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Functional Analyses of Putative PalS (Palindromic Self-recognition) Motifs in Bacterial Hfq

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Abstract

The bacterial protein Hfq has been linked to nucleic acid metabolism and signaling, however its explicit role has been elusive. Recently it was proposed that the C-termini of Hfq subunits in Hfq₆ complexes could be involved in functional interactions with other Hfq hexamers and/or nucleic acids. To test the proposed model of the native Hfq complex experimentally, we genetically engineered chimeric Hfq₆ complexes, in which C-termini of bacterial Hfq subunits were substituted with a sequence derived from human histone H2B (*h*H2B) that includes multiple functionally significant amino acids whose modifications have been linked to carcinogenesis. We demonstrate that this substitution results in an enhanced formation of dodecameric assemblies by the Hfq–*h*H2B hybrid – a result pointing to the possibility of a (functional) homology between these motifs in proteins from distant kingdoms. We hypothesize that these putative Palindromic Self-recognition (PalS) motifs could act as proteins' 'cohesive ends' that could allow the protein complexes carrying such motifs to interact dynamically and dissociate–reassociate in response to stress and/or growth phase-specific changes. We provide experimental support to the latter hypothesis and demonstrate that in *E. coli* the dodecameric Hfq assemblies are formed in a growth stage-specific manner. We describe a refined system – consisting solely of purified Hfq, polynucleotide phosphorylase (PNP) and ADP – that allows reconstitution *in vitro* of characteristic 'SDS-insensitive' Hfq₆–Hfq₆ assemblies observed in experiments with whole-cell extracts obtained from exponentially-growing cells. We also optimized conditions for the extraction of intact native dodecameric Hfq complexes.

Keywords

Hfq; histone; nucleosome; carcinogenesis; polyadenylation; amyloid

INTRODUCTION

Eukaryotes compact their genomic DNA by wrapping it around a spool-shaped complex of histone proteins, which are postulated to have flexible or disorderly C-termini [1–2]. The resulting protein–nucleic acid assembly (termed nucleosome) undergoes significant

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conformational changes during transcription and other cellular events, some of which presumably require for DNA to be fully or partially unwrapped from the histone core. These conformational transitions – often referred to as nucleosome remodeling – are poorly understood, and it is not known to what extent (if at all) histone proteins participate in the management of nascent RNA. It is thought that bacteria compact their DNA in a somewhat dissimilar manner, and no close equivalents of eukaryotic nucleosomes have been described for prokaryotes. Bacterial DNA is compacted within a superstructure termed nucleoid, with the help of a set of DNA-binding or ‘histone-like’ proteins [3]. Bacterial protein Hfq has been linked functionally to metabolism of nucleic acids [4] and signaling [5]. *In vitro* studies with Hfq have proven to be challenging due to the purified recombinant and native Hfq proteins often displaying dissimilar properties [6], which could be due to the artificial self-association/stacking of recombinant *His*₆-tagged and C-terminally truncated Hfq (resulting in protein association/dissociation-dependent masking/unmasking of nucleic acid- and nucleotide-binding sites [6]) and a dissimilar makeup of protein modifications/accessory molecules in the native and recombinant Hfq proteins (with the former protein carrying hydrophobic modifications [6] which could contribute to anchoring of nucleic acid–Hfq complexes in cell membranes). It was established with certainty that the N-terminal domains of Hfq subunits form a disc-shaped hexameric assembly [7], with multiple nucleic acid- and nucleotide-binding sites on both sides of the disc and at its rim [8–12]. C-termini of Hfq subunits in hexameric complexes (obtained through a purification protocol that included 80°C heat treatments of unrefined cell extracts) were postulated to be disorderly [13], however our prior study indicated that in native Hfq complexes these presumably flexible C-termini could be involved in the formation of dodecameric (Hfq₆–Hfq₆) assemblies [6], which we hypothesized could be stabilized by shared C-terminal Hfq polypeptides capable of palindromic self-recognition (PaS) and/or nucleic acid ([6] and Fig. 1B). Also, a recent study has demonstrated that these motifs are capable of self-association resulting in the formation of amyloid fibers [14]. In this work, to test the proposed model of the native Hfq complex experimentally (without using Hfq truncation mutations and/or protein tags, which have been shown to result in the formation of artificial protein aggregates possessing nucleic acid-binding properties dissimilar to those of wild-type native Hfq [6]) we genetically engineered chimeric Hfq₆ complexes, in which C-termini of bacterial Hfq subunits were substituted with a sequence derived from human histone H2B (*h*H2B), whose presumably disorderly C-termini (which do not share sequence homology with C-termini of Hfq) could play, hypothetically, a functionally similar role (Fig. 1D). (Interestingly, Hfq and H2B do share some partial sequence homology [Fig. S1A], which – based on the available structural data – appears to be limited to the clusters of positively charged amino acids stabilizing a nucleic acid coil wrapped around a protein core [Fig. S1B, amino acids highlighted as blue spheres]). We demonstrate that the purified chimeric Hfq^{H2B}₆ complexes are capable of forming dodecameric assemblies and, in side-by-side comparisons with purified wild type Hfq, appear to be more ‘sticky’ and prone to self-association. Taken together, our results point to a novel mode of interaction between disc-shaped oligomeric protein complexes bearing extruded flexible strands; we hypothesize that this mode of interaction could be functionally significant for modulating the extent of compaction of nucleic acid coils wrapped around (dynamically-formed) protein cores. Also, we describe reconstitution of Hfq₆–Hfq₆ and Hfq^{H2B}₆–Hfq^{H2B}₆ complexes *in vitro* in a refined model system that

