. coli* and *Aquifex aeolicus* (45, 75). Additionally, there is evidence that the rim of *E. coli* Hfq interacts with dsRNAs and dsDNA (32, 40), indicating how stems in sRNAs may be bound (49).

In Proteobacteria, Thermotogae, Aquificae and Euryarchaeota, the rim of the Hfq hexamer tends to be strongly electropositive (57, 72, 75–77), with a cluster of arginines and lysines

positioned along a groove that connects the proximal and distal sides of the Sm ring. For example, the rim of *E. coli* Hfq contains a patch of 3 arginines and a nearby lysine (Figure 1D). Like the distal face, however, residues on the rim of Hfq have diverged in Firmicutes and Proteobacteria. In Firmicutes, the rim of the hexamer is generally neutral (56), with the mildly basic rim of *L. monocytogenes* Hfq being an exception (59). While Hfqs from cyanobacteria possess several basic residues on the rim (78), these residues are distributed more sparsely than basic residues in *E. coli* Hfq. Some archaeal Hfqs contain an extensive "strip" of basic residues, running from the proximal edge to the distal edge of the rim (72). It is currently unknown whether the basic rim residues of cyanobacterial and archaeal Hfqs participate in RNA binding.

Chaperone cycle of Hfq

Its similarity to Lsm proteins (79), role in phage Qβ replication (80), and the direct demonstration that Hfq forms stable ternary complexes with sRNA and mRNA suggested that an important function of Hfq is to facilitate sRNA-mRNA interactions (8, 10). This was followed by the demonstration that *E. coli* Hfq speeds sRNA-mRNA base pairing *in vitro* (21, 81), through RNA interactions with the basic patch on the rim (39). As a chaperone for sRNA-mRNA interactions, Hfq must bind a complementary pair of RNAs, accelerate base pairing, and release the annealed duplex to begin the cycle anew (Figure 2). A panoply of recent *in vivo* and *in vitro* studies has started to reveal how Hfq overcomes multiple barriers that limit the efficiency and specificity of sRNA-mRNA annealing *in vivo*. As explained below, these barriers include electrostatic and entropic costs for nucleating RNA double helices (82), self-structure that may mask the complementary region in either RNA, and the marginal stability of some sRNA-mRNA pairs. Recent work is also shedding light on how Hfq can form stable complexes with its RNA substrates, while permitting rapid and dynamic exchange of non-cognate RNAs.

sRNA-mRNA complex stabilization

The 5′ end of an sRNA target binding site is often called the "seed region" because it is most important for target recognition (86). The seed regions of sRNAs in *E. coli* and other enteric γ-proteobacteria are typically less than 10 nucleotides in length and imperfectly complementary to the target mRNA (87–89), leading to poor stability of cognate duplexes and small differences between cognate and non-cognate interactions. *E. coli* Hfq circumvents the former by creating long-lived ternary complexes that reinforce otherwise thermodynamically unstable sRNA-mRNA duplexes, enabling the sRNA to complete its regulatory activity (24, 90, 91). The thermodynamic stability of the RyhB–*sodB* mRNA duplex was shown to correlate exponentially with the strength of sRNA regulation (92). Hfq may also amplify the difference between cognate and non-cognate pairs, through their competition for the same RNA binding surface of Hfq. In other words, annealing between two RNAs in the presence of Hfq no longer relies simply on their sequence complementarity, but also on the complementary disposition of their Hfq binding sites.

RNA restructuring

Another way that Hfq can facilitate sRNA-mRNA binding is by refolding one or both RNAs. The complementary regions of many sRNAs and their mRNA targets are partially sequestered in secondary or tertiary structure, inhibiting their binding to each other. Structure probing revealed that *E. coli* Hfq generally restructures both bound sRNAs and mRNAs (10, 93–99), such that the complementary seed and target regions are made more accessible or amenable to annealing. Restructuring can occur through transient non-specific interactions with Hfq, as postulated for other RNA binding proteins with chaperone activity (100, 101), or through specific distortion of the RNA by Hfq (97). Restructuring might explain why some long RNAs bind Hfq more slowly (~10^6 $M^{-1}s^{-1}$) (21, 91) than short, unstructured RNAs that bind near the diffusion-controlled limit (~10^8 $M^{-1}s^{-1}$) (83).

