

Investigating the Roles of Biomolecular Condensates in Bacterial Stress Response

by

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Abstract

Subcellular organization is essential for the survival, stress response and adaptation of all cellular organisms. Due to the lack of endomembrane, it is suggested that bacteria might rely on phase-separated, membraneless compartments known as biomolecular condensates for cytoplasmic and genomic organization. However, only a handful of such compartments have been characterized in bacteria, and their physiological significance remains largely obscure. In my thesis work, I expanded our understanding of bacterial subcellular organization by revealing the formation mechanisms and biological functions of two types of stress-responsive bacterial biomolecular condensates. I show that nitrogen-starved *Escherichia coli* produces long-chain polyphosphate (polyP) that scaffolds the RNA chaperone Hfq into high molecular weight complexes, which eventually phase separate with components of the RNA translation and processing machinery. Presence of polyP in condensates controls Hfq function by selectively stabilizing polyadenylated RNAs involved in transcription and protein translation and by promoting interactions with translation- and RNA-metabolism-associated proteins involved in *de novo* protein synthesis. Lack of polyP significantly impairs condensate formation, increases cell death, and hinders recovery from N-starvation. In functional analogy, my work shows that polyP contributes specifically to the formation of processing (P)-bodies in mammalian cell lines, revealing that a single, highly conserved and ancestral polyanion serves as a modulator for functional phase-separated condensate formation across the tree of life. In the second part of my thesis work, I demonstrate that H-NS and StpA, the gene silencing protein homologs in Gram-negative bacteria, fine-tune the material properties of nucleoid associated compartments. Whereas H-NS forms dynamic condensates *in vitro*, StpA assembles into stable insoluble fibrils. However, together the two proteins form liquid-like droplets, whose fluidity and stability are tunable by their relative stoichiometry. By increasing the levels of StpA over H-NS, bacteria stabilize heterochromatin-associated compartments, thus preserving gene repression and optimizing bacterial growth under stress conditions. Structural differences at these proteins' dimerization sites help explain their distinct phase behaviors. These findings reveal a novel mechanism in which intragenomic

homologs with highly distinct phase behaviors can fine-tune subcellular organization to promote survival in fluctuating environments. In summary, my work reveals the important implication of biomolecular condensates in bacterial subcellular organization and stress response. Moreover, my research lays the foundation for future studies on similar biomolecular condensates beyond *E. coli*.

Chapter 1 The Role of Biomolecular Condensation in Cellular and Genomic Organization

1.1 Cytoplasmic and genomic organization through biomolecular condensation

Spatial organization of cellular content is essential for the survival of all organisms. While lipid-based endomembranes have long been regarded as the canonical model for subcellular organization, membraneless organelles formed by proteins and/or nucleic acids have recently emerged as a new organizing principle (1, 2). Despite the absence of delimiting membranes, these supramolecular assemblies compartmentalize and concentrate specific biomolecules and are therefore collectively referred to as biomolecular condensates (condensates hereafter) (2). So far, well-characterized *in vivo* condensates include processing (P-) bodies and stress granules in the eukaryotic cytoplasm, nucleoli in the eukaryotic nucleus, as well as several ribonucleoprotein bodies in bacteria (1, 3).

Protein condensates behave like liquids, hydrogels or solids, and often transition between these material states (4). However, most condensates studied so far display liquid-like properties, as evidenced by their droplet-like morphology, ability to fuse and divide, and diffusion of constituent molecules both internally and across the condensate boundary (5, 6). Regardless of material properties, it is generally accepted that condensate formation requires multivalent interactions (e.g., electrostatic, hydrophobic, *pi-pi* interactions), which are mediated by folded domains connected by disordered linkers or interacting motifs within intrinsically disordered regions (IDRs) (7, 8). This “sticker-spacer” framework suggests that noncovalent interactions between stickers govern intermolecular interaction strength, while spacers define macromolecular solubility (9, 10). In dilute, sub-saturated solutions, multivalent interactions spontaneously give rise to heterogeneous mesoscale clusters (11). When the concentration of multivalent polymers reaches a threshold known as saturation concentration (c_{sat}), these clusters undergo solvent release and density transition. This process, also known as phase separation, typically gives rise to two distinct yet coexisting phases: the dense phase and the dilute phase (4, 8). Concentration of constituent multivalent molecules in the dense phase is often orders of magnitude higher than that in the surrounding dilute phase. Intermolecular interactions in such crowded environment results in

strong increase in bulk viscosity (12). At the macroscopic level, the resulting dense phase morphologically resembles droplets of thick liquids.

Condensates function as unique regulators of cellular activities due to their distinct properties. The transient nature of multivalent interactions (1, 12) make condensates more responsive to changes in environmental conditions such as temperature and osmolarity compared with membrane-bound organelles (4). Furthermore, sequestration of enzymes and their substrates in condensates increases enzymatic reaction rates within these compartments (1). For example, co-enrichment of NADH-oxidase and its cofactor within condensates significantly increases its enzymatic activity (13). On the other hand, recruiting select molecules into condensates can inhibit their interactions with other molecules in the dilute phase (1). This is evidenced by virus-infected cells forming stress granules that sequester viral proteins and mRNAs, thus disrupting the viral replication cycle (14). Moreover, condensates can be involved in aggregate formation. High protein concentration in the dense phase and the specific chemical environment at the phase boundary can facilitate aggregate seeding and growth (15, 16). Taken together, it is not surprising that many mutations associated with cancer, neurodegeneration and other diseases affect condensate formation, while aberrant condensates are often observed in patient-derived cell lines (17, 18).

Despite decades of research, questions remain over the function of condensates. First, for any given biomolecule, dissecting its condensate-associated functions from other functions is challenging. Protein domains and motifs contributing to multivalency are often involved in interactions with DNA, RNA and/or other proteins. Disrupting these domains will inevitably affect intrinsic protein properties other than condensation and cause misleading results in functional studies. Second, observations of aberrant condensation in pathological conditions are often correlative and the exact contribution of these condensates to disease progression is obscure (17). For example, while stress granule assembly is increased in many cancer cells, it is unclear whether this is a cause or consequence of cancer development (17, 19, 20). Finally, bacteria are thought to rely more on condensates for subcellular organization due to the absence of endomembrane systems, yet few bacterial condensates have been identified so far (21). The mechanistic reasons underlying such differences remain poorly understood. Addressing these outstanding questions will provide valuable insight into the regulation and evolution of subcellular organization and will likely open new opportunities for drug development.

1.2 Mechanisms of bacterial genome organization

Despite the absence of nuclear envelope, electrostatic repulsion between negatively charged DNA and ribosomes compacts the bacterial chromosome into a distinct, phase separated compartment known as the nucleoid (22-24). Though traditionally viewed as structurally disordered, recent studies revealed the presence of hierarchical spatial organization in the nucleoid across a wide range of bacterial species (25-28). In *Escherichia coli*, genome DNA supercoils and forms braided structures known as plectonemes (29). At a larger scale, the nucleoid is spatially organized by the protein complex MukBEF, which forms a ring-like structure that traps or extrudes DNA, producing large DNA loops (27, 30). This enables long-range DNA interactions and segments the nucleoid into larger self-interacting domains known as chromosomal interaction domains (tens to hundreds of kb) and macrodomains (~1 Mb) (27, 29, 31-33). Loss of MukBEF in *E. coli* disrupts long-range DNA contacts and further leads to temperature sensitivity and formation of anucleate cells during rapid growth (27, 34).

1.2.1 Nucleoid associated proteins (NAPs)

Apart from MukBEF, bacterial nucleoid structure is also maintained by a set of small DNA binding proteins collectively referred to as nucleoid-associated proteins (NAPs), which are considered as functional analogs to eukaryotic histones (29). NAPs can bind DNA in both sequence-specific and non-specific manner, thus allowing them to serve as dual function regulators (29, 35). Sequence-specific binding allows NAPs to function as positive and negative transcriptional regulators. For example, when the *E. coli* NAP Fis binds to promoters, it suppresses gene expression by excluding the RNA polymerase (36). However, at sites upstream of the promoter, Fis binding changes DNA supercoiling, which disrupts DNA duplexes at the promoter, and allowing transcription complexes to form (37). For many NAPs, however, their protein copy number is orders of magnitude higher than that of their specific genome binding sites (29). This leads to their non-specific association with the genome, which often causes changes in DNA conformation and leads to nucleoid compaction and higher-ordered genome structures (29). For example, the *E. coli* NAP HU binds DNA in a non-specific manner yet has some weak preference towards distorted DNA such as four-way junctions (38, 39). At low concentrations, HU binding introduces bends in DNA and reduces the latter's rigidity, while at high concentrations it stiffens DNA and gives rise to a bundled DNA network (40, 41). Several NAPs can form condensates *in vitro* or *in vivo*. HU forms dynamic submicron domains *in vivo* and liquid condensates in the presence of four-way junction DNA *in*

vitro (42). Dps (DNA protection during starvation protein) is proposed to form nucleoid-spanning condensate in stationary phase *E. coli* that compacts the nucleoid but does not interfere with transcription (43). It forms condensates *in vitro* with DNA and can assemble into multiphasic droplets with HU (42).

Functional investigation of NAPs *in vivo* has been challenging, mainly due to the combined effects of sequence-specific and non-specific binding, concentration-dependent conformational changes, as well as overlapping binding landscape between different NAPs. Additionally, some NAPs such as Hfq (host factor for phage φ) also function as RNA binding proteins (44), which further complicates their *in vivo* roles. Finally, although NAPs are mainly implied in nucleoid architecture while transcription factors are involved in tuning the expression of specific genes, it should be noted that the line between the two groups is blurred. In fact, the two types of proteins lie along a functional spectrum, and most proteins display some degree of structural/regulatory duality (35, 45).

1.2.2 Bacterial functional heterochromatin

A particularly notable example of NAP's functional duality is the formation of extended protein occupancy domains (EPODs), sometimes referred to as bacterial functional heterochromatin (46). EPODs are multi-kb genomic regions featuring dense NAP binding and transcriptional silencing (46, 47). In *E. coli*, over 200 EPODs are distributed across the genome, with a fractional coverage of 10-15% depending on growth conditions (47, 48). While the precise protein composition of EPODs is unclear, a significant portion of these regions overlap with binding sites of the NAP H-NS (histone-like nucleoid structuring protein) (46, 48). H-NS functions as global gene silencer by preferentially binding AT-rich DNA and forming nucleoprotein filaments that interfere with RNA polymerase activity (49-52). In *E. coli*, nearly half of H-NS binding motifs fall within horizontal gene transfer regions which have a higher AT-content than the resident genome (50). These regions are often enriched in virulence genes and mobile genetic elements (MGEs) such as prophages (53). As expected, loss of H-NS profoundly affects bacterial virulence and can cause prophage activation, which in some cases is lethal (48, 53-55).

Although the majority of EPODs overlap with H-NS-bound loci, a significant portion of EPODs is formed through the interaction with other NAPs (46). Fis and Hfq suppress a highly overlapping set of prophages in *E. coli*, and a $\Delta fis \Delta hfq$ strain is not viable unless in a genetic background devoid of all MGEs (48). EPODs are also identified in other species such as the Gram-positive bacterium

Bacillus subtilis, where the H-NS-like protein Rok mediates gene silencing in densely protein-bound regions (48). It should be noted that although EPODs are referred to as bacterial heterochromatin, they are fundamentally different from eukaryotic heterochromatin in terms of structure and the lack of nucleosome-like assemblies. Nevertheless, the formation of heterochromatin-like regions with suppressed gene expression is clearly a conserved functional feature of both eukaryotic and prokaryotic genomes.

1.3 Bacterial ribonucleoprotein (RNP) condensates

Precise control over RNA synthesis, modification, translation and degradation is essential for survival of bacteria. Some ribonucleoprotein (RNP) complexes involved in these processes have been reported to form condensates *in vivo* and *in vitro* over the past decade. Their mechanisms of formation, biophysical properties and biological functions have been the subject of intensive investigation.

1.3.1 RNase E and BR-bodies

Bacteria must rapidly remodel their transcriptome to cope with fluctuating environments. A key to fulfilling this requirement is the modulation of mRNA stability. The half-life of mRNA in rapidly growing bacteria is in the range of minutes (56-58). This fast turnover contrasts with tRNA and rRNA, whose median half-life can be several folds higher than the doubling time of the resident cell (59, 60). Bacterial mRNA decay is facilitated by a large set of ribonucleases (RNases), one of which is the essential endoribonuclease RNase E, which preferentially cleaves mRNA at AU-rich regions (61). RNase E is composed of a well-folded catalytic core and an intrinsically disordered C-terminal domain (CTD). The latter is responsible for binding polynucleotide phosphorylase (PNPase), RNA helicase RhlB and the glycolytic enzyme enolase, which together form a complex known as the RNA degradosome (62). RNase E-mediated cleavage increases the number of mRNA termini available for exoribonucleases including PNPase, and it is responsible for initiating the decay of over 60% of all mRNAs in *E. coli* (63). RNase E activity can be regulated by multiple factors. RNase E preferentially binds and degrades 5'-monophosphorylated mRNAs (64). Therefore, alternative 5'-end capping such as nicotinamide adenine dinucleotide (NAD) globally stabilizes mRNA (65) while converting 5'-triphosphates to 5'-monophosphates promotes mRNA decay (66). RNase E activity can also be controlled by internal or terminal RNA secondary structures, which can be resolved by RhlB. Furthermore, RNase E accessibility to 3'-ends can be

improved by poly(A) polymerase I, which adds polyadenylated tails to mRNAs, thus providing a binding site for exoribonucleases (67). RNase E activity can also be regulated by other means, including post-translational modifications and access restriction by ribosomes (68, 69).

While RNase E's intrinsically disordered CTD implies its ability to form condensates, *E. coli* RNase E only forms small, transient puncta at the membrane periphery (70, 71). In stark contrast, RNase E in *Caulobacter crescentus* constitutively form condensates known as bacterial RNP-bodies (BR-bodies) (72). BR-body formation is mainly driven by RNase E's disordered CTD (72), which is also required for sequestration of RNA degradosome components, as well as RNase D, Hfq and RNA helicase RhlE, into BR-bodies (72, 73). BR-bodies serve as a selective and stress responsive hub for RNA decay and storage. During exponential growth, they enrich and degrade poorly translated mRNAs yet exclude tRNAs and rRNAs (72, 74). Associative activity of RNase E and exoribonucleases in BR-bodies ensures complete degradation of substrates, which prevents harmful accumulation of RNA decay intermediates (74). As cells enter stationary phase, however, accumulation of untranslated mRNA causes BR-bodies to mature and rigidify into a dynamically arrested state. This transition reduces RNase activity and shifts BR-body function from mRNA decay to mRNA storage. Upon nutrient replenishment, BR-bodies disassemble and release the stored mRNAs, allowing them to be immediately translated during recovery growth (75). Not surprisingly, disrupting BR-body formation by truncating RNase E's CTD increases global mRNA half-life during stationary growth and reduces translation of essential transcripts upon recovery from stationary phase (72, 74, 75). However, it should be noted that disruption of RNase E condensation by truncating its CTD also inhibits assembly of the RNA degradosome. Therefore, the biological significance of RNase E condensation is still not fully understood. Notwithstanding, BR-bodies are the best characterized bacterial RNP condensate to date.

1.3.2 RNA chaperone Hfq and H-bodies

Another critical post-transcriptional pathway regulating mRNA decay is mediated by small RNAs (sRNAs) and RNA chaperone Hfq. sRNAs are small non-coding RNAs that bind mRNA through perfect or imperfect base pairing (76). sRNA-mRNA association can be dual-functional depending on the binding site and structure of the resulting duplex. While sRNA binding near the ribosome binding site (RBS) can block translation initiation, binding to the 5'-untranslated region (5'-UTR) can disrupt secondary structures which otherwise render RBS inaccessible to ribosomes. Similarly, sRNA can either stabilize mRNA by forming secondary structures resistant to RNases or stimulate

mRNA decay by recruiting RNases to exposed target motifs (76-78).

sRNA-mRNA binding often requires RNA chaperones such as Hfq. Hfq is a small DNA/RNA binding protein that typically forms a toroidal hexamer (44, 79). This gives rise to three distinct RNA binding surfaces: the proximal face where the N-terminal α -helix resides, the distal face enriched in antiparallel β -sheets and the rim region (44). Structural studies have revealed different nucleic acid binding preferences among three surfaces. The distal face preferentially binds RNAs bearing ARN repeats (R stands for purine, N stands for any nucleotide) including poly(A) RNA, the proximal face binds DNA and U-rich RNA, while the rim binds AU-rich RNA (79-81). sRNAs interact with Hfq in at least two ways. While all sRNAs contain a U-rich motif that binds the proximal face, class I sRNAs contain an AU-rich motif that binds the rim and class II sRNAs contain ARN repeats that has high affinity to the distal face (82). Consequently, class I sRNAs target mRNAs that contain ARN repeats, while class II sRNAs target those with AU-rich motifs (82). Hfq association has profound implications in mRNA stability. Hfq can directly interact with RNase E's non-catalytic region, which recruits the latter to mRNAs targeted for degradation (83). On the other hand, Hfq can bind certain mRNA motifs that would otherwise be targeted by RNase E, thereby increasing their stability (84, 85). Therefore, loss of *hfq* often leads to pleiotropic phenotypes (86).