included purified Hfq, PNP, and ADP. Importantly, we demonstrate that in *E. coli* the dodecameric Hfq assemblies are formed during active growth. The mechanisms of growth phase-specific changes in bacterial nucleoids are poorly understood; our results suggest that Hfq – capable of binding both DNA and RNA – could contribute to these changes by bringing DNA strands together and/or tethering them to hydrophobic scaffolds in response to RNA synthesis.

MATERIALS AND METHODS

Synthetic DNA primers and DNA amplification

Synthetic DNA primers were obtained from Invitrogen. DNA amplification was carried out with OneTaq[®] DNA polymerase obtained from New England Biolabs (M0480) under conditions specified by the manufacturer.

Plasmid constructs

The restriction endonucleases and T4 DNA ligase were obtained from New England Biolabs. Gel extraction of DNA fragments, enzymatic digests, and ligations were carried out as described elsewhere. Wild-type *hfq* was amplified (from MG1655 *E. coli* chromosomal DNA) using the primers MS1301 (5′-AAAGGAGGATCCAAGGGGCAATCTTTACAAGATCCG) and MS80 [15]. DNA encoding the chimeric Hfq-*h*H2B protein (Hfq^{H2B}) was amplified using the primers MS1301 and MS1302 (5′-GAGCTTAAGCTTTTAAGAGGTGTACTTGGTAACAGCCTTGGTACCTTCAGAAACAGCATGCTTTGCCAGTTCGCCGCCACCGGCGTTGTTACTGTGATG). DNA encoding the C-terminal part of Hfq was amplified using the primers MS1303 (5′-TCCGGGATCCCATCACAGTAACAACGCCGGTGGCGGTACC) and MS1304 (5′-TATCAAGCTTCTATTATTCGGTTTCTTCGCTGTCCTGTTG). The amplified DNA fragments were gel-purified, digested with *Bam*HI and *Hind*III, and ligated into the *Bam*HI/*Hind*III sites of the expression vector pQE60 (Qiagen). (Thus, the utilization of the *Hind*III site and the introduction of stop codons into the DNA primers eliminate C-terminal hexahistidine tags in the resulting plasmid-borne Hfq proteins). The plasmid constructs were verified by DNA sequencing (Figs. S2 and S3). The single constructed pQE60-Hfq^{H2B} clone encoded the chimeric Hfq-*h*H2B protein with a single amino acid substitution (V to I) in its histone tail (Fig. S2).

Purification of the recombinant Hfq^{wt} and Hfq^{H2B} proteins

The Hfq proteins (overproduced in NEB[®] 5-alpha *E. coli* cells from pQE60-Hfq^{wt} and pQE60-Hfq^{H2B}) were isolated in a manner similar to that described in [6] (*Purification Method A*) with the modifications specified in the legend to Fig. 2C.

PAGE/Immunoblotting protocol is described in Fig. S5.