Helix nucleation

Although Hfq can promote sRNA-mRNA association by co-localizing and refolding the RNA, this RNA refolding activity of Hfq is not in itself sufficient to achieve maximum annealing rates (97). Biophysical experiments on DsrA sRNA and RNA oligomers revealed that Hfq accelerates base pairing between complementary RNAs, after the ternary complex has formed. For oligonucleotides, the rate of helix formation was 10–10,000 times faster with *E. coli* Hfq (83). Trapping intermediates with a photo-caged RNA indicated that Hfq stabilizes a helix initiation complex that base pairs rapidly once the protecting group is removed (85). Once base pairing is complete, Hfq cycles off the newly formed dsRNA (21), reducing the rate of the reverse reaction (unzippering) and freeing Hfq to interact with another substrate. These observations led to a working model for RNA annealing by *E. coli* Hfq, in which RNAs are initially recruited to Hfq through rapid binding to the rim and the proximal or distal face. Formation of the ternary complex is followed by slow helix nucleation, rapid zippering, and dsRNA release (Figure 2) (39, 83).

Replacing the rim arginines with alanine eliminates Hfq's annealing activity in these assays, although RNA binding to the proximal or distal faces is unaffected (39, 85). Exactly how these arginines facilitate RNA base pairing remains unclear. Basic residues on the rim and in the flexible NTDs interact with the RNA phosphate backbone (45, 102), which may reduce electrostatic repulsion between the two strands, in a manner similar to the RNA matchmaker protein gBP21 (103). Lysine residues are less active than arginines (39), raising the possibility that the arginine guanidinium group hydrogen bonds with the RNA base pairs. Interestingly, conserved acidic residues flanking the basic patch may be important for correctly orienting the seed and target RNAs at the active site (104). Finally, the geometry of the Hfq hexamer may alleviate the entropic penalty for helix initiation by restricting the conformations of natural RNA substrates (48). Although the rim arginines are essential for RNA annealing in vitro, it is not clear whether this activity of Hfq is always needed for sRNA regulation in the cell (31, 50).

Product release

When the Hfq concentration greatly exceeds the concentration of dsRNA, *E. coli* Hfq melts short dsRNA oligomers (102, 105) and sRNA-mRNA duplexes (96), particularly when the duplex is unstable or A/U-rich. This destabilization of dsRNA suggests that rather than

accelerate annealing by binding and stabilizing duplexes (106), the rim of Hfq may instead bind partially annealed or "frayed" reaction intermediates. Several mechanisms may work in concert to prevent Hfq from improperly destabilizing sRNA-mRNA complexes and disrupting sRNA regulation. For example, the total concentration of substrate RNA is normally high relative to Hfq in the cell (84), preventing Hfq from rebinding ternary complexes (91). As discussed below, the CTDs of Hfq also facilitate the release of dsRNA by competing for electrostatic interactions with Hfq rim residues (32, 40). Finally, coupled turnover of many sRNA-mRNA pairs (9, 31) prevents reversal of the regulatory signal and ensures a rapid recovery of mRNA activity once sRNA transcription is halted.

Intrinsically disordered domains of Hfq

Like many RNA binding proteins (107), Hfq contains intrinsically disordered regions in addition to its stably folded Sm core domain (Figure 1A). In most bacterial Hfqs, disordered NTDs and CTDs protrude from the proximal face and rim of Hfq hexamers respectively (53, 54). Because each hexamer has 6 NTDs and CTDs, these disordered regions can stabilize or occlude RNA binding to the Hfq core (Figure 3A). Similar to disordered regions of other proteins (108–110), the NTD and CTD have diverged at a faster rate than the Sm core, via non-conservative substitutions and indels. This variability has made it more difficult to elucidate the function of the NTD and CTD. A recent model for the *E. coli* Hfq CTD (40) suggests how variations in these disordered regions fine-tune the function of Hfq in different bacterial genera.