In *E. coli*, Hfq has recently been reported to form a large polar condensate termed as the H-body during nitrogen (N) starvation (87). H-body sequesters the RNA degradosome, which is localized to the cell membrane under non-starved conditions, via its interaction with RNase E's CTD (88). In contrast to *C. crescentus*, *E. coli* does not require RNase E for H-body formation and Hfq condensates that do not colocalize with RNase E are formed under other stresses (88, 89). H-bodies also colocalize with the polar-localized protein TmaR, a master regulator of carbohydrate metabolism (90). TmaR forms co-condensates with Hfq *in vitro*, and deletion of *tmaR* completely abolishes H-bodies (89). Since H-bodies have not been isolated to sufficient purity, the Hfq-bound RNAs that specifically associate with H-bodies have not been identified. Nevertheless, the Hfq-RNA interactome changes dramatically during N starvation, and a large number of sRNA-mRNA pairs are identified only in long-term N starved bacteria (91). Considering that >40% of Hfq molecules partition into H-bodies in N starved *E. coli* (87), it is tempting to speculate that H-bodies serve as a hub for RNA storage and/or decay in stressed *E. coli*, similar to the role of BR-bodies in *C. crescentus*.

1.3.3 Conservation of RNP condensates across evolution

BR-bodies and H-bodies bear notable similarities with stress-induced eukaryotic RNP bodies such as P-bodies and stress granules. Both bacterial RNP bodies contain the endoribonuclease RNase E, while eukaryotic RNP bodies harbor the major exoribonuclease Xrn1 (92). Furthermore, P-bodies harbor the decapping activator Lsm1-7 complex, which belongs to the same protein superfamily as Hfq (93, 94). Finally, whereas eukaryotic RNP bodies enrich RNases, they are not required for mRNA decay. Instead, it is suggested that they can facilitate mRNA storage during stress and, like BR-bodies, release stored transcripts for translation during recovery (95-97). These similarities suggest a functional convergence between eukaryotic and bacterial RNP condensates and imply other conserved features yet to be discovered.

1.4 Polyphosphate as a novel modulator of condensates¹

Inorganic polyphosphate (polyP) is a linear, non-branched polymer of phosphate groups covalently linked by high-energy phosphoanhydride bonds (98). It is suggested to originate prebiotically via volcanic activities and has been identified in all organisms examined so far (99, 100). PolyP's high charge density, combined with its structural flexibility, allows it to interact with a great array of molecules (101-103). Not surprisingly, polyP is involved in a wide range of biological activities including bacterial biofilm formation, quorum sensing, neurodegeneration and blood clotting (98). However, the precise mechanism by which polyP participates in so many processes is still unclear.

1.4.1 PolyP metabolism in bacteria

In many bacteria including *E. coli*, polyP is synthesized by the conserved polyP kinase 1 (PPK1), which reversibly transfers the γ -phosphate from ATP to polyP (104, 105). Some bacteria, such as *Pseudomonas aeruginosa*, encode an additional polyP kinase 2 (PPK2), which has no sequence or structural homology with PPK1 (106-108). PolyP degradation in bacteria, on the other hand, is almost universally mediated by the conserved exopolyphosphatase PPX, which hydrolyzes polyP starting at the end of the chains, and releases phosphate ions in this process (109). Despite knowing the relevant enzymes, we still know surprisingly little about how polyP levels are regulated in bacteria. In *E. coli*, polyP accumulation is induced by oxidative stress, heat shock, hyperosmolarity

¹ Partial contents of this section were published in Guan, J. & Jakob, U. (2024) The protein scaffolding functions of polyphosphate. *Journal of Molecular Biology*, 436(14), 168504. The publisher permits the reproduction of articles by the author for the purpose of an academic thesis.

and, most robustly, nutrient limitation (110-112). A longstanding model proposes that upregulation of polyP is the result of PPX inhibition by the alarmone (p)ppGpp, a signaling molecule produced by the stringent response (113, 114). This is based on the observation that (p)ppGpp is a potent inhibitor of PPX activity *in vitro* (115). However, it has been undermined by a more recent study showing that a strain unable to produce (p)ppGpp still accumulates polyP under starvation (116). Moreover, *ppx* deletion mutants of *E. coli* fail to accumulate detectable amounts of polyP in the absence of stress (117), suggesting that activation of PPK might be the more likely mechanism underlying stress-induced polyP accumulation. Indeed, several newly identified PPK mutants, which carry point mutations in the monomer interface of the PPK tetramer, display a population shift from mainly tetramers to dimers and monomers (118). Many of these mutants show increased resistance to substrate and product inhibition *in vitro* (118) and support significant *in vivo* polyP accumulation even under nutrient-replete conditions (117). Although much more investigation is needed, these observations point to a possible scenario where binding factors and/or post-translational modifications that disrupt PPK tetramers allow polyP synthesis to occur in cellular environments that are otherwise inhibitory (118).

1.4.2 PolyP as a non-proteinaceous chaperone

The accumulation of polyP in heat or oxidatively stressed bacteria had long been associated with a cytoprotective effect (110-112), yet the underlying mechanism(s) had long remained elusive. Our lab later discovered that polyP could function similarly as molecular chaperones, which protect unfolding proteins against irreversible aggregation during proteotoxic stress. Our findings suggest that during proteotoxic stress, polyP specifically interacts with protein unfolding intermediates as they transiently adopt a β -sheet-rich conformation, which stabilizes the latter in a soluble state. Once stress is relieved, polyP-bound proteins regain their native conformation assisted by protein-based, ATP dependent chaperones (111, 112). This allows polyP to effectively prevent irreversible protein aggregation upon near-boiling temperatures or in the presence of harsh oxidants, conditions that will inevitably inactivate protein-based chaperones (111, 112). While these studies likely explain polyP's involvement in a wide range of stress responses, many questions remain. The chaperone function of polyP is often chain length-dependent, with longer polyP chains disproportionately more active than shorter ones (111). This excludes a simple charge-based function of polyP and supports the idea of polyP serving as a multivalent folding scaffold. It is also entirely unclear whether polyP interacts with client proteins via their side chains,

backbone, or a combination thereof. Approaches such as solid-state NMR would ultimately provide answers to these important questions. However, the significant heterogeneity among the formed complexes (112) presents a major challenge.

1.4.3 PolyP as a scaffold for condensation

Organismal polyP was first observed in bacteria and algae as spherical, electron dense granules (119). As these granules do not have defined boundaries or crystal-like internal order (120-123), it is suggested that they are likely condensates (3, 124). Indeed, polyP forms *in vitro* condensates with numerous metal ions, especially Mg^{2+} (125-129), the most abundant cation in bacterial polyP granules (122, 123, 130). *In silico* simulation suggests that Mg^{2+} preferentially interacts with non-bridging oxygen atoms that protrudes from the polyP backbone, leading to polyP chain compaction and interchain networking that favors condensation (125). PolyP condensates can also enrich DNA and proteins (126, 129), which agrees with the observation that bacterial polyP granules are often embedded in or attached to the nucleoid (124).

The presence and morphology of bacterial polyP granules differ among species and growth conditions. While *C. crescentus* constitutively contain polyP granules, *P. aeruginosa* accumulates polyP granules only upon certain stresses (122). Notably, *E. coli* does not typically produce polyP granules even when cytoplasmic polyP concentration is very high (131). As ectopic expression of *EcPPK* in *ppk*-deleted *C. crescentus* results in wild-type-like polyP granules (131), it is apparent that such distinction is due to some other factor(s) present in the cytoplasm, rather than the activity of PPK itself. Characterization of the polyP granule proteome will likely provide valuable insight into the identity of such factor(s). However, due to technical challenges only a handful of granule-associated proteins have been identified so far (132), including PPKs, DNA binding protein AlgP and several conserved histidine α -helical domain (CHAD) proteins (131, 133-135). Limited by imaging resolution, it is unclear whether these proteins are partitioned within polyP granules or peripherally associated. Furthermore, none of these granule-associated proteins (except for PPKs) are required for the formation of polyP granules, and their deletion has negligible impact on bacterial fitness (134, 135).

Despite the ubiquitous presence of bacterial polyP granules, their biological function is poorly understood, largely due to the inability to uncouple polyP granule formation from polyP synthesis (124). However, based on current evidence, we propose that polyP granules at least serve to “safely” store phosphate. Considering its multivalent nature, high concentrations of polyP

dispersed in the cytoplasm will likely cause promiscuous interactions with proteins and unchecked sequestration of metal ions, both of which have profound effects on bacterial survival. Therefore, condensing polyP into a phase-separated compartment limits cytoplasmic free polyP concentration to a low and relatively stable level (136), thereby only allowing specific polyP-protein interactions to occur.

1.5 Thesis overview and impact

Biomolecular condensates provide significant advantage over membrane-bound organelles in remodeling subcellular organization upon acute stress. The sensitivity of c_{sat} to environmental factors such as temperature and osmolarity allows multivalent biomolecules to bypass the need for gene expression and protein translation, which are innately slow and could be compromised by stress, and rapidly phase-separate into condensates that may serve as hubs for enzymatic activity, storage and signaling. Over the past two decades, stress-responsive condensates have been widely described in eukaryotes, yet their presence in bacteria has received little attention. In my thesis, I characterized the formation mechanisms, material properties and functions of two stress-responsive bacterial condensates. In Chapter 2, I show that nitrogen starved *E. coli* accumulates high levels of polyP, which scaffolds the RNA chaperone Hfq into higher oligomers, thus reducing the latter's c_{sat} and allowing it to phase separate into condensates known as H-bodies. PolyP facilitates the recruitment of RNA degradosome components and translation-related proteins into H-bodies as clients. It also allows H-bodies to specifically enrich and stabilize polyadenylated RNAs, especially those related to survival under and recovery from nitrogen starvation. Consistent with the functional and compositional similarities between H-bodies and P-bodies, polyP is also shown to participate in the formation of P-bodies in mammalian cells. In Chapter 3, I show that *E. coli* NAP homologs H-NS and StpA display distinct phase behaviors. StpA forms long fibrillar oligomers that pack into amorphous aggregates, while H-NS can dissolve and convert it into condensate-like droplets. In exponentially growing bacteria the two proteins readily form co-condensate-like compartments, upon heat stress bacteria accumulate higher levels of StpA, which is more stress-resistant, to stabilize H-NS condensates and maintain proper gene silencing. Together, my work reveals the extensive involvement of biomolecular condensates in bacterial stress response and adaptation. These findings help us better understand how bacteria survive and grow in diverse and often challenging environments and could potentially serve as the basis for future investigations in antimicrobial research.

Chapter 2 Polyphosphate Modulates the Stress-Responsive Formation of Functional RNA-Protein Condensates in Bacteria and Mammalian Cells²

2.1 Introduction

Membrane-less organelles, also referred to as biomolecular condensates, arise from liquid-liquid phase separation (LLPS), where biomolecules in a homogeneous solution separate into a condensed liquid phase surrounded by a dilute phase (4, 137). Condensates serve as a critical addition to the endomembrane system in the subcellular organization of physiological processes as they promote specific segregation of biochemical pathways and local enrichment of cellular components (2). The rapid formation of condensates and its independence from membrane synthesis provides cells with rapid and effective means to reorganize cellular processes in response to stress (138). Indeed, several stress conditions lead to functional condensate formation in eukaryotes and, as shown more recently, also in prokaryotes (139). For instance, oxidative stress, such as that elicited by arsenite treatment, triggers the formation of cytosolic stress granules (SGs) in mammalian cells, which sequester essential components of the protein translation machinery to globally prevent translation initiation during stress (140, 141). Similarly, select stress conditions increase the size and number of Processing (P)-bodies in mammalian cells or Bacterial Ribonucleoprotein (BR)-bodies in *Caulobacter crescentus* (72, 74, 142, 143), both types of condensates aiding in RNA surveillance and selective RNA degradation (72, 144). Yet, in none of

² Contents of this chapter were published in Guan, J., Hurto, R. L., Rai, A., Bhattra, J., Azaldegui, C. A., Ortiz-Rodríguez, L. A., Liu, Q., Biteen, J. S., Freddolino, L., & Jakob, U. (2026) Polyphosphate modulates the stress-responsive formation of functional RNA-protein condensates in bacteria and mammalian cells. *PLoS Biology*, 24(4), e3003775. The publisher permits the reproduction of articles by the author for the purpose of an academic thesis. A preprint is available at bioRxiv titled as “HP-bodies—ancestral condensates that regulate RNA turnover and protein translation in bacteria.” I performed all the bacterial and *in vitro* experiments in this chapter except single-molecule tracking, bioinformatic analysis, co-immunoprecipitation and mass spectrometry analysis. Preparation of sequencing samples were performed by R.L.H. Bioinformatic analysis was performed by L.F. J.G., Q.L., L.F. and U.J. performed quantitative analysis of microscopy images. A.R. performed cell culture experiments. J.B. performed protein pull down. C.A.A. and L.A.O-R. performed single molecule tracking experiments. The study was supervised by J.S.B., L.F. and U.J. Recombinant proteins were expressed and purified by Ke Wan from the Bardwell lab. Mass spectrometry was performed by Hye Kyong Kweon at U-M Chemistry. All authors contributed to the research design, data analysis, and writing.

these cases, the precise mechanism that leads to the rapid stress-induced formation and/or expansion of functional biomolecular condensates is fully understood.

Recent studies in *Escherichia coli* revealed that in response to nitrogen (N) starvation, hyperosmolarity or stationary growth, the conserved RNA chaperone Hfq assembles into a single, polar-localized condensate (i.e., H-body) (87-89, 145). Hfq is a small hexameric protein that shares structural features with eukaryotic LSm proteins, one of the major components of P-bodies (146). Best known for its function as an RNA chaperone, Hfq facilitates sRNA-mRNA pairing, thus regulating mRNA stability and translation (44, 147). In addition, about 10-20% of cellular Hfq is nucleoid-associated and involved in the silencing of mobile genetic elements (148-150). Based on the observed recruitment of members of the RNA degradosome to the condensates, Hfq foci have been suggested to be critical for post-transcriptional gene regulation (62, 88, 89, 151). Yet, what leads to Hfq's phase separation under stress conditions that stall *de novo* protein synthesis is not known. A recent study revealed that the sugar regulator TmaR, which itself forms a polar-localized condensate in *E. coli*, is necessary for H-body formation (89, 151). However, because TmaR condensates are also present under non-stress conditions (90, 151), it is unlikely that this protein directly initiates Hfq foci formation during stress. Given the known involvement of polyanions in condensate formation (152-154), we thus wondered whether polyphosphate (polyP), the most ancient, ubiquitous, and highly negatively charged polymer known, might contribute to stress-induced Hfq foci formation (100). We based this reasoning on the fact that stress conditions that cause Hfq condensate formation also trigger the accumulation of long-chain polyP (110) as well as on the recent finding that polyP supports the phase separation of Hfq *in vitro* and Hfq functionality *in vivo* (150). Here, we demonstrate that the stress-induced formation of long-chain polyPs plays an important role in Hfq foci formation. The ability of polyP to scaffold Hfq into high molecular weight assemblies appears to reduce the saturation concentration (c_{sat}) of Hfq for phase separation, thus promoting liquid droplet formation at existing Hfq levels. We find that Hfq-polyP condensates sequester and selectively stabilize polyadenylated transcripts that are critical for bacterial survival of and recovery from N starvation. Extending to the extreme opposite end of the tree of life, we demonstrate that polyP accumulation also contributes to the formation of mammalian P-bodies but not stress granules. Based on these findings, we propose that polyP plays a conserved role in shaping functional condensates throughout evolution, marking it as one of the most ancient modulators of condensate formation across biology.