RESULTS AND DISCUSSION

It is generally accepted that bacterial DNA-binding proteins – in which pool Hfq is casually included [16] – can alter the architecture of the nucleoid. The long-known growth phase-specific manner of expression of some of the *E. coli* histone-like proteins [17] is consistent with this notion. The conformational transitions of the nucleoid can also be affected by the synthesis of RNA [18]. Recent studies with *E. coli* Hfq suggested that its C-termini could be involved in interactions with nucleic acids [19] and contribute to the formation of dodecameric (Hfq₆–Hfq₆) assemblies ([6], some of the data described in our prior work also indicated that such assemblies could be stabilized by an accessory nucleic acid). When chemically synthesized peptides with sequences matching those found in the C-termini of Hfq₆ complexes were analyzed, their self-association was observed only at relatively high protein concentrations [14]. However, in the native Hfq₆ complexes, C-terminal domains of individual subunits are attached to the disc-like assembly formed by the subunits' N-terminal domains, so the self-association reaction between two distinct Hfq₆ complexes – with each complex bearing six potential low-affinity interactor-motifs (Figs. 1B–C) – would be quite dissimilar to the reaction between individual, untethered peptides bearing the same interactor-sequence (Fig. S4 highlights some of the differences between the two reactions and potential challenges in monitoring such interactions using untethered peptides). Therefore, we sought alternative methodologies that could allow surveying the function(s) of the Hfq C-termini integrated into an oligomeric protein complex. First, to test if modifications in Hfq₆ C-termini could have a measurable effect on the formation of dodecameric assemblies, we constructed the *E. coli* Hfq–hH2B chimera (referred to below as Hfq^{H2B}). *In vivo*, overproduction of (non-tagged) wild-type Hfq (Hfq^{wt}) resulted in arrested cell growth (Fig. 2A, the pQE60-Hfq^{wt} plasmid construct). Normal cell growth could be restored by substituting the C-termini of plasmid-borne Hfq with the C-terminal sequence derived from human histone H2B (Fig. 2A, the pQE60-Hfq^{H2B} plasmid construct) and also was observed with the construct overproducing the individually-expressed C-terminal domain of wild-type Hfq (Fig. 2A, the pQE60-Hfq^{CTD} control). This set of experiments suggests that the functional interactions of the Hfq C-termini integrated into an oligomeric complex are unlike those of the same protein sequence expressed separately.

Next, we obtained partially purified Hfq^{H2B} protein and monitored the dodecamer/hexamer ratios at varying protein dilutions in Hfq^{H2B} and Hfq^{wt} protein samples fractionated by PAGE. (To minimize nonspecific Hfq–[rA]_n binding/aggregation [6] these experiments were performed in the presence of an ionic detergent). As seen in Fig. 2C, when the two proteins were compared side-by-side at comparable concentrations, the chimeric Hfq^{H2B} protein formed characteristic (SDS-insensitive) 120-kDa assemblies (Fig. 2C, bottom panel, lanes 8 and 9, 11 and 12). However, with wild-type Hfq, such assemblies were not observed in the examined range of protein concentrations (Fig. 2C, respectively, lanes 2 and 3, 5 and 6). These results are consistent with the Hfq–hH2B chimera being more prone to forming the Hfq₆^{H2B}–Hfq₆^{H2B} assemblies. We carried out an additional set of experiments, in which we subjected the samples of mutant and wild-type Hfq to treatment with various nucleases (such as RNase I, RNase A, and DNase I). These experiments have demonstrated a dramatic resistance of dodecameric Hfq assemblies to nuclease digestion (data not shown), suggesting

a near-complete compartmentation of the enclosed nucleic acid (if any present) and/or the primary role of protein-protein interactions in the formation of such assemblies.