The NTDs are typically short (mode of 4 residues), but range from 0–49 residues in length (Figure 1A). These flexible segments protrude from the proximal face of Hfq, which could allow even short NTDs to interact with RNA bound to this surface. Indeed, in the first co-crystal structure of an sRNA with Hfq, residues in the NTD of *E. coli* Hfq provide further stabilizing contacts with RNA bound at the rim of a neighboring monomer (45). Furthermore, the basic, 15 residue NTD of *Methanococcus jannaschii* Hfq has been shown to bind RNAs (55). Nevertheless, additional studies are needed to determine the NTD's effects on sRNA and mRNA binding and annealing.

The typically longer CTDs range from 0–185 residues (mode of 9), and protrude from the rim of the Hfq hexamer (53) (Figure 3A). In γ-proteobacteria, the beginning of the CTD tends to pack along the core of Hfq, increasing the stability of the hexamer (76, 111). Nevertheless, most of the *E. coli* Hfq CTD is disordered in solution NMR (112) and small angle X-ray scattering (54, 112, 113) experiments, and are unresolved in crystal structures.

CTD autoinhibition

The subtle and sometimes conflicting effects of the CTD on RNA binding (111, 115–117), annealing (32, 45, 112, 116) and sRNA-mediated post-transcriptional regulation (32, 45, 115, 118–120) suggested to us that the CTD modulates the function of the Hfq Sm core, either as an autoregulator or by recruiting partner proteins in the cell (32). Biophysical assays for RNA binding and annealing, as well as genetic reporters in *E. coli*, showed that the 30 aa CTD of *E. coli* Hfq is not needed for RNA annealing (32). In fact, Hfq lacking the entire CTD anneals RNA more actively than full-length Hfq. In the full-length protein, the

CTD restricts Hfq's chaperone activity by inhibiting non-specific ssRNA binding to the rim of the hexamer. In addition, the results of stopped-flow FRET and fluorescence anisotropy experiments showed that the CTD displaces dsRNA from the rim after annealing is complete. dsRNA was released from full-length Hfq in a few seconds, whereas dsRNA remained bound to Hfq lacking the CTD for many minutes.

Thus, the intrinsically disordered CTDs convert the stable Sm RNA binding domain of Hfq into a dynamic chaperone for RNA. In an updated model for Hfq's chaperone activity (32), the flexible CTDs continually sweep RNA from the rim and the proximal surface of the Sm ring (Figure 3B). Consequently, only RNAs that stably bind the proximal pore or distal face form long-lived ternary complexes. Once two complementary RNAs base pair, the CTDs displace the newly formed duplex from the rim of the hexamer, preventing strand separation and making the rim sites available for the next RNA.

Nucleic acid mimicry

Autoinhibition and dsRNA release are accomplished by transient interactions between the acidic ends of the CTDs and the basic patches on the rim of Hfq (32, 40). This self-interaction was consistent with NMR chemical shift anisotropy measurements on *E. coli* Hfq, which suggested that CTD residues transiently interact with residues on the rim of the hexamer (112). *De novo* modeling with ROSETTA and intermolecular binding of CTD peptides with the Hfq core showed that the acidic CTD tip competes with sRNA and DNA for binding to the basic patch. This competition prevents Hfq from binding RNA or DNA indiscriminately, which is likely important in the cell where many nucleic acids compete for a limited quantity of Hfq protein. Mimicry of nucleic acid by an acidic polypeptide is widespread in nucleic acid binding proteins, such as HTLV-1 NC (121), *E. coli* gyrase (122), SSB (123), and mammalian high-mobility group B1 (124).