2.2 Results

2.2.1 Loss of polyphosphate negatively affects stress-induced Hfq foci formation

To investigate the potential influence of polyP on Hfq foci formation *in vivo*, we replaced the chromosomal copy of *hfq* with a fully functional and previously validated *hfq-mCherry* gene fusion (155) in either *E. coli* MG1655 wild type (WT) or a mutant strain that lacks polyphosphate kinase (Ppk), the only known polyP-synthesizing enzyme in *E. coli* (98). When grown in defined media supplemented with limiting amounts of nitrogen (N), both strains enter N-starvation at ~5 h (N-0) (Fig. S2.1A). At this point, WT bacteria begin to accumulate polyP in the form of very long (>300 P_i units) chains (i.e., polyP-300) (Fig. 2.1A). Within 3 hours of N-starvation (N-3), over 80% and within 24h (N-24), close to 100% of all WT bacteria contain one polarly localized Hfq focus (Fig. 2.1B, C). Incubation of N-24 WT cells with 1,6-hexanediol, a known disrupter of *in vivo* protein condensates, dissolves these foci, consistent with their previously reported liquid-liquid phase properties (Fig. S2.1B) (156). In contrast, the Δppk strain, which does not produce any detectable polyP (Fig. 2.1A), shows a significant delay in the appearance of Hfq foci with only ~ 50% of bacteria containing a detectable focus at N-6 (i.e., 6h after onset of N-starvation) and ~75% of cells presenting foci at N-24 (Fig. 2.1B, C). Single-molecule trajectory measurements of Hfq endogenously labeled with photoactivatable mCherry (PAmCherry) confirmed these results by revealing that, though the Hfq molecules have the same dynamics in N-24 Δppk cells compared to Hfq molecules in N-24 WT *E. coli*, these slow-moving molecules are confined to a single focus in WT cells but spread out throughout the cell in Δppk cells (Fig. 2.1D, E). Moreover, incubation with 1,6-hexanediol caused a faster disappearance of Hfq foci in the *ppk* deletion cells as compared to WT cells, suggestive of fluidic and/or compositional differences between the Hfq foci formed in the presence and absence of polyP (Fig. S2.1B). Automated image analysis also revealed that Hfq foci formed in the Δppk strain are, on average, between 40 to 50% smaller than foci formed in the WT strain (Fig. 2.1F). These latter results were particularly surprising given that the N-24 Δppk strain accumulates almost twice the amount of total Hfq compared to N-24 WT *E. coli* (Fig. S2.1C). Consistent with these results, the ratio of Hfq intensity within the foci *versus* the cellular background in the Δppk strain was reproducibly about 60% of the WT ratio at all measured times during N-starvation (Fig. S2.1D). We also observed significant differences in focus formation between WT and Δppk strains when we expressed a 3×FLAG tagged Hfq instead of Hfq-mCherry. Upon fixing and staining the bacteria with anti-FLAG antibodies, we detected focal accumulations

of Hfq in about 30% of WT bacteria (consistent with published results (89)) as compared to less than 2% in Δppk bacteria (Fig. S2.1E, F). Based on these results, we concluded that the Hfq foci that form in response to N-starvation in the absence of polyP are fewer, smaller, less intense and more 1,6-hexanediol sensitive than Hfq foci that form in the presence of polyP.

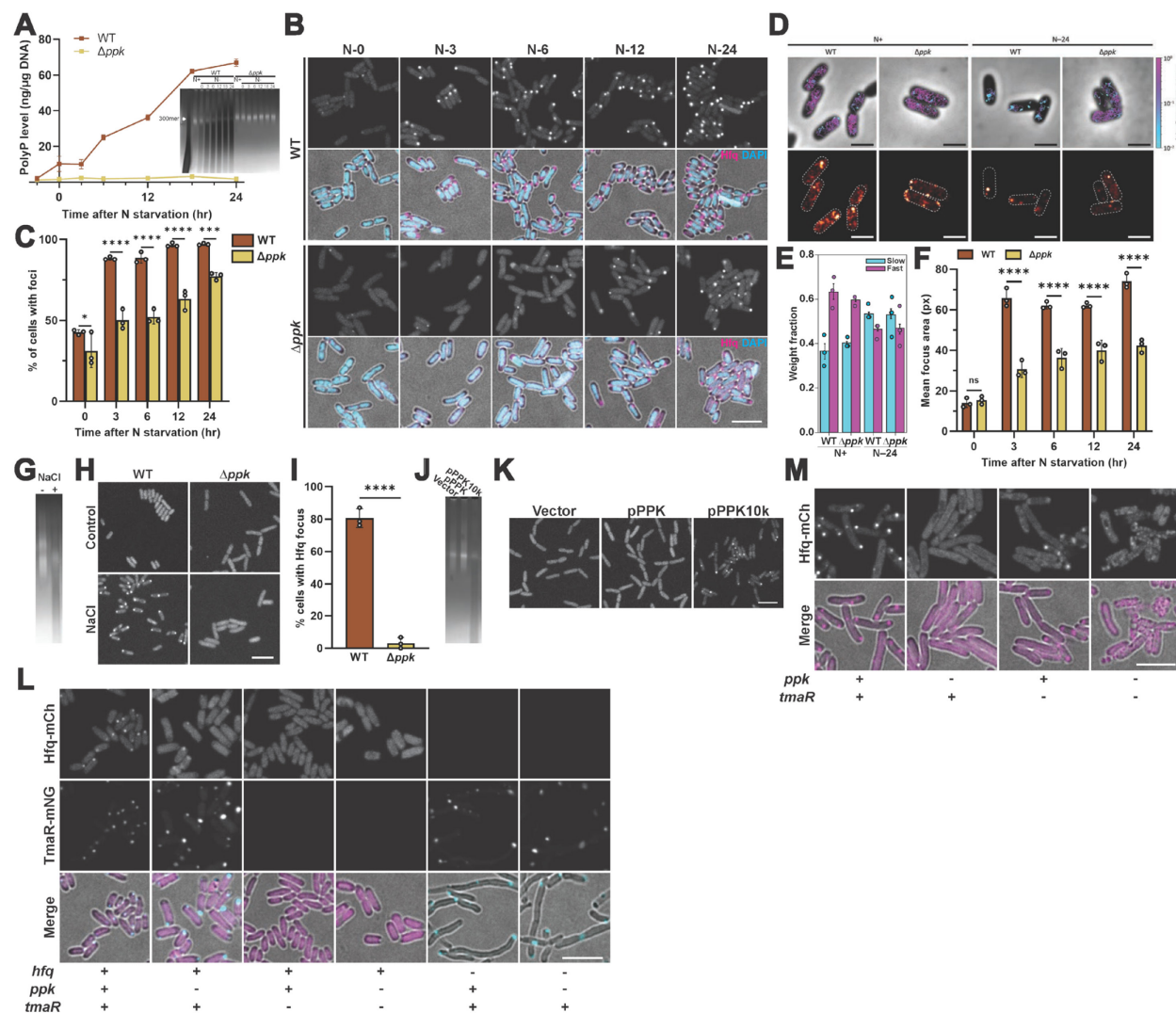


Figure 2.1 PolyP drives foci formation of Hfq in *Escherichia coli*.

(A) Normalized polyP levels in WT (MG1655) and Δppk *E. coli* 2 h before and select timepoints upon entry into N-starvation (N-0). Error bars indicate SD ($n = 3$). **Inset:** PolyP extracted under the same conditions and visualized on TBE gel by DAPI staining and photobleaching, which will appear bright for nucleic acid and dark for polyP. Purified polyP 300mer was used as reference (lane 1). (B) Subcellular localization of Hfq-mCherry (magenta) and nucleoid (stained by DAPI, cyan) in *hfq::hfq-mCherry* WT (MG1655) or Δppk strains at select times (in hours) after the onset of N-starvation (N-). (C) Quantification of cells containing visible Hfq-mCherry foci after entering N-starvation. A minimum of 200 cells were quantified for each of three replicates under each time point. Comparisons were made by two-way ANOVA. Error bars show SD. $*p < 0.05$, $***p < 0.001$, $****p < 0.0001$. (D) Upper panel: Representative single molecule trajectories of Hfq-PAmCherry in indicated strains and conditions overlaid with phase contrast images. Trajectories are color-coded based on the apparent diffusion coefficient. Lower panel: Reconstructed super-resolution PALM images based on images above. Cell boundaries are indicated by dashed lines. Scale bars: 2 μm . (E) Percentage of single-molecule Hfq-PAmCherry trajectories in the fast (magenta) and slow (cyan) populations

for cells in each of the indicated conditions. Data points are shown for each of at least three biological replicates. **(F)** Mean area of Hfq foci for cells under each of the indicated conditions; data points are shown for each of three biological replicates, with each such data point arising from joint quantification of four imaging fields at each time point; see Methods for details. Comparisons were made by two-way ANOVA. Error bars show SD. **** $p < 0.0001$. **(G)** PolyP extracted from *E. coli* MG1655 *hfq::hfq-mCherry* after 4 h of mock treatment (–) or upon osmotic stress treatment with 1.17 M NaCl (+) in LB medium was visualized on TBE gel by DAPI staining and subsequent photobleaching. **(H)** Subcellular localization of Hfq-mCherry in *hfq::hfq-mCherry* WT (MG1655) or Δppk after 4 h of osmotic stress as in **(G)**. **(I)** Quantification of cells containing visible Hfq-mCherry foci after osmotic stress. At least 100 cells were quantified in each of three replicates. Comparisons were made by unpaired *t*-tests. Error bars indicate SD. **** $p < 0.0001$. **(J)** PolyP extracted from exponentially growing Δppk *E. coli* *hfq::hfq-mCherry* carrying an empty plasmid or a plasmid expressing WT *ppk* or *ppk10k* was visualized on TBE gel by DAPI staining and subsequent photobleaching, which darkens the polyP signal. **(K)** Subcellular localization of Hfq-mCherry in strains shown in **(H)**. **(L)** Subcellular localization of Hfq-mCherry (magenta) and/or TmaR-mNG (cyan) in indicated *E. coli* strains at N-24. **(M)** Subcellular localization of Hfq-mCherry in indicated *E. coli* strains after 4 h of osmotic stress as in **(G)**. Scale bars: 5 μ m unless otherwise indicated.

2.2.2 Increased polyP levels are sufficient for Hfq foci formation

We next wondered whether the decrease in foci number and size that we observed in the N-24 *ppk* deletion mutant is directly caused by a lack of polyP or due to potentially increased levels of cellular ATP, the substrate of *E. coli* polyphosphate kinase and a known biological hydrotone that prevents condensate formation *in vitro* (157). However, comparison of ATP concentrations in WT and *ppk* deletion bacteria revealed that the N-24 WT bacteria had even slightly higher levels of ATP compared to the N24 Δppk mutant, making this explanation unlikely (Fig. S2.1G). A second stress condition that has been shown to cause a significant increase in Hfq foci formation is exposure to osmotic stress. We chose 4h of treatment with 1.17 M NaCl because these conditions had previously been shown to significantly increase polyP (158). We confirmed those results and found a substantial accumulation of long and medium-chain polyP in these bacteria (Fig. 2.1G). Of note, under these stress conditions, absence of *ppk* had even more dramatic effects; whereas ~80% of WT *E. coli* cells displayed a single, clearly visible and polar-localized Hfq focus, fewer than 5% of the Δppk bacteria showed any detectable focal accumulation of Hfq (Fig. 2.1H, I). These results now raised the interesting possibility that Hfq foci formation might be directly linked to the stress-induced accumulation of polyP. To investigate whether this is indeed the case, we expressed a plasmid-encoded polyP-overproducing mutant variant PPK10k (117) in the Δppk strain and compared Hfq foci formation with strains expressing either the vector alone or vector-encoded WT Ppk. We confirmed that the expression of PPK10k causes a substantial increase in the steady state levels of polyP under exponential growth conditions compared to WT Ppk-expressing bacteria (Fig. 2.1J). More relevant, however, we observed that most of the Ppk10k expressing cells contain one Hfq focus whereas neither of the other two Δppk strains shows any

detectable Hfq foci under these growth conditions (Fig. 2.1K). Based on these results, we concluded that the stress-induced increase in cellular polyP concentrations contributes to the phase separation of Hfq during N-starvation and osmotic stress.

2.2.3 Both polyP and TmaR are necessary for Hfq foci formation during N-starvation

Previous studies reported that the formation of Hfq condensates in response to N-starvation, stationary growth or osmotic stress depends on the bacterial sugar metabolism regulator TmaR, which forms co-condensates with Hfq (89, 90). Intriguingly, however, these studies also revealed that before stress, significantly more bacteria harbor TmaR foci than Hfq foci, and that during N-starvation, the formation of Hfq foci lags significantly behind the increase in TmaR foci (89, 151). These results led to the conclusion that TmaR condensates, while necessary for Hfq foci formation, might not be sufficient and that other, potentially metabolic changes act downstream of TmaR to promote Hfq condensation (151). We now wondered whether polyP is one such downstream factor, which would make the stress-induced synthesis of polyP likely a rate-limiting step for Hfq foci formation. Alternatively, it was also conceivable that polyP impacts condensation of TmaR, thereby only indirectly affecting Hfq phase separation. To distinguish between these possibilities, we expressed Hfq-mCherry in $\Delta tmaR$ and $\Delta tmaR/\Delta ppk$ strains as well as TmaR-mNeonGreen (mNG) in WT, Δppk , Δhfq and $\Delta hfq/\Delta ppk$ strains and monitored stress-induced Hfq and/or TmaR foci formation. As expected, the formation of Hfq foci under N-starvation conditions was significantly impaired in the Δppk strain and lost in the $\Delta tmaR$ and $\Delta tmaR/\Delta ppk$ strains (Fig. 2.1L), the latter also not rescuable by the expression of PPK10k (Fig. S2.1H). In contrast, lack of polyP, Hfq or both did not appear to affect the overall number of TmaR foci but caused heterogeneity in the size and intensity of individual TmaR foci (Fig. 2.1L). While these results suggested some level of feedback regulation between Hfq and/or polyP on TmaR condensate formation, they also implied that polyP acts more likely downstream of TmaR and hence directly on Hfq. These conclusions were further supported when we compared Hfq focus formation under strong osmotic stress conditions, where, as noted above (Fig. 2.1G) deletion of *ppk* caused an over 90% reduction in Hfq focus formation, whereas deletion of *tmaR* only led to a ~60% reduction in visible Hfq foci (Fig. 2.1M, S2.1I). The $\Delta ppk/\Delta tmaR$ strain revealed a highly aberrant cellular Hfq distribution, distinctly different from the Hfq distribution in the individual *ppk* and *tmaR* deletion mutants. Our observations differ from the earlier study by Goldberger et al. (89), where the authors reported that deletion of *tmaR* resulted in complete loss of Hfq foci when bacteria were treated with 0.3 M NaCl.

However, it should be noted that we used much higher NaCl concentrations (i.e., 1.17 M) to induce osmotic stress because these concentrations trigger substantial polyP accumulation while lower NaCl concentrations fail to do so (Fig. S2.1J). We therefore conclude that under mild osmotic stress conditions (i.e., presence of 300 mM NaCl), TmaR is likely the main factor supporting Hfq foci formation. At higher osmotic stress conditions, however, TmaR and polyP appear to act as independent entities, both necessary yet individually not sufficient for the proper generation of *in vivo* condensates of Hfq.