To better understand the nature of ‘SDS-insensitive’ 140-kDa Hfq assemblies observed in cell extracts derived from actively growing *E. coli* cells ([6], Figs. 3C and S2B therein; also, Fig. 4 below), we sought to reconstitute these assemblies *in vitro*. Our initial trials with purified Hfq^{wt} and Hfq^{H2B} indicated that mixing purified proteins with RNA had little or no effect on hexamer/dodecamer equilibria (Fig. 2C, compare lanes 1–3 with lanes 4–6, lanes 7–9 with lanes 10–12). However, because in our prior work we described the native complexes (that included PNP and Hfq) whose fractionation by SDS-PAGE yielded 140-kDa Hfq species ([6], Fig. 6 therein), we specifically wanted to explore the Hfq–PNP model system in order to test if PNP could be responsible for the formation of these complexes. Fig. 3 shows a representative set of experiments in which the kinetics of formation of Hfq₆–Hfq₆ and (Hfq₆–Hfq₆)₂ complexes – triggered by the synthesis of (rA)_n by PNP – was monitored at varying ratios of Hfq^{wt} and Hfq^{H2B}. (In Fig. 3C the ratios of the two proteins were adjusted [with Hfq^{wt} in excess to Hfq^{H2B}] to obtain comparable levels of dodecamers in the samples). Importantly, in these experiments we were able to reconstitute *in vitro* – using solely purified Hfq, PNP, and ADP – the dodecameric complexes arguably similar to the assemblies observed in whole-cell *E. coli* extracts ([6]; also, Fig. 4 below).

Next, we sought (i) to determine the optimal conditions for solubilization of the native Hfq₆–Hfq₆ assemblies and (ii) to test if growth phase-specific changes in *E. coli* cells could affect the formation of such assemblies. To this aim, we carried out the next set of experiments, in which *E. coli* cell extracts – derived from cells harvested in exponential and stationary phases – were subjected, side-by-side, to five alternative cell lysis protocols (which tested the effect of four common non-ionic and ionic detergents on the solubilization of native Hfq) and the amounts of ~140-kDa dodecameric Hfq complexes in PAGE-fractionated cell extracts were then determined (Fig. 4). As seen in Fig. 4, strong and medium-strength ionic detergents were particularly effective in solubilization of Hfq, in accord with [6]. (In Fig. 4B, note that the solubilization profile of the control protein [‘soluble’ protein GroEL] indicated that the inability of Extraction Methods 1–3 to solubilize Hfq was not due to an incomplete cell lysis). Importantly, the apparent levels of ~140-kDa Hfq complexes in extracts derived from exponentially growing *E. coli* cells greatly exceeded the levels of similar complexes present in extracts of cells harvested in the stationary phase (Fig. 4C, graph; there was only a moderate change in overall Hfq expression levels in the two sets of samples, in accord with [17]). Further analyses indicated that these higher-order Hfq assemblies were labile and sensitive to repeated cycles of freezing and thawing in the presence of SDS (Figs. 4B, 4C, and 4D).

Taken together, our data are consistent with the proposed involvement of the Hfq₆ complexes in the formation of dynamically-formed superstructures in actively growing *E. coli* cells. While it is clear that additional studies with the dodecameric Hfq complexes reconstituted *in vitro* are needed in order to determine their ‘functionality’, the available data – obtained with minimally processed cell extracts – indicate that such assemblies exist in actively growing *E. coli* cells (Fig. 4). It seems plausible that the native Hfq assemblies formed in actively growing *E. coli* cells could be more functionally complex than previously

thought and include both RNA (which could interact with the proximal and distal surfaces of the Hfq₆ discs and traverse the pore[s]) and DNA (which could be wrapped around the spool-shaped [Hfq₆]₂ complex in a manner vaguely reminiscent to that of eukaryotic nucleosomes). Our utilization of fast methods of cell lysis/sample fractionation and varied methods of protein extraction – the elements that differentiate our work from previously conducted studies – likely explain why these labile assemblies were not identified in prior studies with Hfq. Experiments with the *E. coli* Hfq–hH2B chimera demonstrate that modifications in the Hfq C-termini can alter the hexamer– dodecamer equilibria (Figs. 2C and 3). These results are consistent with the proposed involvement of Hfq’s C-termini in the formation of (Hfq₆)_n complexes. Furthermore, this set of experiments – demonstrating an apparent stabilization of the Hfq dodecamers after the introduction of the putative interactor-motif derived from human histone H2B into the C-terminus of Hfq – is broadly significant and hints that histones’ C-termini could play a previously unrecognized role as mediators of low-affinity interactions between nucleosomes. The innovative aspect of our research methodology was to engineer chimeric protein complexes in which putative low-affinity interactor motifs are tethered in a position-specific manner to a multi-subunit, ring-shaped protein assembly. This approach arguably made possible observing interactions that otherwise could be more technically challenging to detect in studies with individual, untethered proteins/peptides (Fig. S4); the overall design was also dictated by a need to avoid the Hfq truncation mutations that could lead to the formation of artificial protein aggregates possessing nucleic acid-binding properties dissimilar to those of wild-type Hfq [6]. Taken together, our results point to the possibility of a novel mode of interaction between oligomeric protein complexes through short extruded protein strands capable of palindromic self-recognition (PalS). While such mode of interaction has not been previously explicitly recognized, the chemistry of interactions between individual amino acids in such assemblies is not new, as some known amyloid fibers contain interactor-motifs reminiscent of those found in the Hfq C-termini (specifically, E–X[positively charged amino acid] links that integrate short antiparallel protein strands into a sheet [21]).