CTD-RNA interactions

Although the *E. coli* Hfq CTD peptide does not measurably bind RNA on its own, the CTDs may stabilize certain RNA-Hfq complexes (45, 53, 67), however the mechanism of stabilization is unknown. For example, several studies reported a modest decrease in binding affinity of sRNAs to Hfq in the absence of the CTD (115, 117), while the binding of short ssRNAs was unaffected and short dsRNAs was improved (32). We envision several explanations for apparent stabilization of RNA binding by the CTD. First, substrate RNAs (45, 51) and the Hfq CTDs (40) both occupy large volumes around the Hfq core. This may limit the number of RNA segments that can simultaneously interact with the rim of the Hfq hexamer, while destabilizing low affinity, non-specific complexes. As a result, the remaining complexes are more uniform. Second, weak hydrogen bonding or aromatic stacking interactions between CTD residues and the RNA may only occur when the RNAs are tethered to the proximal or distal faces of Hfq. These interactions are likely to be transient, as regular folding of the CTD was not discerned in a co-crystal of RydC sRNA with Hfq (45). Although the CTDs were reported to increase the on-rate of RNA by surface plasmon resonance (45), no difference in on-rate was seen by stopped-flow FRET (32). Finally, it is uncertain that neutral or negatively charged CTDs of many Hfqs could attract RNA (40) by "fly-casting" or by other mechanisms (126).

Active cycling and sRNA competition

Competition among sRNAs for access to Hfq determines the efficiency of sRNA regulation in the cell (28, 29). The number of Hfq hexamers in an *E. coli* cell ranges from 1,400–10,000, depending on the growth medium and the stage of the bacterial growth cycle. These estimates equate to an intracellular concentration of 2–15 μM Hfq hexamer, which is 50–10,000 times greater than the dissociation constant of Hfq for most sRNA and mRNA substrates (127–129). RNA substrates are in turn estimated to be in large excess over Hfq (84). Therefore, all Hfq hexamers in the cell are thought to be bound to sRNA or mRNA, and surrounded by a large pool of additional, free RNA substrates (28, 29). Free sRNA is rapidly turned over by cellular ribonucleases, as evident from the decreased lifetimes of sRNAs in the absence of Hfq (28, 29, 50, 130, 131). Thus, competition for Hfq also affects sRNA accumulation (28). However, the fraction of sRNA and mRNA-bound to Hfq *in vivo* has not been quantitatively determined, which would assist the examination of how regulatory networks rely on Hfq under different conditions.

Remarkably, newly synthesized sRNAs can rapidly gain access to saturated Hfq, such that sRNA-mediated degradation of an mRNA target is typically complete within 3–5 minutes of sRNA induction from its chromosomal locus (9), or a plasmid (132). Slower regulation has been reported for some sRNA-mRNA pairs, such as SgrS-dependent degradation of *manX* mRNA (133). Certain sRNA-mRNA pairs, such as SgrS-*manX* or RyhB-*sodB*, may anneal independently of Hfq (92, 134), but nevertheless rely on Hfq for sRNA stability (92) or downstream regulation (17).

sRNA cycling

Fast sRNA-mediated regulation demands that Hfq search out complementary sRNA-mRNA pairs among a large quantity of competitor RNA. This responsiveness seems at first inconsistent with the tight affinity of Hfq for its substrates, which can form long-lived ($t_{1/2}$ ~ 100 min) Hfq-RNA complexes *in vitro* (91, 135). This paradox is solved by the ability of sRNAs to actively cycle on and off Hfq (28, 84, 91, 135, 136). Active cycling, in which a free sRNA displaces one bound to Hfq, may be enabled by the multiple RNA binding surfaces on Hfq hexamers which can be shared by two or more RNAs (48, 84). By sweeping RNA from the proximal face and rim, the CTDs amplify sRNA competition and may drive active sRNA cycling (32).

Hfq CTD heightens sRNA competition

Variance in the kinetic competition between different sRNAs enables a more nuanced cross-talk between signaling pathways that regulate the same target, because sRNAs that do not bind Hfq are rapidly turned over. A strongly competing RNA can either gain access to Hfq when present in very low amounts relative to other RNA substrates, or can dominate Hfq-dependent pathways when present at equimolar amounts. Biochemical (33) and *in vivo* competition experiments (28, 29) showed that sRNAs differ widely in their ability to compete for Hfq, although they have similar binding affinities. The broad hierarchy of competition indicates the importance of kinetic effects, such as active cycling, on sRNA regulation.