2.2.4 PolyP regulates the subcellular organization of Hfq during and after N-starvation

To investigate how polyP affects the subcellular distribution of Hfq during N-starvation, we measured the relative diffusion rates and localization of the various Hfq subpopulations using single-molecule super-resolution microscopy (see Methods for details). We classified Hfq-PAmCherry trajectories by their overlap with Hfq foci and subsequently assigned the relative quantities of Hfq diffusive populations into three states: inclusion in a slow-diffusing condensate C-state, slow-moving non-condensate B-state or fast-diffusing free F-state (Fig. S2.2). Because Hfq has been previously identified as a nucleoid-associated protein (148-150), we assign the slow-moving B-state as nucleoid-bound; the Hfq molecules included in the C-state may also be nucleoid-bound. Consistent with the bulk observations noted above and fully supported by recently published results (151), we found that 47% of all Hfq molecules coalesce into one distinct, slow-diffusing C-state (i.e., the Hfq focus) in N-24 WT *E. coli* (Fig. 2.2A) compared to only ~27% of Hfq molecules in the N-24 *ppk* deletion strain. Furthermore, we noted that in WT bacteria, the Hfq molecules that form the slow-diffusing C-state are balanced by a reduction in the pool of highly mobile “free” (F) Hfq molecules (~23%) and, to a lesser extent (~14%), in the pool of nucleoid-bound (B) Hfq molecules (Fig. 2.2A). In the absence of polyP, however, the increase in Hfq in the condensate is primarily due to a reduction in the free pool, thus leaving close to 50% of non-focus associated Hfq molecules bound to the nucleoid. These results agree well with previous *in vitro* findings, which showed that, at sufficiently high concentrations, polyP competes with DNA for Hfq binding (150). The results furthermore suggested that polyP might be involved in increasing the concentration of cytosolic Hfq molecules that can be recruited to the focus by releasing Hfq molecules from the nucleoid. To next test whether polyP also affects the reverse process (i.e., focus dispersal), we replenished N-24 WT and Δppk strains with a fresh N source, which causes the dissolution of Hfq foci within a few hours (87) (Fig. 2.2B, C). We observed that the Hfq foci

disappear from WT *E. coli* at a rate that coincides with the decline in cellular polyP levels (shown in Fig. 2.2D). Moreover, Hfq foci formed in the Δppk strain disappeared significantly faster than those in WT *E. coli*, consistent with their observed differences in stability upon hexanedial

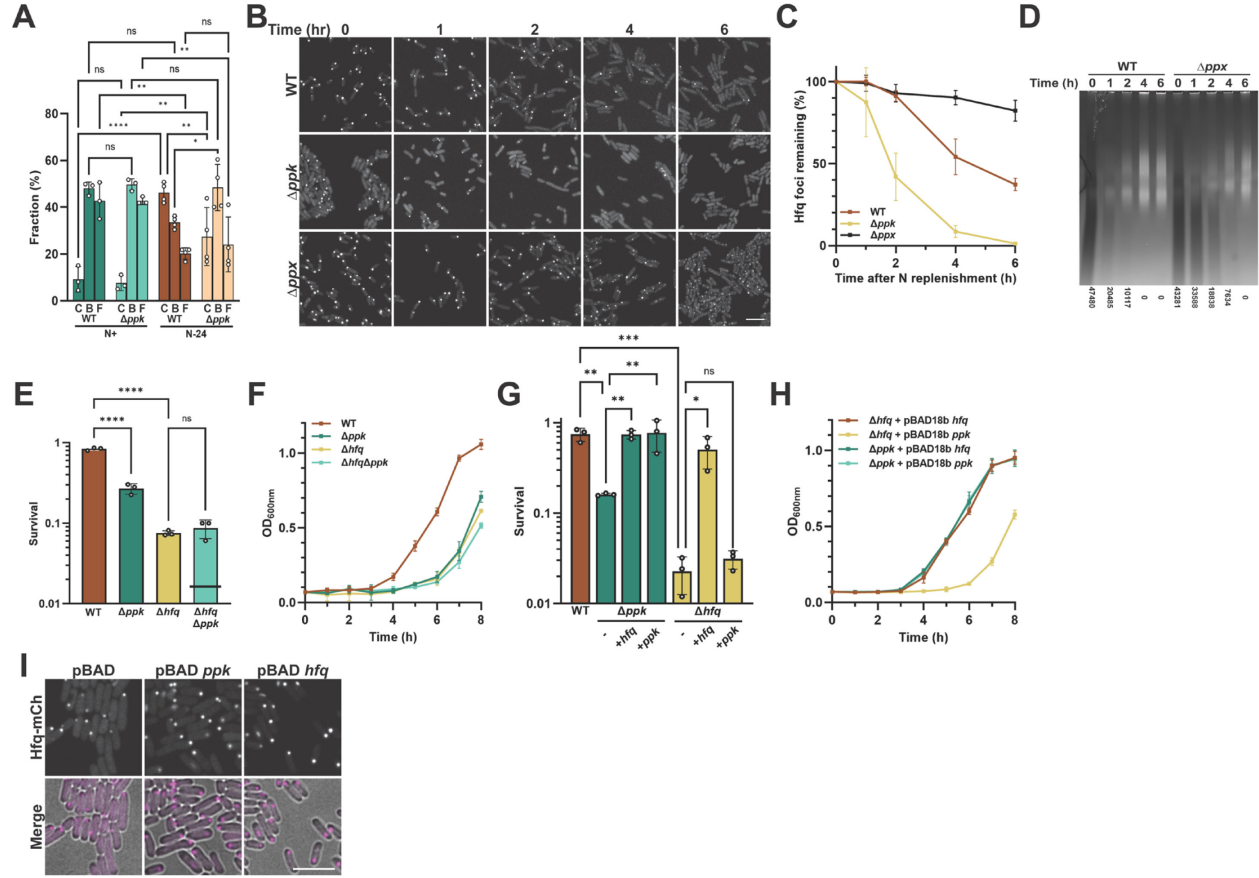


Figure 2.2 In vivo interplay between Hfq and polyP during N-starvation.

(A) Fraction of Hfq-PAMCherry present in condensates (C), found in the nucleoid-bound (B) form or free (F) in the cytosol of N+ and N-24 WT (MG1655) or *ppk* deletion strains as assessed by single-molecule localization. >1,500 trajectories were analyzed per replicate ($N \geq 3$) of each condition. Comparisons were made by 2-way ANOVA ($n \geq 3$), $*p < 0.05$, $**p < 0.01$, $****p < 0.0001$. Error bars indicate SD. (B) Subcellular localization of Hfq-mCherry in *hfq::hfq-mCherry* N-24 WT (MG1655), Δppk , or Δppk *Escherichia coli* at indicated time points after supplementing the media with fresh N source. (C) Percentage of Hfq-mCherry foci remaining calculated from (B). The number of condensates at N-24 (i.e., $t = 0$ h) is set to 100% and used as a reference point. A minimum of 200 cells were quantified for each of three replicates under each time point, error bars indicate SD. (D) PolyP levels at the indicated timepoints after supplementing the growth media of N-24 *hfq::hfq-mCherry* WT (MG1655) or Δppk with fresh N source. PolyP was visualized on a TBE gel using DAPI staining and subsequent photobleaching, which darkens the polyP signal. Relative polyP levels (A.U.) are shown below. (E) Survival of WT (MG1655), Δppk , Δhfq , and $\Delta hfq\Delta ppk$ *E. coli* at N48. Similar results were obtained in the respective MG1655 *hfq::hfq-mCherry* strains. The black bar in the double mutant indicates the expected phenotype if *ppk* and *hfq* deletion behaved log-additively. (F) Recovery growth of N-24 WT, Δppk , Δhfq , and $\Delta hfq\Delta ppk$ *E. coli* after dilution into fresh Gutnick medium supplemented with 3 mM NH_4Cl . (G) Survival of WT (MG1655), Δppk , or Δhfq *E. coli* carrying empty vector, *ppk* or *hfq* expression vectors at N-48. (H) Recovery growth of N-24 Δppk and Δhfq *E. coli* carrying *ppk* or *hfq* expression vectors after dilution into fresh Gutnick medium supplemented with 3 mM NH_4Cl . Error bars indicate SD ($n = 3$). One-way ANOVA with was used in panels A and C; $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$. (I) Subcellular localization of Hfq-mCherry in N-24 *hfq::hfq-mCherry* Δppk MG1655 carrying empty vector or overexpressing either *ppk* or *hfq*. Scale bars: 5 μm .

treatment (Fig. 2.2B, C). We observed that the Hfq foci disappear from WT *E. coli* at a rate that appears to coincide with the decline in cellular polyP levels (shown in Fig. 2.2D). Moreover, Hfq foci formed in the Δppk strain disappeared significantly faster than in WT *E. coli*, consistent with their observed differences stability upon hexanediol treatment (Fig. 2.2B, C). In contrast, foci that formed in a mutant that lacks the *E. coli* polyphosphatase Ppx—and hence contains higher polyP levels during stress and shows a slower polyP decline upon N-replenishment compared to WT *E. coli* (Fig. 2.2D)—revealed a significant delay in the disappearance of Hfq foci (Fig. 2.2B, C). For example, while less than 40% of WT and 0 % of Δppk *E. coli* contained any clearly visible Hfq foci after 6h of N-replenishment, over 80% of Δppx bacteria still contained discrete Hfq foci (Fig. 2.2C). These results supported our conclusion that changes in the cellular levels of polyP affect the formation as well as the dispersal of Hfq foci presumably by partitioning Hfq molecules across various cellular regions (cytoplasm, condensate and nucleoid).

2.2.5 PolyP-mediated Hfq-foci formation correlates with improved stress resistance

One of the most crucial questions in the field of biological condensates concerns their physiological function(s). Previous studies revealed that the deletion of *hfq*, while not affecting *E. coli* growth under exponential conditions (Fig. S2.1A), significantly reduces bacterial survival during N-starvation (recapitulated in Fig. 2.2E) (87) and delays the restart of bacterial growth upon N-replenishment (Fig. 2.2F). Yet, it had been unclear whether these phenotypes are connected to the lack of free Hfq, which functions as RNA chaperone and/or nucleoid associated Hfq, which acts as suppressor of gene expression, or are due to the absence of foci-associated Hfq species. Based on our observation that the N-24 *ppk* deletion strain accumulates almost twice as much total Hfq compared to the N-24 WT strain (Fig. S2.1C), the vast majority of which in either the free or nucleoid-bound form (Fig. 2.2A), we now reasoned that the phenotypic analysis of the Δppk strain should shed light into the physiological role of Hfq foci during N-starvation. Analysis of N-starvation induced cell death revealed that while the deletion of *ppk* causes a slightly weaker phenotype compared to the phenotype of the Δhfq strain (Fig. 2.2E), both strains show an equally pronounced delay in the recovery upon N-replenishment (Fig. 2.2F). Moreover, and even more importantly, we found that the combined deletion of both *ppk* and *hfq* genes phenocopied the growth defects of the *hfq* deletion alone (Fig. 2.2E, F), implying that polyP and Hfq are likely part of the same pathway that protects *E. coli* against N-starvation stress. Rescue of the phenotypes by plasmid-borne expression of *ppk* or *hfq* confirmed that the observed growth defects are mediated

by the respective gene deletions and not caused by any polar effects (Fig. 2.2G, H). Importantly, overexpression of *ppk* in the absence of Hfq was insufficient to restore the growth defects, indicating that the growth defects are mediated primarily by Hfq. Yet overexpression of Hfq was able to fully rescue the phenotypes of the Δppk strain (Fig. 2.2G, H) and, fully consistent with this activity, restored WT levels of Hfq foci formation in the Δppk strain (Fig. 2.2I). Based on these findings, we now propose that the observed stress-induced accumulation of polyP acts on Hfq foci formation by reducing the critical concentration of Hfq for phase separation (i.e., the saturation concentration, c_{sat}). This model would explain why upregulation of Hfq levels is not necessary for its phase separation and can take place even under stress conditions that inhibit *de novo* protein synthesis. Moreover, it would imply that the observed upregulation of Hfq levels in the *ppk* deletion strain might serve as a compensatory mechanism to partially offset the requirement for polyP for phase separation and allow for some foci formation even in the absence of polyP.

2.2.6 PolyP is a hitherto unknown component of H-bodies

Our results provided strong evidence for the conclusion that the stress-induced increase in polyP levels contributes to the phase separation of Hfq *in vivo*. To test whether polyP constitutes an integral component of Hfq foci, we fixed N-24 Hfq-mCherry expressing WT and Δppk strains and co-stained the cells with an antibody against mCherry to detect Hfq and a GFP-fused probe derived from the C-terminal polyP-binding domain of *E. coli* Ppx (159) to visualize polyP (Fig. 2.3A). We found a modest but significant colocalization of the two components in N-24 WT bacteria (Fig. 2.3B, C), implying that polyP is a previously unrecognized component of stress-induced Hfq foci. Of note, however, only a subset of Hfq foci showed strong polyP enrichment. This result could be explained by inefficient permeability of the Ppx-GFP probe into some Hfq foci or indicate that some Hfq foci form independently of polyP, consistent with our previous observations (Fig. 2.1B). It is also important to mention that we observed a diffuse polyP signal in the cytoplasm/nucleoid region of the cells but did not observe any discrete polyP foci that were also not associated also with Hfq foci (Fig. 2.3A, B). This polyP population might be associated with nucleoid-bound or free-diffusing Hfq (Fig. 2.2A), or other charged proteins that might interact with polyP. To determine whether the presence of polyP affects the *in vivo* fluidic properties of Hfq condensates, we next compared the rate and extent of fluorescence recovery after photobleaching (FRAP), targeting similarly sized Hfq foci in both WT and Δppk strains (Fig. S2.3). We noted that while the kinetics of the Hfq-mCherry fluorescence recovery were similar and proceeded on the order of

tens of seconds, the relative extent of recovery was significantly lower for Hfq-mCherry foci that formed in the absence of polyP compared to those formed in WT *E. coli* (Fig. 2.3D). Subsequent Hfq solubility fractionation revealed that over 15% of Hfq molecules in the N-24 Δppk strain were insoluble compared to less than 5% of Hfq molecules in the N-24 WT strain (Fig. 2.3E). This increased insolubility might contribute to a decrease in Hfq solubility within the foci or within the dilute phase, potentially affecting the diffusion of Hfq across the phase boundaries. In either case, these results suggested that polyP affects the fluidic properties of Hfq condensates.

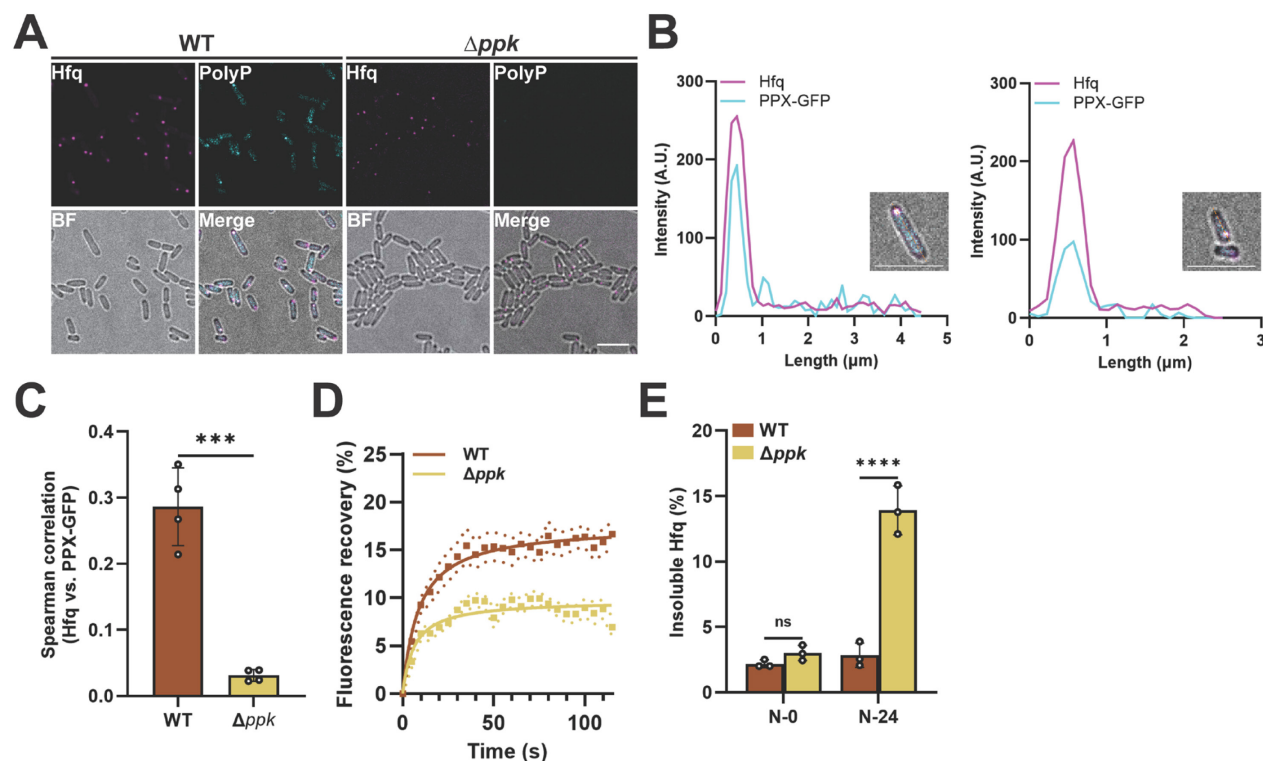


Figure 2.3 PolyP is a hitherto unknown component of H-bodies.