What is the plausible functional significance of the formation of PalS motifs-stabilized protein complexes? We hypothesize that such interactions could be functionally significant for modulating the extent of compaction of nucleic acid coils wrapped around dynamically-formed protein cores (Fig. S6; thus, PalS motifs could act as ‘stabilizers’ for macromolecular ‘springs’ – coiled nucleic acids). Also, the resulting ring-like protein assemblies (Fig. 1C) could create enclosures for transmembrane and/or cytosolic transport of macromolecules – including the transport/channeling of RNA.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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- The bacterial protein Hfq has been linked to nucleic acid metabolism and signaling
- We show that Hfq₆-Hfq₆ complexes are formed in actively growing *E. coli* cells
- Rapid extraction protocols are critical to isolation of intact native Hfq complexes
- We show alterations of hexamer-dodecamer equilibria with the Hfq-*h*H2B chimera
- Our data imply that Hfq C-termini could play a role in intermolecular interactions

Figure 1. Structure of the complex of Hfq subunits' N-terminal domains and the hypothetical structure of the native Hfq₆-Hfq₆ assembly

A. Structure of the disc-shaped complex formed by the Hfq subunits' N-terminal domains [7]. **B** and **C.** Hypothetical structure of the native Hfq complex proposed in our prior work [6]. **D.** Human nucleosomes [22] with the highlighted C-terminal sequences of histone H2B (amino acids highlighted as yellow and olive spheres) – which, we hypothesize, could act as Palindromic Self-recognition (PalS) motifs – are shown. Note that the putative self-recognition sequence of *h*H2B (panel at the top) is not homologous to that of Hfq (Fig. 1C, panel at the top). To test the hypothesis that these motifs could play functionally homologous roles – as adaptors for low-affinity interactions between disc-shaped protein complexes – we replaced the putative PalS motif in *E. coli* Hfq with that of human histone H2B.

Figure 2. Comparative analyses of wild-type and mutant Hfq

A. Functional effects of overproduction of Hfq^{WT}, Hfq^{H2B}, and Hfq^{CTD}. Reduced cell growth in the presence of excess Hfq^{WT} (but not Hfq-*h*H2B chimera or Hfq^{CTD}) suggests that the functional interactions of the Hfq C-termini integrated into an oligomeric complex are unlike those of the same protein sequence expressed separately. **B.** Expression of the chimeric Hfq protein carrying the C-terminal motif derived from the human histone H2B (Hfq^{H2B}) in NEB[®] 5-alpha *E. coli* cells. Schematic of the plasmid construct for the expression of non-tagged Hfq^{H2B} is shown at the left; the DNA sequence of the construct is shown in Fig. S2. Immunoblot at the right shows the expression of Hfq^{H2B} in whole-cell *E. coli* extracts. PAGE/Immunoblotting (PAGE/IB) utilizing Hfq-specific antibodies [6] was carried out as described in Fig. S5. Whole-cell *E. coli* extracts were made as described in Extraction Method 2 [6]. Note the dissimilar electrophoretic mobility of Hfq₆ and Hfq₆^{H2B} complexes; the altered electrophoretic mobility of the latter complexes was likely due to a lack of accessory C-terminal fragments from adjacent hexameric Hfq assemblies and/or accessory RNA [6]. Lanes 1 and 2, 3 and 4, 5 and 6 contained, respectively, 2, 4, and 8 μl of whole-cell *E. coli* extract. **C.** Formation of dodecameric assemblies by purified Hfq^{H2B}. Purified wild-type Hfq and Hfq^{H2B} were present at 0.07 mg/ml (lanes 1, 4, 7, and 10), 0.14 mg/ml (lanes 2, 5, 8, and 11), and 0.28 mg/ml (lanes 3, 6, 9, and 12). When indicated, purified synthetic RNA oligonucleotide rA₂₀ (Dharmacon) was at 0.15 μg/μl (lanes 4–6 and 10–12). With Hfq^{H2B}, note the formation of assemblies with a molecular mass consistent