At least one method for instituting a hierarchy of regulatory pathways through sRNA competition seems to have arisen via the co-evolution of sRNAs and the Hfq CTD. In *E. coli,* a minor group of sRNAs (Class II, see Box 2) often outcompete the more abundant Class I sRNAs. These Class II sRNAs contain AAN motifs as well as U-rich sequences, enabling them to bind both the distal and proximal faces of Hfq (31, 33), resulting in increased accumulation of Class II sRNAs (31). Although the Hfq CTDs sweep RNA from the rim and proximal side of Hfq, AAN RNA bound to the distal face of Hfq is immune to this displacement (Figure 3B). As a result, the CTDs selectively destabilize Class I sRNA-Hfq interactions, while handing Class II sRNAs a competitive advantage (32, 40). Intriguingly, the CTD of *E. coli* Hfq is thought to have expanded concomitantly with the acquisition of new sRNAs (30).

Search for complementary targets

Finally, it is not enough that a given sRNA or mRNA successfully competes for Hfq; the same hexamer must also bind a cognate RNA for regulation to occur. How Hfq searches out cognate sRNA-mRNA pairs, which are greatly outnumbered by non-cognate pairs, is poorly understood at present. One proposition is that positive or a negative cooperativity in ternary complexes favors co-binding of cognate pairs, resulting in an increased efficiency of duplex formation between cognate pairs (137). However, there is currently little experimental support for cooperativity or anti-cooperativity in RNA binding. The fuzziness of trans-acting sRNA-target pairing may allow for the sampling and crystallization of new sRNA and mRNA binding sites (138), which can arise over relatively short timescales (30, 138). Finally, as outlined below, the structures of individual sRNA and mRNA substrates may increase the specificity of their base pairing interactions.

It has been proposed that high efficiency and robustness of duplex formation by Hfq could be achieved by a high rate of RNA dissociation, combined with a high rate of duplex release (83, 102, 139, 140). Increased RNA dissociation from Hfq is likely achieved via kinetic competition from additional substrate RNAs (84), enabled by the CTD (32). The importance of increased RNA dissociation and duplex release is thought to manifest in networks containing multiple sRNAs and mRNAs (137), such as that found in *E. coli* (18). Nevertheless, many sRNA·Hfq·mRNA ternary complexes are known to be stable *in vitro* (23, 91, 141). Perhaps the complementarity between these sRNA-mRNA pairs is unusually strong, or there may be annealing-dependent conformational changes that occur in the RNAs, which prevent the duplex from interacting with the rim again. The stability of certain ternary complexes is particularly important in some cases of negative regulation, in which RNase E is recruited for mRNA degradation (12, 142).

Three-dimensional organization of ternary complexes

sRNA-mRNA annealing involves not only the recruitment of cognate sRNA-mRNA pairs to the same ternary complex, but also the refolding of RNA substrates and the proper orientation of seed and target regions at nearby sites on the lateral rim of Hfq. The locations of $(AAN)_x$ and U-rich Hfq binding motifs, plus the size and secondary structure of the RNA, dictates how a particular RNA folds around the Hfq hexamer. The architectures of Hfq-RNA

complexes, which arise from the multiple RNA binding surfaces of the Hfq hexamer (51), may provide an additional layer of specificity for sRNA-mRNA recognition. Specific orientation of the RNA on Hfq is also likely important for interactions with downstream effectors such as the degradosome (142, 143), or co-recognition by other RNA-binding proteins (66, 144–146).

Proximal face binding sites on sRNAs are typically 3′ to the seed sequence, whereas distal face binding sites on mRNAs are typically 5′ to the target sequence (66). This organization poises the seed and target regions to find each other in the correct 5′-3′ orientation at the arginine-rich rims of Hfq. It is not currently clear how this orientation is achieved for Class II sRNAs and their mRNA targets, especially as some mRNAs are regulated by both types of sRNA. Footprinting studies have shown that Hfq can restructure its mRNA targets, and these interactions may reorganize during sRNA annealing (24, 99, 147). Interactions between single-stranded A/U-rich regions and RNA stem-loops to the rim of Hfq are particularly dynamic (31, 32, 40, 48, 49, 65). In the *rpoS* mRNA leader, Hfq binding to an upstream $(AAN)_x$ motif and downstream U-rich loop folds the RNA into a more compact structure that strains the complementary target region, preparing it to base pair with incoming sRNA (51) (Figure 4). The multiplicity of interactions may be required for the coordination and folding of large substrates, such as 5′ mRNA leader sequences, around the Hfq core (51, 99).