(A) Co-localization of polyP and Hfq-mCherry in fixed N-24 WT (MG1655) and Δppk *hfq::hfq-mCherry*. PolyP is visualized by immunofluorescence using a -PPXPD-GFP probe while Hfq is visualized with endogenous mCherry fluorescence. (B) Plot profiles of Hfq-mCherry and PPXPD-GFP in select cells (A), showing colocalization of Hfq and polyP. (C) Comparison of Spearman correlations between Hfq-mCherry and Ppx-GFP for nitrogen-starved cells of the indicated genotypes, demonstrating strong enrichment of polyP in the Hfq foci of WT cells. Comparison was made by unpaired *t* test, error bars show SD. ****p* < 0.001 (D) Analysis of in vivo FRAP measurements using similarly sized Hfq-mCherry foci at N-24 in *hfq::hfq-mCherry* WT (MG1655) and Δppk cells. Ten cells were analyzed per sample. FRAP traces were fitted against a one-phase association curve: WT $t_{1/2}$ = 8.8 s (95% CI 6.0 to 12.5 s), Δppk $t_{1/2}$ = 6.2 s (95% CI 3.9 to 9.2 s), with solid lines representing fitted FRAP curves, square dots average fluorescence intensities at indicated time points and round dotted lines indicating the SEM. (E) Proportion of insoluble Hfq-mCherry at N-0 and N-24 in WT (MG1655) and Δppk deletion strain as determined by quantitative western blot using antibodies against mCherry (*n* = 3, error bars indicate SD, *****p* < 0.0001). Scale bars: 5 μ m.

2.2.7 PolyP – A modulator of phase separating Hfq-RNA complexes

To investigate the interaction between Hfq and polyP in more detail, we purified Hfq and analyzed

its phase separation properties in the absence and presence of polyP-300. Under our buffer conditions (i.e., HEPES buffer, 50 mM NaCl, no crowding reagents), Hfq forms very few if any detectable droplets at protein concentrations below 25 μ M but reproducibly phase separates at higher protein concentrations (Fig. 2.4A). In the presence of polyP, droplet formation was significantly increased and clearly detectable at Hfq concentrations as low as 1 μ M. A phase diagram revealed that while molar ratios of polyP (in Pi units) to Hfq of 2:1 to 3:1 promote droplet formation, further increase in the polyP concentration causes the re-entry of Hfq droplets into the solute phase (Fig. 2.4B). Co-incubation of fluorescently labeled Hfq and polyP confirmed that the two biomolecules co-partition into the droplets (Fig. 2.4C). Inspired by previous work, which demonstrated that Hfq binds polyadenylated mRNAs and helps to prevent their degradation by the RNA degradosome *in vitro* (85), we next wondered whether the presence of polyP would affect RNA-Hfq interactions within the condensates. Therefore, we differentially fluorophore-labeled Hfq, polyP and rA₃₀ RNA (a known interactor of Hfq (80)) and co-incubated the three components. While the addition of either polyanion increased the relative number of droplets that Hfq forms at any given concentration and co-localized with Hfq, their combined presence further enhanced *in vitro* droplet formation and led to the formation of ternary condensates (Fig. 2.4C). These results implied that in contrast to DNA binding, which appears to be mutually exclusive with polyP binding (150), Hfq simultaneously binds to both rA₃₀ RNA and polyP-300. We independently confirmed this simultaneous interaction by using an electrophoretic shift assay (EMSA), which demonstrated that addition of increasing amounts of polyP-300 to discretely migrating pre-formed Hfq-rA₃₀ RNA complexes causes the formation of ternary complexes that stain positive for all three components (Fig. 2.4D). The migration pattern of the ternary complexes was similar to the ladder-like migration of Hfq-polyP complexes (Fig. 2.4D, last lane), suggesting that polyP-300 scaffolds multiple rA₃₀ RNA-binding Hfq hexamers into larger oligomers that phase separate *in vitro* and potentially also *in vivo*.

2.2.8 PolyP mediates higher oligomerization of Hfq under N-starvation *in vivo*

To test whether the observed *in vivo* Hfq foci formation also involves the polyP-dependent formation of high molecular weight (HMW) Hfq assemblies, we prepared lysates from Hfq-mCherry expressing WT and Δ *ppk* bacteria grown under N-0 and N-24 conditions and analyzed the migration of Hfq-mCherry on native western blots. As expected, Hfq-mCherry migrated as a low molecular weight (LMW) species in lysates prepared from either set of N-0 bacteria. In

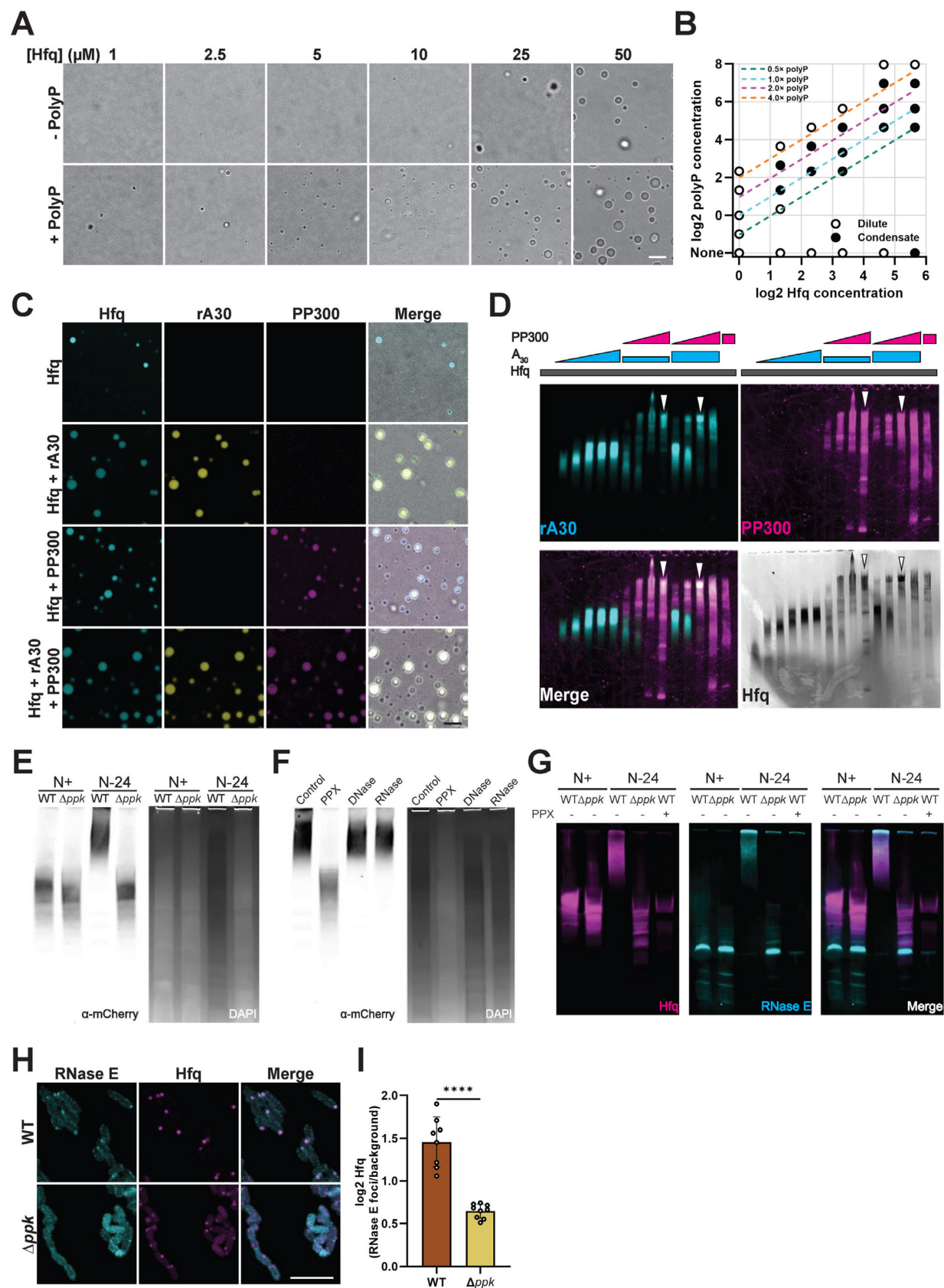


Figure 2.4 PolyP promotes Hfq condensate formation *in vitro*.

(A) Brightfield images of Hfq condensates reconstituted with the indicated Hfq concentrations in the presence or absence of $1 \times$ polyP-300 (in Pi units). Samples were prepared in 50 mM NaCl and 20 mM HEPES pH 7.0. **(B)** Phase diagram of Hfq and polyP-300 in the same buffer as (A). Formation of condensates was computationally determined by quantitative image analysis as detailed in the Methods section. Lines corresponding with specific polyP:Hfq ratios are highlighted. **(C)** Fluorescence and brightfield images of condensates reconstituted with 50 μ M Hfq (supplemented with 4% Cy3-Hfq S65C) in the presence or absence of 1 μ M FAM-labeled rA₃₀ or 100 μ M AF647-labeled polyP-300. Samples were prepared in the same buffer as (A). **(D)** Electrophoretic mobility shift assays (EMSA). Lanes 1–6: 25 μ M Hfq with 0, 0.5, 1, 2, 3, 4 μ M FAM-rA₃₀. Lanes 7–10 and lanes 11–14: 25 μ M Hfq with 1 μ M or 4 μ M FAM-rA₃₀, respectively, +25, 62.5, 125, 250 μ M AF647-polyP300. Lane 15: 25 μ M Hfq + 125 μ M AF647-polyP300. A representative gel ($n = 2$) is shown. Hfq was stained with Coomassie blue. Hfq-polyP-RNA complexes indicated by vertical arrowheads. **(E)** Left panel: Native western blot of bacterial lysates from MG1655 *hfq::hfq-mCherry* WT and Δ *ppk* at N+ and N-24 using antibodies against mCherry. Right panel: UV-bleached DAPI stain of native gel shown in left panel. PolyP is shown as dark areas. **(F)** Left panel: Native western blot of cell lysates from N-24 MG1655 *hfq::hfq-mCherry* WT (MG1655) using antibodies against mCherry. Lysates were treated with the indicated enzymes for 2 h prior to electrophoresis. Right panel: UV-bleached DAPI stain of native gel as shown in left panel. **(G)** Native western blot of N+ and N-24 lysates from *hfq::hfq-mCherry rne::rne-mTQ2* WT (MG1655) relative to Δ *ppk* using antibodies against mCherry (Hfq) and GFP (RNase E). N-24 samples were left untreated (–) or digested with Ppx for 2 h (+) prior to electrophoresis. **(H)** Subcellular localization of Hfq-mCherry and RNase E-mTQ2 in N24 WT (MG1655) or Δ *ppk*. Scale bars: 5 μ m. **(I)** Distribution of log₂ ratios of RNase E intensities in Hfq foci (see Methods for details) vs. cellular background. Each data point shows a separate imaging field taken across two separate biological replicates. Scale bars: 5 μ m.

contrast, the majority of Hfq-mCherry in N-24 WT lysates migrated in form of HMW assemblies (Fig. 2.4E), reminiscent of *in vitro* formed complexes between Hfq, polyP and rA30 (Fig. 2.4D). In the N-24 Δ *ppk* lysate, however, most of the Hfq-mCherry signal remained in the LMW region, suggesting that the smaller Hfq foci that form in the Δ *ppk* strain are unstable and dissociate during sample preparation. Incubation of N-24 WT lysates with the polyP-degrading yeast polyphosphatase (yPpx) (*l60*) -- but not with DNase I nor RNase A -- prior to the native PAGE analysis caused the dissociation of the majority of HMW complexes into LMW Hfq species (Fig. 2.4F), further supporting the idea that polyP serves as an *in vivo* scaffold for HMW complex formation of Hfq. To test whether the HMW complexes of Hfq that we observed in N-24 WT lysates correspond to foci-associated Hfq, we next analyzed the migration behavior of RNase E, a known component of N-starvation induced Hfq foci (88) (see below). For these experiments, we tagged RNase E with mTurquoise2 (mTQ2) at its endogenous loci, expressed it in the Hfq-mCherry expressing WT or Δ *ppk* strains and prepared lysates from N-0 and N-24 cells. As shown in Fig. 2.4G, we found that part of the RNase E-mTQ2 signal associates with the HMW species of Hfq in N-24 WT lysates but not in the Δ *ppk* strain or in Ppx-treated WT lysates. Live-cell fluorescent imaging of WT and Δ *ppk* strains co-expressing RNase E-mNG and Hfq-mCherry confirmed our native PAGE results and demonstrated that upon N-starvation, RNase E forms membrane-enriched foci that colocalize with Hfq foci to a significantly higher extent in WT *E. coli* compared to the Δ *ppk* strain (Fig. 2.4H, I). These results collectively suggest a potential

mechanism by which polyP promotes Hfq foci formation. While Hfq hexamers have the intrinsic propensity to form smaller oligomers that can further phase separate into condensates, polyP facilitates this process by scaffolding Hfq hexamers into larger oligomers, which favor phase separation due to their lower mixing entropy and stronger attractive interactions (161).

2.2.9 H-bodies – Control centers for protein translation and RNA processing

Based on these results, we now reasoned that the proteomic analysis of the gel region encompassing the HMW complexes of Hfq in WT lysates and its comparison to the same gel region of the Δppk lysates might provide an opportunity to characterize the protein composition of Hfq foci without the need of developing techniques to isolate the foci, an often extremely challenging endeavour (162). We thus excised the respective gel regions and conducted comparative tandem mass spectrometry (MS/MS) analysis (Fig. 2.5A). We obtained highly reproducible results and identified 76 proteins which are significantly enriched in the gel region corresponding to the HMW Hfq complexes of N-24 WT lysates compared to the corresponding gel region of the Δppk lysate (Table S2.1, full results on all detected proteins are in Supplementary Dataset 1). Importantly, and, very similar to our results with Hfq and RNase E (see above), we found that the treatment of the WT lysates with yPpx but not RNase A depleted many of these proteins from the HMW region of the native PAGE (Table S2.1), implying that the identified proteins are indeed associated with Hfq through the action of polyP and the observed enrichment hence unlikely caused by relative differences in the expression levels of these proteins in WT *versus* the *ppk* deletion strain. Moreover, among the list of proteins that we found specifically associated with the HMW complexes of Hfq were two proteins which had previously been shown to colocalize with Hfq foci under N-starvation: enolase (Eno) and the RNA helicase RhlB. In an effort to further validate our results, we endogenously mTQ2-tagged Eno or RhlB in Hfq-mCherry expressing WT and *ppk* deletion strains and monitored their N-starvation induced foci formation and co-localization with Hfq. While we observed a mostly diffuse localization of Eno-mTQ2 that made focus calling impossible (Fig. 2.5B), we confirmed that RhlB-mTQ2 foci strongly coincided with Hfq-mCherry in the presence of polyP but not in its absence (Fig. 2.5C). We note, however, that proteins forming higher oligomers in the presence of polyP but not directly interacting with Hfq could also be identified via the gel extraction method described above. To eliminate these, potentially false positive candidates, we co-immunoprecipitated Hfq from N-0 and N-24 WT and Δppk lysates and focused our analysis on those proteins that we identified in our HMW complexes.