with that of dodecameric ($[\text{Hfq}_6^{\text{H2B}}]_2$) complexes and the lack of such assemblies with purified wild-type Hfq. Note the lack of interaction between Hfq and synthetic RNA under these conditions (lanes 4–6 and 1–12) and the formation of ‘SDS-insensitive’ Hfq-(rA)_n assemblies with enzymatically-synthesized adenylate (Figs. 3B, 3C and data not shown); these differences are possibly due to the dissimilar chemical structure of the 5' termini in chemically- and enzymatically-synthesized adenylate. Purified Hfq^{H2B} was obtained using the purification procedure similar to that described in [6] (*Purification Method A*), except that the step of DNA–Sepharose 4B chromatography was substituted with a step of ion-exchange chromatography on a custom-packed Source Q (10×50 mm) column. Interpretative schematics at the right identify individual protein complexes.

Figure 3. Formation of the ‘SDS-insensitive’ Hfq₆-Hfq₆ assemblies in the Hfq-PNP system

A. Synthesis of polyadenylate from ADP by purified PNP in the presence of Hfq.

Schematics illustrate the *in vitro* reactions shown in Figs. 3B and 3C. In Schematic 1, the position of Hfq-associated polyadenylate (that is shown passing through the Hfq pore[s] and the shared assembly of C-terminal fragments) is conjectural, as it cannot be ruled out that $(rA)_n$ could mediate the formation of Hfq dodecamers by interacting with the distal sides of disc-shaped assemblies in individual Hfq₆ complexes (Schematic 2), as seen in the structure 4HT8 [20]. **B** and **C.** RNA-synthetic reactions similar to those described in Fig. 3A were carried out in the presence of wild-type Hfq (lanes 1, 3, and 5) or Hfq^{H2B} (lanes 2, 4, and 6), at varying Hfq^{wt}/Hfq^{H2B} ratios, and the resulting reaction products were analyzed by PAGE/IB (Fig. S5). Durations of the incubations are specified in the figures. Native *E. coli* PNP was purified as previously described [23]. Reaction buffer D (50 mM potassium acetate, 20 mM Tris-acetate, 10 mM magnesium acetate, 1 mM dithiothreitol, pH 7.8); PNP,

21 nM; ADP (when present), 167 μ M. Figs. 3B (lanes 2, 4, and 6) and 3C (lanes 3–6) demonstrate that the production of nascent polyadenylate by PNP correlates with the formation of ‘SDS-insensitive’ dodecameric Hfq complexes [6].

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Figure 4. *In vivo*, the dodecameric Hfq complexes are formed in a growth phase-specific manner

A. Schematic of a fast cell lysis protocol that was used in the experiments described below (Figs. 4B–4C). **B.** PAGE/Immunoblotting analyses of whole-cell extracts obtained from exponentially grown *E. coli* cells (lanes 1–5; a cell pellet resulting from 25 ml of *E. coli* MG1655 cell culture grown in LB medium and harvested at OD₆₀₀ 0.15 was used in this set of experiments) and stationary-phase *E. coli* cells (lanes 6–10) demonstrates a growth phase-specific manner of formation of the dodecameric Hfq assemblies. Approximately 2.5 µl of whole-cell extract was loaded per well of a gel. **C.** This panel – illustrating a relative instability (and sensitivity to freezing/thawing in the presence of an ionic detergent) of the dodecameric Hfq assemblies – demonstrates the result of PAGE/IB analysis of the same set of samples (described in Fig. 4B), except that the samples were subjected to PAGE/IB after they were frozen (at –72°C) and thawed after approximately 1 week of storage; 5 µl of whole-cell extract was loaded per well of a gel. Note that this treatment of samples resulted in partial proteolysis of the native Hfq₆-Hfq₆ assemblies and the appearance of Hfq₆-Hfq₆ NTD_i species [6] that were not seen in the samples that were analyzed

immediately following the fast cell lysis (Figs. 4A and 4B). **D.** Highlight of the experiments described in Figs. 4B and 4C.

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