Conclusion

Many recent advances have been made in understanding how the structured and disordered components of Hfq participate in RNA binding, competition and annealing. Structural and biochemical studies have started to shed light on how sRNAs and mRNAs contact the many RNA-binding surfaces of Hfq, and how multi-surface contacts lead to conformational strain or restructuring of large RNA substrates. Hfq's multiple RNA binding surfaces are necessary for the formation of ternary complexes, and are likely important for sRNA exchange. Fluorescence spectroscopy has revealed RNA substrate binding, annealing and product release to be a very dynamic process, in part mediated by competition from Hfq's disordered CTDs for RNA binding sites on the rim. Evolution of both the RNA substrates, as well as RNA binding and RNA competing segments of Hfq are relevant to the outcome of crosstalk between sRNA regulatory pathways in the cell.

Several gaps in the literature cry out for further experimentation. Firstly, high resolution structures of Hfq complexed with native sRNA or mRNA are key to understanding how RNA-protein contacts with the Hfq hexamer generate annealing-competent sRNA•Hfq•mRNA ternary complexes, given the symmetry of the Hfq hexamer. It is unknown how many rim sites are needed for Hfq's chaperone activity, and how non-productive intermediates, in which the seed and target regions are positioned at opposite sides of the hexamer, are avoided. Single-molecule studies may assist in examining these questions.

Secondly, most RNA binding and annealing data is derived from observations with *E. coli* Hfq and closely related sequences. Further work is needed to know how substrate

recognition, RNA competition, annealing speed and efficiency of product release differ among bacterial and archaeal Hfq proteins. Nevertheless, some trends can be anticipated from the concepts outlined in this review. For example, sequence and structural analyses of bacterial and archaeal Hfqs suggest that uridine recognition by the proximal pore is strongly conserved (55, 56, 59, 60), and likely to be maintained in most Hfqs. Whereas, the more variable distal face may have different binding partners in other species (44, 46, 55, 59, 67, 70–72). Similarly, the correspondence between annealing activity and the number of arginines in the basic patch (52) suggests that Hfq proteins lacking these arginines may have little chaperone activity, but instead participate in other aspects of RNA metabolism (5–9, 12–17, 92). Biochemical studies of *E. coli* Hfq CTD mutants and computational modelling of other Hfqs (40) suggest that short, acidic CTDs will strongly restrict RNA binding and annealing, whereas variants with flexible or less acidic CTDs will be more permissive chaperones. Competition for Hfq and turnover of free RNA enable a rapid response to new stressors in *E. coli*, but it is not known how these features are balanced in other organisms. Finally, there are now more examples in which Hfq acts together with another RNA binding protein (66, 146, 148); how these proteins modify RNA recognition and sRNA-mRNA annealing is not yet understood.

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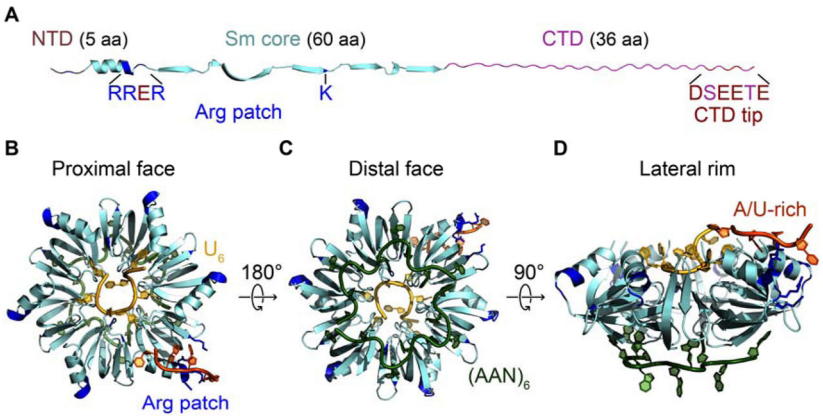