Of the 76 proteins that we identified to co-migrate with Hfq in a polyP-dependent manner on native PAGE, 45% were found quantitatively also in these pulldowns (excluding the mCherry only, non-Hfq-tagged control; see Supplementary Data Set 2). This result was despite us only detecting ~8%

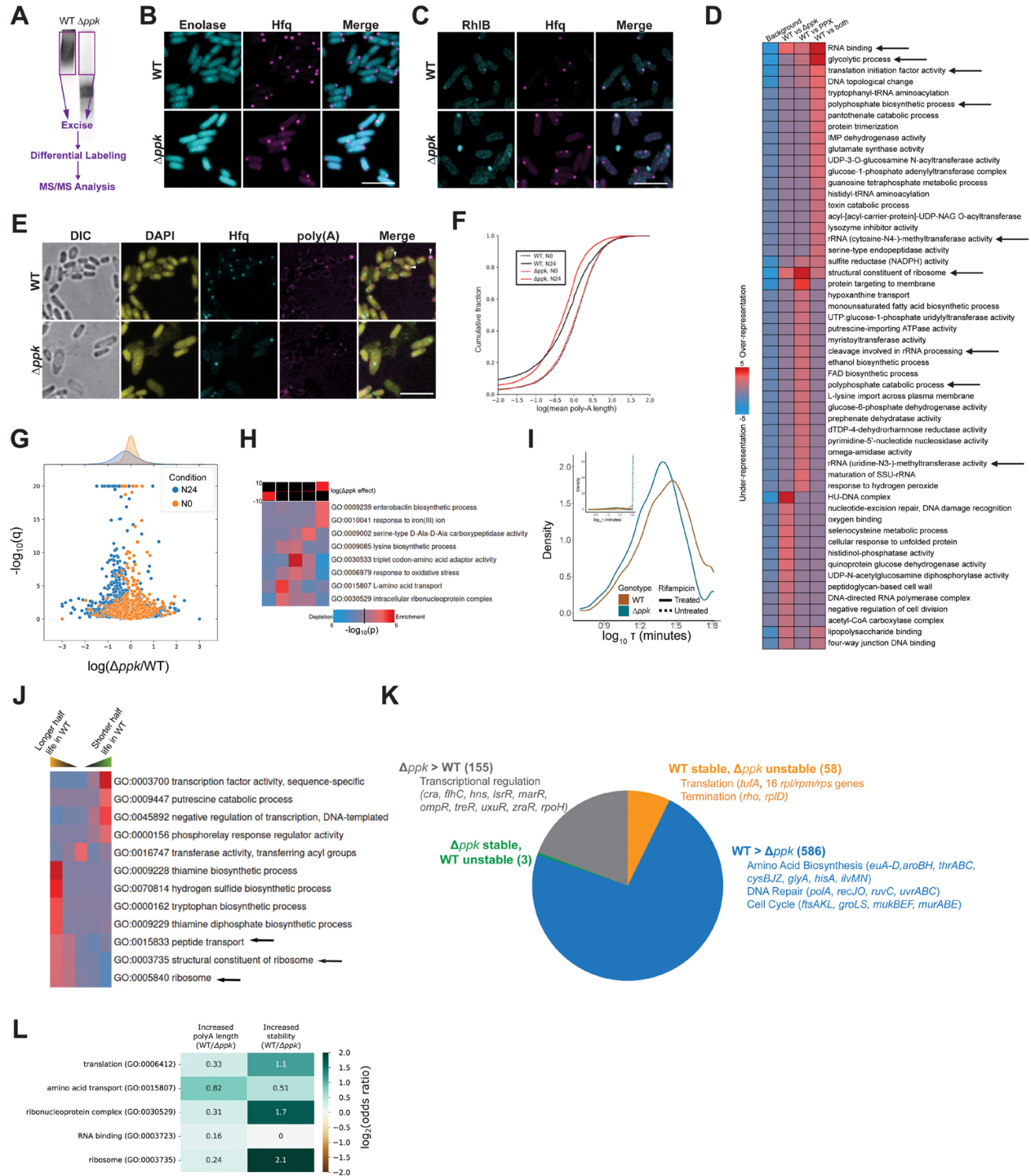


Figure 2.5 Compositional and functional properties of Hfq/polyP-foci.

(A) Diagram showing the workflow of MS/MS on native gel-resolved samples. Excised regions are indicated by purple rectangles. (B) Subcellular localization of Hfq-mCherry and Eno-mTQ2 in N24 WT (MG1655) or Δppk . Scale

bars: 5 μ m. **(C)** Subcellular localization of Hfq-mCherry and RhlB-mTQ2 in N24 WT (MG1655) or Δppk . **(D)** GO term analysis using iPAGE (see Methods for details), indicating GO terms enriched by more than 2-fold in WT vs. Δppk pulldowns, untreated vs. yPpx-treated pulldowns, or both. In each case, red boxes indicate over-representation of genes annotated with a particular GO term in a given group. **(E)** Colocalization of poly(A) RNA and Hfq as shown by fluorescent in situ hybridization against poly(A) RNA in *hfq::hfq-mCherry* WT (MG1655) and Δppk at N24. **(F)** Cumulative distributions of estimated mean untemplated poly(A) lengths across the entire transcriptome in each of the indicated conditions. **(G)** Changes in poly(A) tail length (x-axis) and statistical significance (y-axis) comparing Δppk and WT cells under each of the indicated conditions; the leftward shift in Δppk cells specifically at the N24 time point is clearly apparent. **(H)** GO term enrichments and depletions among the indicated quintiles of changes in poly(A) tail length upon deletion of *ppk*; terms in the lower quintiles show relative decreases in poly(A) tail length upon *ppk* deletion. **(I)** Distributions of the observed transcript half-lives in rifampicin treated *hfq::hfq-mCherry* WT (MG1655) and Δppk . Decay constants outside of the range 5-65 min are clamped for plotting purposes. Inset: Comparison of untreated and treated distributions. **(J)** GO term enrichments and depletions among the indicated quintiles of changes in transcript half-life upon deletion of *ppk*. **(K)** Key GO terms and representative genes enriched in each of four major categories of *ppk* induced stability changes (stable vs. unstable: τ in one genotype >65 min and <55 min in the other; WT > Δppk / Δppk > WT: τ in both genotypes <65 min but τ in one genotype increased by ≥ 10 min) along with representative genes. **(L)** Enrichments of genes in each indicated GO term among genes showing higher poly(A) tail lengths or stability in WT cells relative to Δppk cells in the N-24 condition, shown as log2-scaled odds ratios (small sample method plus Yates' continuity correction, calculated using the epitools R package).

of the proteome in the pulldowns, making it a 5.7-fold enrichment over what we would expect by chance, and hence statistically highly significant ($p < 10^{-15}$, proportion test). These results further increased our confidence that the proteins that co-migrate with Hfq in the native PAGE of WT lysates but not the Δppk lysates (or yPpx-treated WT lysates) are likely proteins that are sequestered into Hfq foci in a polyP-dependent manner and encouraged a deeper analysis of the identified HMW Hfq-associated proteins through gene ontology (GO) enrichment analysis. We found that this set of proteins revealed a strong over-representation for proteins associated with ribonucleoprotein complex assembly, ribosome assembly, and RNA modifications (Fig. 2.5D). Of particular interest were 23 translation-associated proteins that showed a log2-fold enrichment in WT relative to Δppk cells between +1.1 to +6.6 and included all three translation initiation factors (IFs), several ATP-dependent RNA helicases, numerous rRNA modifying enzymes and ribosomal subunits (Table S2.1). Based on these findings, we concluded that polyP affects the protein composition of Hfq foci, which appear to be organizing centers for proteins involved in protein translation, RNA processing and decay.

2.2.10 H-bodies regulate transcript stability during N-starvation

Based on previous reports on Hfq's role in stabilizing poly(A)-mRNAs against degradation by the RNA degradosome (85) and our finding that Hfq foci contain proteins involved in RNA processing, we now wondered whether H-bodies sequester poly(A)-transcripts. To test for the presence of poly(A) transcripts in Hfq foci, we conducted fluorescence *in situ* hybridization (FISH)

experiments in N-24 WT and Δppk strains. Although we observed clear co-localization between poly(A)-transcripts and Hfq foci in about 20% of N-24 WT cells per field of view and no such co-localization in any Δppk cells, the low signal to noise ratio of the polyA probe made quantification of these data challenging (Fig. 2.5E). As an alternative approach, we therefore tracked the lengths of untemplated poly(A) sequences present at the 3' end of transcripts in both WT and Δppk cells at the N-0 and N-24 conditions (see Methods for details). At N-0, absence of *ppk* had no discernible systematic effect on the poly(A) tail lengths of the transcripts. As cells entered deep N-starvation conditions, we observed a general decrease in the mean poly(A) tail lengths which was significantly more pronounced in cells lacking polyP (Fig. 2.5F, G). Given that polyadenylation of *E. coli* mRNA classically leads to reduced transcript stability (163), these results suggested that under N-starvation conditions, H-body formation might aid in sequestering mRNAs with longer poly(A) tails which would otherwise be degraded by the degradosome (164). Of particular interest was the finding that many of the genes with increased poly(A) tail lengths in the WT cells compared to the Δppk cells encode for proteins involved in amino acid uptake and the translational machinery (Fig. 2.5H). These results offered a functional connection to the classes of proteins observed to be enriched in Hfq/polyP-foci (Table S2.1) and suggested the possibility of a concerted regulation and stabilization of those genes by Hfq foci formation at both the transcript and protein levels.

Based on these results, we now wondered whether the observed changes in poly(A) tail lengths might serve to differentially stabilize select mRNA transcripts during N-starvation. To directly monitor transcript stability, we added the transcriptional inhibitor rifampicin and conducted transcriptional shutdown experiments in N-24 WT and Δppk cells over a 60 min time course, using spike-ins for normalization (165) (see Methods for important details on the experimental design and interpretation). In both WT and Δppk cells, we observed clearly normal distributions (Fig. 2.5I; Supplementary Dataset 3), yet with a significant shift toward shorter half-lives for transcripts in the Δppk cells relative to WT (~14% decrease in median half-life; $p < 10^{-15}$, Wilcoxon rank sum test). We calculated that about 33% of all transcripts with identifiable half lives in each condition were substantially less and only about 8% transcripts substantially more stable in N-starved Δppk versus WT bacteria (Fig. S2.4A). Gene set enrichment analysis revealed several categories of genes for which transcript stability was altered by deletion of *ppk* during N-starvation, including systematic stabilization of transcripts encoding genes involved in transcriptional regulation, and

broad destabilization of transcripts encoding genes involved in cell division, amino acid metabolism, and protein translation (Fig. 2.5J). Deeper analysis of specific genes of interest among the transcripts that are highly stable in WT during N-starvation but significantly destabilized in the Δppk bacteria showed a particular enrichment for several components of the translational machinery (i.e., 30S small and large ribosomal subunits) (Fig. 2.5K, S2.4B). These results, and particularly the strong ppk -dependent stabilization of transcripts encoding core translational proteins, suggested that polyP-dependent Hfq foci formation plays a role in preserving transcripts that are necessary to rapidly restart protein synthesis and growth while targeting potentially less-essential transcripts for turnover. It is especially notable that the transcripts encoding Hfq/polyP foci components (as identified by MS on the HMW complexes; see above) showed a median 28% larger increase in half-life (and thus higher stability) in the presence of polyP, relative to other transcripts ($p=5.2 \times 10^{-5}$, Wilcoxon rank sum test), reflecting a concerted preservation of the proteins involved in Hfq foci across all stages of synthesis and degradation. Finally, by cross-referencing our data on poly(A) tail length and transcript stability, we revealed that the transcripts involved in the core polyP-preserved processes such as translation are indeed among the ones with increased poly(A) tail lengths and increased stability in WT compared to the Δppk bacteria (compare Fig. 2.5J and H). Genes contained in the GO terms identified by this analysis generally showed an enrichment for both increased poly(A) tail lengths and increased stability in WT N-24 cells relative to Δppk bacteria under the same condition (Fig. 2.5L). In summary, these results imply that the polyP-dependent sequestration of transcripts into Hfq-foci preserves their poly(A) tails, thereby contributing to their stabilization during N-starvation.

2.2.11 PolyP plays a critical organizational role in eukaryotic P-body formation

At the functional level, we cross-referenced the GO terms with which Hfq/polyP focus associated proteins were annotated, and compared them with the corresponding lists of GO terms for proteins associated with mammalian Processing (P-) bodies, which are involved in regulating RNA turnover, and stress granules (SGs), which sequester the translational apparatus (166). Our analysis revealed that 35% of the Hfq/polyP focus associated GO terms overlapped with SG/P-body GO terms (Fig. 2.6A; Table S2.2) (144). This enrichment was significantly higher than what would be expected by chance ($p = 0.004$, 0.001 , and 0.0004 for the P-body, stress granule, and joint overlaps, respectively, via resampling tests). These results raised the question whether polyP, which is abundantly present also in mammalian cells (167, 168), might be equally involved in eukaryotic

condensate formation. We tested two mammalian cell lines, NIH3T3 (Fig. 2.6B) and HEK293 (Fig. 2.6C) and stained them with the P-body-specific marker protein EDC4 (169) or the SG-specific marker protein G3BP1 (170). As previously noted, both cell lines contain some visible P-

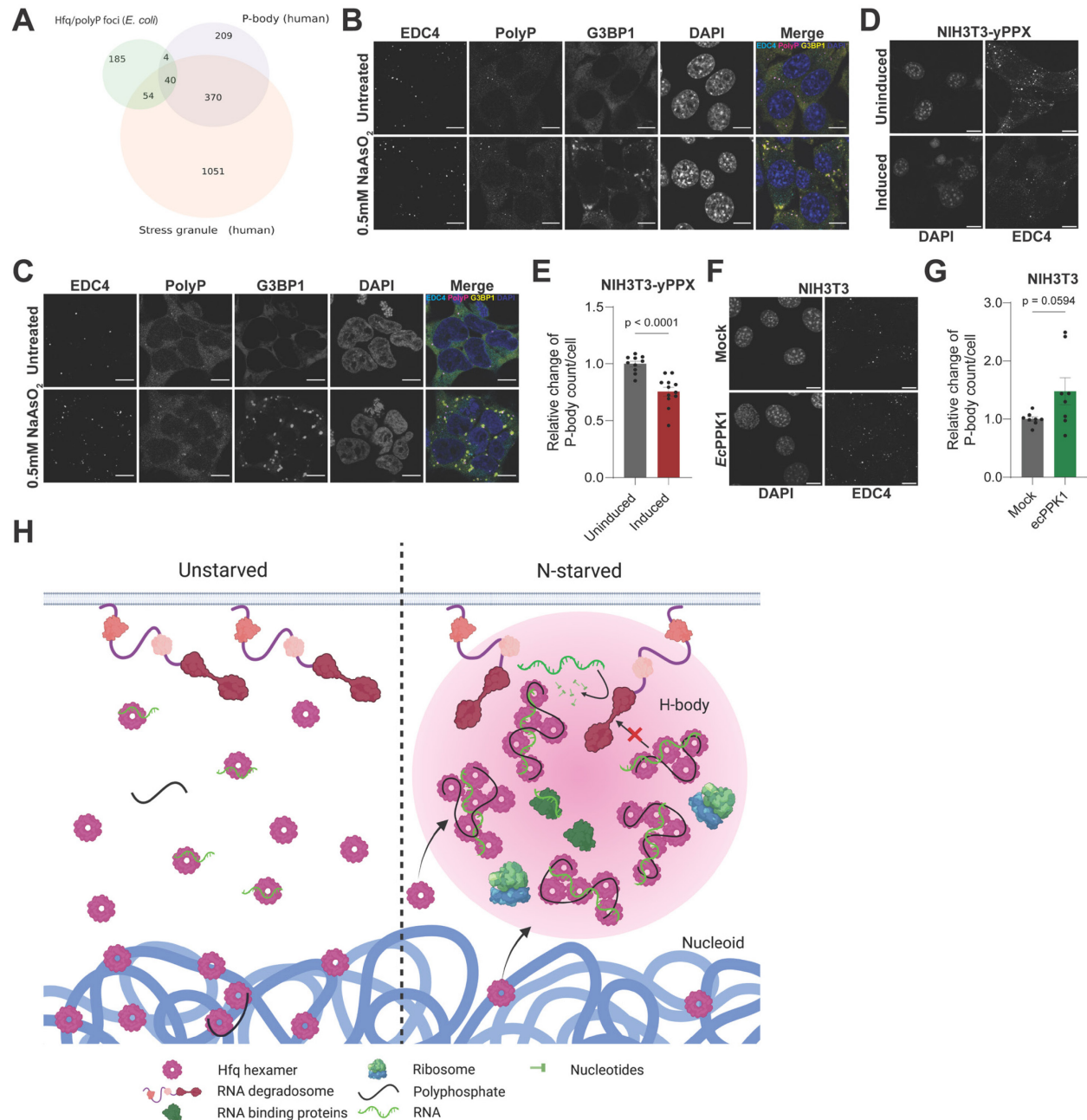


Figure 2.6 PolyP supports P-body formation in mammalian cells.

(A) Euler diagram showing the overlaps of GO terms annotated to proteins identified in the Hfq/polyP foci vs. those in human P-bodies and stress granules. (B) NIH3T3 and (C) HEK293 cells, either untreated or treated with 0.5 mM sodium arsenite (NaAsO₂) for 30 min were fixed and stained for Processing (P)- bodies using antibodies against the marker protein EDC4 (cyan), stress granules (SG) using antibodies against the marker protein G3BP1 (yellow), polyP using the PPXBD-mcherry protein (magenta) and DAPI to visualize the nucleus (blue). A representative experiment is shown ($n = 3$). (D) A stable NIH3T3 cell line expressing an inducible yPpx was fixed after 24h of addition of the inducer shield and stained for the P-body marker EDC4. (E) Quantification of the EDC4 positive punctate structures

shown in (D). The average number of P-bodies counted in the uninduced (D) or untransfected (F) cells was normalized to 1. Image analysis was automated via thresholding to get an unbiased count of P-bodies. An unpaired *t* test was used to assess statistical significance ($n = 3$ biological replicates; data points represent all imaging fields pooled across those experiments). (F) NIH3T3 cells transiently transfected with the *E. coli* Ppk were fixed after 24 h of transfection and stained for the P-body marker EDC4. (G) Quantification of the EDC4 positive punctate structures shown in (F). Each data point represents the average P-body count per cell in an image of at least 20 cells. The average number of P-bodies counted in the uninduced (D) or untransfected (F) cells was normalized to 1. Image analysis was automated via thresholding to get an unbiased count of P-bodies. An unpaired *t* test was used to assess statistical significance ($n = 3$ biological replicates; data points represent all imaging fields pooled across those experiments). Scale bars: 10 μm . (H) Current working model of H-body formation and function. Upon N-starvation, *E. coli* accumulates long-chain polyP, which scaffolds Hfq-hexamers into higher oligomers, giving rise to phase separated condensates. PolyP promotes the selective sequestration of poly-adenylated RNAs and RNA-binding proteins into H-bodies and facilitates their association with the membrane-tethered RNA degradosome. H-body formation confers specific protection of transcripts involved in translation and metabolism while facilitating rapid turnover of transcripts that are likely less critical for survival. Figure panel was created with BioRender, Oleson, B. (2026) <https://BioRender.com/lflmrtp>.

bodies but no distinct stress granules under non-stress conditions (171, 172) and reveal an increase in size and number of both types of condensates upon treatment with arsenite (173, 174). When we co-stained these cells before and after arsenite treatment with the mCherry tagged polyP-binding domain, we found a clear co-localization between polyP and EDC4 but not between polyP and G3BP1 (Figs. 2.6B, C). We did observe some clearly visible polyP-positive P-bodies in close vicinity to stress granules; however, these condensates have been observed before and suggested to be involved in exchanging select proteins and substrates between the different condensates (175, 176). These results strongly suggested that polyP specifically interacts with components of P-bodies but not stress granules. To test whether manipulation of cellular polyP levels affects the formation of P-bodies, we constructed two NIH3T3-based cell lines; we generated stably transfected NIH3T3 cells expressing an inducible version of the yPpx, which significantly decreases cytosolic polyP levels within 24h of induction (Fig. S2.5A, B), or transiently transfected NIH3T3 cells with a vector encoding *E. coli* Ppk, which significantly elevates the levels of polyP compared to non-transfected control cells (Fig. S2.5C, D). Indeed, and similar to our results in *E. coli*, where depletion of polyP decreases and elevated levels of polyP increases Hfq foci formation, we observed a clear relationship between polyP levels and P-body formation in mammalian cells. Cells containing lower levels of polyP showed a significant drop in P-body numbers (Fig. 2.6D, E) whereas cells with higher-than-normal levels of polyP had increased P-body counts compared to non-transfected cells (Fig. 2.6F, G). These results suggest that polyP contributes to, and possibly even also drives, P-body formation in the context of mammalian cells, leading us to conclude that polyP's role in condensate formation has persisted across evolution even if the precise condensates being studied in the systems here may have arisen independently.

2.3 Discussion

After more than a decade of extensive research, biomolecular condensates have been recognized as critical subcellular organizing principles, particularly relevant as rapid response mechanisms to environmental stressors. In the cytosol of mammalian cells, formation of P-bodies and stress granules has been shown to contribute to regulating mRNA turnover, translational repression, and the sequestration of stalled components of the translational machinery during stress, promoting a quick recovery once the stress conditions are over (177). In bacteria, the condensates that have been studied in depth to date seem equally associated with stress response and rapid growth recovery (75, 178, 179). Yet, the common principles of what leads to the sudden phase separation of select proteins under these stress conditions remain unclear. *In vitro* studies demonstrated the strict reliance of condensate formation on the concentration of protein(s) as well as the presence of polyanions, such as RNAs, which promote multivalent interactions, thereby reducing the c_{sat} of the proteins involved (152). However, most known stress conditions that cause protein condensate formation stall *de novo* protein synthesis and do not produce excess levels of RNA (141, 180, 181), raising the question what other stress-responsive mechanisms might be in play that trigger condensate formation. Here we demonstrate that polyP, an ancient and ubiquitous inorganic biopolymer, whose synthesis and degradation are dynamically regulated in response to stress (99, 110), supports the formation and functional regulation of stress-induced condensates in both prokaryotes and eukaryotes (Fig. 3.6H). We discovered that in *E. coli*, the accumulation of long-chain polyP—either naturally produced in response to N-starvation or osmotic stress, or artificially induced by the expression of an “overactive” polyphosphate kinase (i.e., PPK10k) (117) —significantly increases the fraction of bacteria with a single polar-localized and functional Hfq focus, whereas loss of polyP production substantially reduces both the number of cells with Hfq foci, and the sizes/intensities of those foci. We found that polyP appears to play several interdependent roles in both the formation and the function of these condensates: 1) It contributes to the release of nucleoid-bound Hfq, thus increasing the concentration of free Hfq that is available to phase separate; this effect is likely due to the direct competition between polyP and DNA binding to Hfq (150); 2) It scaffolds RNA binding-competent Hfq into higher oligomeric complexes, a feature commonly known to increase phase separating propensities (182); 3) It stabilizes Hfq condensates as reflected by their higher FRAP recovery, higher hexanediol resistance, and longer half-life upon return to non-stress conditions; and 4) It pivots Hfq’s function towards the stabilization of

polyadenylated transcripts essential for translation and cell division while facilitating the decay of those that appear to be less crucial for bacterial survival. These latter findings raise the obvious question as to how polyP might manage this challenging task. The structural similarity between polyP chains and the phosphate-rich backbone of RNA/DNA makes it reasonable to assume that polyP chains interact with one or more of the various nucleic binding surfaces of Hfq (150). In fact, our previous studies, which focused on the role of polyP on nucleoid-associated Hfq, demonstrated that at low polyP concentrations (such as during exponential growth under N-replete conditions), polyP binding to Hfq changes the DNA-binding specificity of Hfq from GC-rich to AT-rich sequences (150). We now posit that at higher polyP concentrations (such as found during N-starvation), polyP binding to other surfaces in Hfq (for instance the DNA binding proximal surface) might similarly alter the RNA binding specificity of Hfq. It is reasonable to assume that polyP binding might compete with low-affinity binding sequences and thus, by default, select for binding to the highest-affinity sequences, such as transcripts with long poly(A) tails. This feature would allow cells to control the sequence specificity of multi-functional nucleic acid binding-proteins simply by changing the relative concentration of polyP. Inevitably, however, detailed structural studies are needed to test this model and dissect the precise mechanisms by which H-body formation leads to the selective binding of polyadenylated transcripts during select stress conditions.

Previous work revealed that polyadenylation of *E. coli* mRNA leads to reduced transcript stability (163), a result that was further supported by very recent reports, which demonstrated that the loss of the *E. coli* poly(A) polymerase PcnB leads to a pervasive transcript stabilization (183). Similarly, transcript stabilization has also been observed upon the interaction of polyadenylated transcripts and Hfq (85), a finding that is largely consistent with our results. Yet, we argue that Hfq likely plays a more nuanced role, especially under stress conditions that lead to the formation of H-bodies. Under these stress conditions, we found that H-bodies preferentially stabilize transcripts with longer poly(A) tail lengths. We furthermore revealed that the transcripts that are enriched for longer poly(A) tails and stabilized in a polyP-dependent manner encode for proteins and cellular pathways that match those enriched in H-bodies at the protein level. Because both Hfq and polyP have been shown to antagonize RNase E-mediated degradation of polyadenylated mRNA *in vitro* (85, 184), we now speculate that polyP-enriched Hfq foci sequester the RNA degradosome in an inactive form, thus serving as a hub for selective mRNA storage. It is important

to note that the polyP-dependent selective sequestration of mRNA transcripts with longer poly(A) tails would also be consistent with N-starvation conditions that might cause a polyP-dependent global increase in polyadenylation. Yet, we consider this possibility unlikely because N-starvation appears to lead to an overall reduction in the average poly(A) tail lengths (Fig. 2.5F). We also wondered whether the activity of PcnB, the enzyme responsible for the formation of poly(A) tails, might be altered. This idea was based on recent results, which showed that PcnB is inhibited at low cellular ATP concentrations (183). However, although we did observe slightly lower ATP concentrations in N-24 *ppk* deletion bacteria compared to N-24 WT *E. coli*, the ATP concentrations of N-starved bacteria did not go below the ATP levels of N+ bacteria. These results make significant differences in PcnB activity due to changing ATP levels also an unlikely explanation for our observations.

Besides knowing how condensates form, another crucial question concerns their specific roles in biological processes. Our results show that the *ppk* deletion mutant, which has twice the levels of Hfq but is defective in the formation of proper Hfq foci, has growth defects that are highly similar to the growth defects of the *hfq* mutant and, even more relevant, not additive when combined with the deletion of *hfq*. Since we cannot fully exclude that the disruption of a major regulator such as *hfq* has effects on various aspects of cellular physiology that could change the needs and/or roles of polyP, suitable separation-of-function mutants of Hfq that bind polyP but do not phase separate and *vice versa* are needed. However, such separation of function mutants may prove impossible to generate, as we suspect that the ability of Hfq to bind polyP and phase separate may be essentially synonymous. Nevertheless, centered on these phenotypical results, we propose that the observed polyP-dependent transcript stabilization and degradosome/translational apparatus sequestration by Hfq foci is the mechanism that promotes improved N-starvation survival. We base this model on several lines of evidence, including our previous results showing that the RNA chaperone activity of Hfq is insensitive to the presence of polyP across a wide range of known client mRNA/sRNA pairs (150), making it unlikely that the beneficial effects of polyP accumulation under N-starvation are connected to altered mRNA/sRNA interactions. Moreover, the observed polyP-mediated dissociation of Hfq from the nucleoid under N-starvation should, if anything, make bacteria more (rather than less) sensitive to stress due to a potential increase in the transcription of prophage genes and mobile genetic elements (150). Finally, the strong consistency of the gene classes that we observe being stabilized at both transcript and protein levels in Hfq foci that contain polyP is

most simply explained by the presence of a system selectively targeting those pathways. Yet, future mechanistic dissections of the relative contributions of different binding surfaces and activities of Hfq to both polyP-dependent phase separation and survival of N-starvation are clearly needed to provide the necessary additional insights into this system and as template for understanding polyP-dependent phase separation in other biological contexts (both in bacteria and in eukaryotic cells).

Finally, our study suggests that polyP might play a potentially equivalent role as modulator for P-bodies, a stress-induced hub for RNA turnover in eukaryotes. We found that H-bodies share many similarities with cytosolic condensates found in many eukaryotic cells: mammalian P-bodies, like H-bodies, form around the Sm-domain containing heptameric protein Lsm1-7, a eukaryotic counterpart of Hfq; accumulate mRNAs with short poly(A) tails; and associate with components of the RNA degradation machinery to regulate turnover (144, 185). Mammalian SGs share with H-bodies the fact that they assemble specifically in response to stress conditions that trigger translation inhibition, and accumulate ribosomal subunits and translation initiation factors, indicative of stalled ribosomes (169, 186, 187). Although considered separate entities, mammalian P-bodies and SGs are often found in close spatial proximity and share select proteins and RNAs (175). Since bacterial H-bodies combine features of both types of condensates on both compositional and functional levels, we propose that they might represent some of the most ancient forms of functional LLPS-driven condensates. Given that polyP concentrations are highly sensitive to environmental conditions that lead to condensate formation, we now speculate that polyP might be the quintessential and, based on its prebiotic origin, the ancestral modulator of condition-dependent protein condensation *in vivo*. While recent studies revealed that select stress conditions induce the synthesis of mammalian polyP as well (159, 188), much still has to be learnt about the system involved in the synthesis and regulation of eukaryotic polyP homeostasis. Nevertheless, our studies suggest that polyP, a simple and ancient biopolymer, potentially carries out a conserved scheme of stress response by triggering and functionally regulating biomolecular condensates in a wide range of organisms.

2.4 Materials and methods

2.4.1 Bacterial strain construction

All strains, plasmids and oligonucleotides used in this study were derived from *E. coli* strain

MG1655 and are listed in Table S2.3. Alleles were deleted or fluorescently labeled via λ Red recombination method (189). Labeled alleles were transduced into various mutant strains using P1 $_{vir}$ bacteriophages from appropriate donors. All plasmids used in this study were constructed with restriction digestion and ligation or Gibson assembly and verified by sequencing.

2.4.2 Bacterial growth conditions

WT and mutant bacteria were grown in Gutnick minimal medium (33.8 mM KH₂PO₄, 77.5 mM K₂HPO₄, 5.74 mM K₂SO₄, 0.41 mM MgSO₄) containing 0.4% (w/v) glucose, 10 mM NH₄Cl and M9 trace elements (N⁺ medium) (190). For N-starvation experiments, overnight cultures in N⁺ medium were inoculated in Gutnick minimal medium containing 0.4% (w/v) glucose, 3 mM NH₄Cl and M9 trace elements. For osmotic stress experiments, cultures at mid-exponential phase in LB medium were spun down, resuspended in equal volume of LB medium containing 1.17 M NaCl, and let grow for 4 hours. Under all conditions, bacteria were grown at 37°C with 200 rpm shaking. During growth in N⁻ medium, the ammonium level in the medium was determined by using the Ammonia/ammonium microplate assay kit following the manufacturer's protocol (#orb545637, Biobyrt). When indicated, kanamycin (100 μ g/ml), ampicillin (200 μ g/ml) and/or chloramphenicol (50 μ g/ml) were added. Optical density of the culture was measured on a UV spectrophotometer. The number of viable cells in the culture were measured as CFU/ml after serial dilutions on LB agar plates and incubation overnight at 37°C.

2.4.3 Fluorescence microscopy and FRAP measurements

Imaging of fluorescently labeled bacteria was performed on a Leica SP8 inverted microscope with 100 $\times$ oil immersion objective, driven by LAS X software (Leica GmbH, Mannheim, Germany). Bacteria were grown under the conditions described above. At the indicated time points, 1 mL of cells were spun down, washed and resuspended in a small volume of Gutnick minimal medium without glucose or NH₄Cl. Cells were loaded onto a cover slip and immobilized on 1% agarose pad made from the same medium (87). For imaging of N⁺ cells, medium with 3 mM NH₄Cl and 0.4% w/v glucose was used for pad preparation. For imaging of the nucleoid, cells were stained with 1 μ g/mL 4',6-diamidino-2-phenylindole (DAPI, #D1306, Thermo Fisher Scientific) before being spun down and washed. For quantification of the foci, a minimum of 100 cells per sample was analyzed. All foci quantifications were performed in a blinded manner with ImageJ. FRAP measurements were performed with the Zoom In mode, using the 405 nm and 587 nm lasers

together at 100% intensity. Three pre-bleach images were acquired, followed by photobleaching and imaging every 5 sec for 115 sec. Fluorescence intensity was measured by LAS X software. Recovery data were plotted and fitted against a one-phase association curve with GraphPad Prism.

2.4.4 Hfq focus area/intensity quantitation

Cells were imaged as described above, acquiring DAPI, mCherry (Hfq), and brightfield channels at each timepoint. Cells were then segmented using omnipose (191) with the `bact_fluor_omni` model applied to the DAPI channel, using a mask threshold of -1 and flow threshold of 0. Each cell mask was then further expanded by four pixels using the `skimage binary_dilation` operator, and a minimal convex hull of the resulting points generated to define the final cell boundaries. A global robust threshold for the pixel-level Hfq intensity needed to constitute an Hfq focus was defined as being at least 3.5 times the median absolute deviation (MAD) above the observed median Hfq signal within cells. We then defined as focal centers all locations that were both local maxima in terms of Hfq signal, and which exceeded the global threshold defined above. Each region exceeding the Hfq threshold was then expanded to encompass all contiguous pixels (using 1-connectivity) that exceeded the threshold and labeled as a focus if it contained at least one potential focus center, and foci containing fewer than three pixels were discarded. We then calculated the areas and intensities of the thus-averaged foci, taking the mean of the indicated quantity across all imaging fields for each replicate separately.