Figure 1. Structure and RNA binding surfaces of Hfq

(**A**) Secondary structure of *E. coli* Hfq, showing the ordered Sm core (cyan) with arginine patch (blue) necessary for annealing (39). Unstructured N-terminal (NTD; dark violet) and C-terminal (CTD; violet) domains are indicated schematically with the autoregulatory acidic CTD tip (40) in red. A previously described set of 985 non-redundant bacterial Hfq sequences (40) were analyzed by DISOPRED (41, 42) to estimate the range of NTD (0–49 aa) and CTD (0–185 aa) lengths. (**B–D**) Superposition of crystal structures of Hfq bound to RNA oligomers. (**B**) Proximal face U-rich RNA (yellow) bound to inner pore (3RER; (43)). (**C**) Distal face bound to A18 RNA (green) (3GIB; (44)). (**D**) View of lateral rim (4V2S; (45)) with A/U-rich RNA (orange) bound to the outer edge of the proximal side.

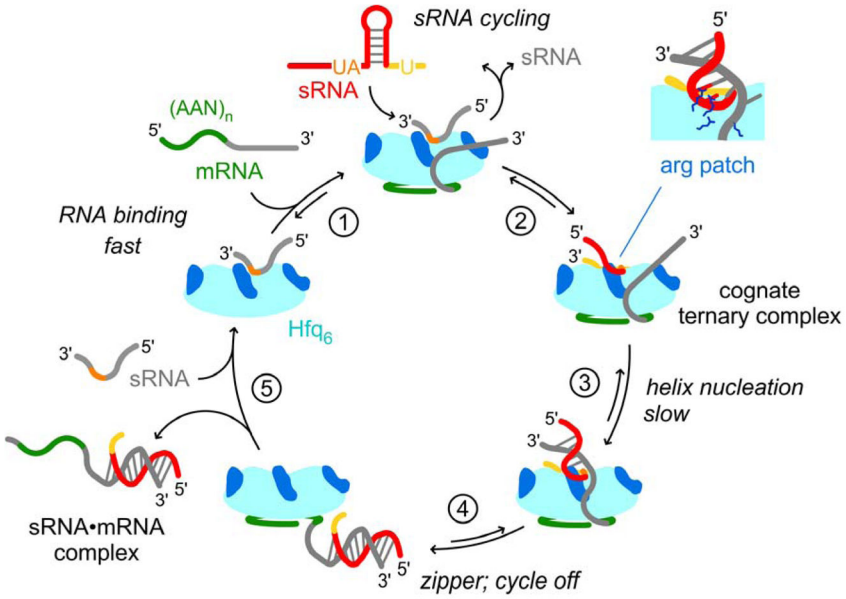

Figure 2. Cycle of RNA binding, annealing and release from Hfq

A working model for RNA annealing by *E. coli* Hfq. [**1**] sRNA (red) and mRNA (green and gray) rapidly bind the Hfq hexamer in random order to form ternary complexes. Short RNAs bind near the diffusion-controlled limit (83); longer RNAs that change structure bind more slowly. Non-cognate ternary complexes are unstable due to active cycling of excess Class I sRNAs on the proximal face of the hexamer (84), until [**2**] a cognate sRNA eventually binds to form a cognate ternary complex. [**3**] In the slow step of the reaction, nucleation of a helix between complementary regions of the sRNA and mRNA is facilitated by arginine-rich patches on the rim of the hexamer (blue). [**4**] Remaining base pairs rapidly zipper into a fully annealed sRNA·mRNA pair (85). CTDs efficiently displace dsRNA from the arginine-rich patches, preventing destabilization of the annealed segment (32, 40). [**5**] sRNA cycling, recruitment of a new mRNA or Class II sRNA, or RNase E turnover, may assist complete dissociation of annealed sRNA·mRNA complex from Hfq core. Hfq NTDs and CTDs are omitted from the pictograms for clarity.

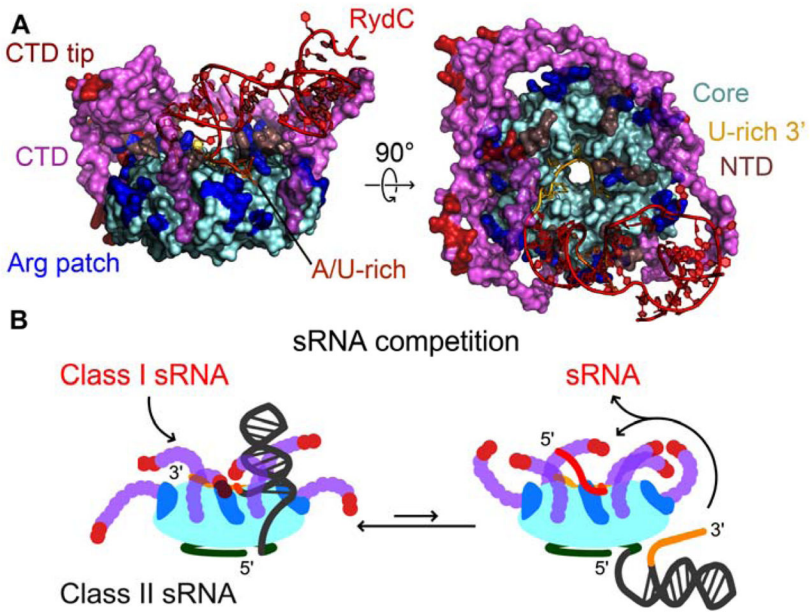

Figure 3. Binding of CTD to rim drives kinetic competition between sRNAs

(**A**) Model of Class I sRNA RydC (red cartoon) bound to full-length Hfq. Crystallographic structure of RydC-Hfq complex (4VQS; (45)) was superimposed on a ROSETTA model of full-length Hfq (40). Clashes introduced by the superposition were alleviated by gradient-based energy minimization and side-chain repacking with backbone coordinates kept constant (114). Shown is only one possible model for the flexible CTDs (violet shades), which can also sample the space on the distal side of the Hfq ring. Basic residues in the core and acidic residues in the CTD tip are colored blue and red, respectively. Some arginine patches are inaccessible due to electrostatic interactions with acidic CTD tips (40). The U-tail of RydC (gold) binds the inner proximal pore, while an A/U-rich motif upstream of the terminal stem-loop interacts with the arginine-rich patch (45, 48, 49). The body of RydC sRNA may weakly interact with the CTDs (45). (**B**) Class II sRNAs outcompete Class I sRNAs for access to Hfq, despite having similar binding affinities (31, 33). Class I sRNAs bind the proximal face and rim (31), and are readily displaced by acidic CTD tips (32, 40). Class II sRNAs contain an AAN motif (green) that binds the distal face (50) and resists displacement by CTDs, leading to a hierarchy of sRNA competition for binding to Hfq (32, 40).

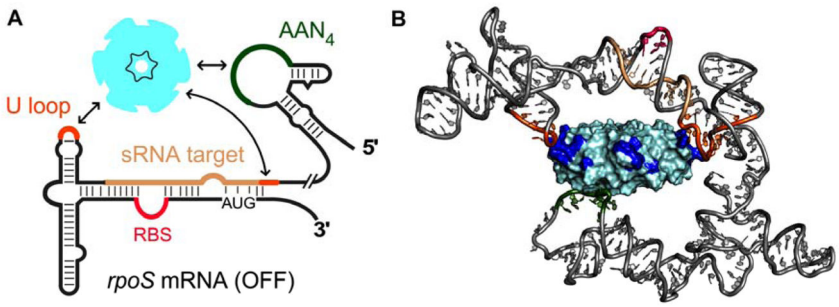

Figure 4. Hfq springloads *rpoS* mRNA for sRNA entry

(**A**) The long (572 nt) leader of *rpoS* mRNA contains Hfq binding motifs upstream (green) and downstream (gold) of the sRNA binding site (tan), which are necessary for sRNA regulation of *rpoS* translation (23, 51). (**B**) SHAPE footprinting and SAXS showed that *rpoS* mRNA contacts every RNA binding surface of Hfq, wrapping the RNA into a compact conformation that partially unwinds the sRNA target site and facilitates base-pairing with complementary sRNAs (51). Hfq NTDs and CTDs are omitted for clarity.