2.4.5 Bacterial immunofluorescence microscopy in fixed cells

N-24 cultures of WT and $\Delta ppk\ hfq::hfq\text{-}mCherry$ strains were cultivated as described. Cells equivalent to 1 ml $OD_{600}=1.5$ were collected and fixed with fresh 4% paraformaldehyde (#1578100, Electron Microscopy Sciences) for 15 min at room temperature followed by 30 min on ice. Cells were spun down and washed three times in phosphate buffered saline (PBS), resuspended in 1 ml GTE buffer (50 mM glucose, 10 mM EDTA pH 8.0, 20 mM Tris-HCl pH 7.5) containing 20 $\mu\text{g}/\text{ml}$ lysozyme, and incubated for 1 min. 20 μl of cells were loaded onto a poly-L-lysine (#P8920, Sigma-Aldrich) coated coverslip (#72230-01, Electron Microscopy Sciences) for 15 min. Coverslips were washed three times with PBS, and dried at room temperature. After rehydrating with PBS, samples were blocked with 2% bovine serum albumin (#A3059, Sigma-Aldrich) in PBS for 20 min. A PPXBD-GFP probe (159) was diluted in blocking solution to 10 $\mu\text{g}/\text{mL}$, and samples were incubated at room temperature overnight. After five washes in PBS,

IRDye 680RD-labeled goat anti-mouse antibody (#926-32210, LI-COR Biosciences) was added in a 1:1,000 dilution and incubated for 2 hours at room temperature. Coverslips were washed three times in PBS, sealed on a glass slide with a drop of antifade reagent (#9071S, Cell Signalling) and imaged as previously described. Four separate imaging fields were imaged and analysed for each genotype, taking the mean Spearman correlation across all cells for each imaging field as a summary statistic for that field. and imaged as previously described. Foci quantification was performed in a blinded manner with ImageJ.

2.4.6 Single-molecule fluorescence microscopy

WT and Δppk Hfq-PAmCherry strains colonies were cultured in LB medium for 6 hours at 37°C and 200 rpm. Subsequently, the cells were diluted 1:400 into N+Gutnick and grown overnight under the same conditions. To induce nitrogen-starvation, the overnight culture was diluted in N-Gutnick to an OD₆₀₀ ~0.02 and grown as above. About 5h after inoculation, the cells entered N-starvation (N-0). Images were taken before (N+), at N-0 and at select timepoints after N-starvation. For imaging preparation, 0.5 ml of cells were centrifuged and resuspended in filtered spent N-Gutnick media. The cells were immobilized on 2% (w/v) agarose:spent N-Gutnick medium pads and imaged at room temperature on a wide-field Olympus IX71 inverted microscope equipped with a 100x 1.40 NA oil immersion objective. Hfq-PAmCherry molecules were photoactivated with 50 to 100-ms pulses of 405-nm laser (Coherent Cube, 405-100; 2 W cm⁻², followed by imaging under excitation by a 561-nm laser (Coherent Sapphire, 561-50; 0.34 kWcm⁻²). Frames were captured at 50 Hz using a 512 × 512-pixel Photometrics Evolve EMCCD camera.

2.4.7 Single-molecule diffusion analyses

The recorded Hfq-PAmCherry movies were subsequently analyzed using the SMALL-LABS algorithm (192) for single-molecule localization and tracking.

To determine the diffusion coefficient of individual trajectories, tracks of at least 7 frames analyzed and their mean-square-displacement to time lag $\tau = 5$ were fit to the diffusion model $MSD = 4D\tau + 4\sigma^2$, where D is the associated apparent diffusion coefficient of the trajectory and σ is the localization uncertainty. Construction of representative PALM images was performed as previously described (193). Briefly, Hfq-PAmCherry localizations with a 95% confidence interval less than 80 nm were used. A Gaussian kernel was applied to each localization, with the degree of blurring (σ) set to the confidence interval of that localization.

To determine the subpopulation of Hfq molecules associated with H-bodies, we classified Hfq-PAmCherry trajectories by their overlap with the H-body foci. To identify H-body foci, all frames for each movie were summed to generate a composite image; regions in this image where Hfq molecules are stably bound form distinct bright foci. The foci present in this image were detected by the Laplacian of Gaussian algorithm ($min_sigma=3$, $max_sigma=5$, $threshold = 0.1$), segmented, and assumed to be H-bodies. Putative foci were filtered to determine if they were indeed stable H-bodies: a focus was kept if it contained at least ten trajectories of at least ten frames each, and where at least 70% of the trajectory overlapped with the focus. The median trajectory length in our dataset is approximately ten frames; therefore, this number was chosen for the minimum threshold of trajectory length in the filtering. To identify the different states of Hfq, single-molecule trajectories were then classified based on their overlap with foci areas. Trajectories were classified as ‘In’ if they completely overlapped with a focus area for their entire duration, ‘In/out’ if they overlapped with a focus for 25% to 99% of their duration, and ‘Out’ if they overlapped for less than 25% of their duration (194). The squared displacement, r^2 , was calculated for consecutive localizations within a trajectory of at least 4 frames ($\tau = 20$ ms). The cumulative probability of the squared displacements in the observation period τ ($P(r^2, \tau)$) was generated from the pool of squared displacements across multiple tracks for each sample replicate by counting the number of squared displacements less than or equal to r^2 normalized by the sample size. The CDF of r^2 was fit to analytical functions describing the diffusive processes with three dynamic states. D_1 , D_2 , and D_3 are the diffusion coefficients for the different states, and α describes the relative fraction between the states.

$$(\text{Three-state}) P(r^2, \tau) = 1 - \left(\alpha_1 e^{\frac{-r^2}{8D_1\tau}} + \alpha_2 e^{\frac{-r^2}{8D_2\tau}} + (1 - \alpha_1 - \alpha_2) e^{\frac{-r^2}{8D_3\tau}} \right)$$

Here, we assumed the D_3 population corresponds to freely diffuse molecules as $D_3 > 1 \mu\text{m}^2/\text{s}$, such that the fraction of freely diffuse molecules is $F_{free} = \alpha_3$. We assumed the two slower populations, D_1 and D_2 , correspond to a combination of nucleoid-associated and within-condensate Hfq molecules. To identify the specific contributions from within-condensate molecules, we first analyzed the trajectories that were classified as “In”. The same diffusion analysis was performed as above but using a two-state model.

$$\text{(Two-state)} P(r^2, \tau) = 1 - \left(\alpha e^{\frac{-r^2}{\frac{8}{3}D_1\tau}} + (1 - \alpha) e^{\frac{-r^2}{\frac{8}{3}D_2\tau}} \right)$$

The resulting D_1 and D_2 were consistent with the D_1 and D_2 from the entire trajectory dataset. Next we fit the combined “In” and “In/out” subsets to a three-state model and used the combined weight fractions $\alpha_1 + \alpha_2$ as the within condensate contribution for this subset as D_3 was consistent with freely diffuse molecules. The total fraction of Hfq in condensates is then defined as:

$$F_{\text{condensate}} = \frac{N_{\text{In}} + N_{\text{In/out}}}{N_{\text{total steps}}} \cdot (\alpha_1 + \alpha_2)$$

Since it is possible for a condensate focus to overlap with the nucleoid, we cannot exclude that some amount of $F_{\text{condensates}}$ is also nucleoid-associated. We defined the fraction of Hfq that is only nucleoid-associated and not within a condensate as:

$$F_{\text{nucleoid}} = 1 - F_{\text{free}} - F_{\text{condensate}}$$

We note that the reported population fractions are percentages of the total Hfq displacements analyzed.

2.4.8 PolyP extraction and detection

PolyP was extracted as previously described (195) with modifications. Briefly, WT and *Appk* bacteria cultured overnight in N+ medium was reinoculated into N- medium and grown at 37 °C. At exponential phase (OD₆₀₀=0.5), N-0, N-3, N-6, N-12, N-18 and N-24, cultures equivalent to 4 mL OD₆₀₀=1.0 were collected, spun down, washed in PBS and resuspended in 350 µL AE buffer (50 mM sodium acetate, 10 mM EDTA). Suspension was sonicated on ice, and 300 µL phenol and 40 µL 10% SDS was added. The mixture was incubated at 65 °C for 5 min, and then on ice for 2 min. 300 µL chloroform was then added, and the mixture was spun down. The upper aqueous phase was mixed with 350 µL chloroform, spun down, and aqueous phase was again collected. After measuring sample volume, 3 times volume of 100% ethanol, 10% volume of 3 M sodium acetate and 5 µL 5mg/mL glycogen (#AM9510, Thermo Fisher) was added. The mixture was vortexed and incubated on ice for 1 hr. DNA, polyP and glycogen was pelleted, washed with 70% ethanol and air dried. Dried samples were dissolved in 500 µL MP buffer (125 mM NaCl, 5 mM MgCl₂, 25 mM Tris-HCl pH 7.5). Per 50 µl sample, 1 µl M-SAN nuclease (#70950-202, ArcticZymes) was added and incubated at 37 °C for 2 hr. To examine polyP on gel, 10 µL sample was mixed with 2 µL 20% glycerol and 2 µL gel loading dye, loaded onto a 4-20% TBE gel

(#EC6225Box, Invitrogen), and ran for 4 hr at 100V. PolyP 300mer and 130mer were loaded as chain length markers. To measure polyP concentration (in phosphate units), 30 μ L M-SAN digested samples were added with 1 μ L *Saccharomyces cerevisiae* exopolyphosphatase (yPpx) and incubated at 37 °C for 2 hr. Potassium phosphate solutions ranging from 0 to 250 μ M was used as standards. In a microplate, 25 μ L of each standard was mixed with 25 μ L MP buffer, 15 μ L 5x yPpx buffer (100 mM Tris-HCl pH 7.5, 25 mM MgCl₂, 250 mM ammonium acetate), 10 μ L water and yPpx. 25 μ L of each sample was mixed with 50 μ L water. Molybdate working solution was prepared by mixing 91.2% total volume of detection base (0.6 mM antimony potassium tartrate, 600 mM H₂SO₄, 2.4 mM ammonium heptamolybdate) and 8.8% total volume of 1 M ascorbic acid. 25 μ L working solution was added to each standard and sample well, rested for 2-5 min, and absorbance at 882 nm was measured.

2.4.9 ATP measurements

About ~1 OD mL culture of MG1655 WT or Δ *appk* was collected at N+ or N-24, boiled at 95°C for 20 minutes and spun down. The CellTiter-Glo 2.0 Assay kit (#G9241, Promega) was used to determine ATP concentration in the supernatant. Briefly, supernatant was mixed with pre-equilibrated CellTiter-Glo 2.0 Reagent at a 1:1 ratio in an opaque walled 96 well plate. Plate was incubated at room temperature for 10 mins to allow the signal to stabilize after which luminescence was measured on an Infinite M1000 (Tecan). To extrapolate ATP concentrations, a standard curve was generated using purified ATP (#R0441, Thermo Scientific) in the same media that the cells were cultured in. ATP concentrations were normalized to protein concentrations in the pellets.

2.4.10 Immunoblotting

Strains of interest were grown to various stages of N-starvation, and cultures equivalent to 1 ml OD₆₀₀=0.6 were collected, spun down, washed and resuspended in 50 μ L SDS-polyacrylamide gel loading buffer, and lysed by incubating at 95 °C for 10 min. Protein samples were then run on a 4-12% NuPAGE Bis-Tris gel (#NP0336BOX, Thermo Fisher) at 175V for 45 min. The gel was then transferred onto a polyvinylidene difluoride membrane (#1620174, Bio-Rad) and blocked with 5% milk in TBST for $\geq$ 1 hour. The membrane was incubated in a dilution of 1:1000 anti-mCherry monoclonal antibody (#M11217, Thermo Fisher) or 1:1000 anti-GFP polyclonal antibody (#632592, Takara Bio) in blocking solution for $\geq$ 1 hour, rinsed five times with TBST, and then incubated in 1:10000 diluted anti-rat or anti-rabbit secondary antibodies labeled with IRDye

680RD or 800CW (#926-68076 and #925-32211, LI-COR Biosciences). The membrane was rinsed three times in TBST, once in TBS, and imaged on a LI-COR Odyssey CLx imager (LI-COR Biosciences). All washes and incubations were done at room temperature. A gel was run in parallel and stained with Coomassie blue as normalization control. Image quantification was performed using ImageJ, and three biological replicates were included in each experiment.

2.4.11 Native western blot

Bacterial cultures equivalent to 40 ml OD₆₀₀=1.0 were collected, spun down, and lysed by sonication in 5 mL native lysis buffer (20 mM Tris-HCl pH 8, 1 mM β -mercaptoethanol, 1 mM EDTA, 2% glycerol and cOmplete protease inhibitor (#5056489001, Sigma-Aldrich)). Samples were centrifuged to remove insoluble debris, and supernatant was mock treated or digested with DNase I (#18068015, Invitrogen), RNase A (#10109169001, Sigma-Aldrich) or yPpx for 2 hours at 37°C before running on a 4-20% TBE gel (#EC6225Box, Invitrogen) at 100V for 16 hours. The gel was incubated in SDS-PAGE running buffer for 20 min and was subjected to western blotting following standard protocols described above.

2.4.12 Fractionation of soluble and insoluble proteins

As previously described (196), cultures equivalent to 4 ml OD₆₀₀=1.0 were collected by centrifugation. The pellet was resuspended in 50 μ L ice-cold lysis buffer (10 mM potassium phosphate pH=6.5, 1 mM EDTA, 20% sucrose, 1 mg/mL lysozyme, 50 u/ml benzonase), incubated for 30 min on ice, and frozen at -80 °C. After thawing on ice, 360 μ L ice-cold buffer A (10 mM potassium phosphate pH=6.5, 1 mM EDTA) was added. The mixed sample was transferred to a 2 mL tube containing 200 μ L 0.5 mm glass beads and was incubated for 30 min at 8 °C with 1,400 rpm shaking. After settling down the beads, 200 μ L lysate was centrifuged at 16,000 $\times$ g for 20 min, and supernatant (soluble proteins) was collected for further use. The pellet (insoluble proteins) was washed with buffer A, buffer B (buffer A containing 2% Nonidet P-40), and again with buffer A. Previously collected supernatant was mixed with ¼ volume of 100% trichloroacetic acid, incubated for 10 min at 4 °C and spun down at 21,000 $\times$ g, and precipitates were washed three times with ice-cold acetone. After washing acetone was removed from samples by heating at 37 °C. Both soluble and insoluble proteins were resuspended in 100 μ L 1 $\times$ reducing SDS buffer (6.5 mM Tris-HCl pH=7.0, 10% glycerol, 2% SDS, 0.05% bromophenol blue and 2.5% β -mercaptoethanol), boiled at 95 °C for 10 min, and wash subjected to further analysis as described above.

2.4.13 Protein purification

As described previously (150), *E. coli* BL21 (DE3) carrying pET-21a-*hfq*, or pET-21a-*hfqS65C* were grown in LB + ampicillin at 37°C to OD₆₀₀ = 0.5. Protein expression was induced with 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). After 16 hours at 22°C, cells were pelleted (5000g, 20 min, 4°C) and resuspended in lysis buffer (25 mM Tris, 300 mM NaCl, DNase I (5 mg/ml), 5 μL of Benzonase nuclease (Merck), 100 μM MgCl₂, and cOmplete (Roche) (pH 7.5)) and lysed by a 3 minutes sonication at 4°C. Lysates were incubated at room temperature for 30 min, boiled in water for 20 min to precipitate most bacterial proteins, and transferred to room temperature water bath for 15 minutes. Lysate was spun at 30000g, 30 min, 4°C, and supernatant was loaded onto two connected 5 mL HisTrap columns (Sigma-Aldrich). The columns were washed with 20 mL of buffer A (300 mM NaCl, 25 mM Tris pH 7.5), 60 mL of 6 M guanidinium hydrochloride in buffer A, a 120 mL gradient from 6 to 0 M guanidinium hydrochloride in buffer A, followed by 60 mL of buffer A. Hfq was eluted with a 70 mL gradient from 15 to 300 mM imidazole in buffer A. The fractions containing Hfq were pooled and incubated for 1 hour with 100 μM MgCl₂ and 4 μL of Benzonase before overnight dialysis against buffer A. After dilution the sample 1:1 into 25 mM Tris pH 7.5, the sample was loaded onto a 5 mL HiTrap SP HP column (Sigma-Aldrich) and washed with 5% buffer B (100 mM NaCl, 25 mM Tris pH 7.5). Hfq was eluted with a gradient from 6 to 40% buffer C (1 M NaCl, 25 mM Tris pH 7.5). Purified samples were pooled, dialyzed against buffer A, and stored at -80°C.

2.4.14 PolyP and Hfq labeling

PolyP-300 was provided by T. Shiba (RegeneTiss, Japan). All chemicals were from Sigma-Aldrich unless stated otherwise. PolyP-300 was labeled with AF647 as previously described (150). Briefly, 37.5 mM polyP-300 was incubated with 1 mg AF647 cadaverine (Life Technologies) and 200 mM 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (Invitrogen) in 100 mM NaCl and 20 mM MOPS pH 8.0 at 45°C for 1 hour before quenching on ice. Free dye was removed using a PD-10 column (GE Healthcare) equilibrated with the same buffer. Flow through was cleaned up using Zeba desalting column (Qiagen). PolyP concentration was determined in 100 μL samples containing the same buffer and 1 μg of DAPI, using excitation and emission wavelengths of 415 nm and 550 nm, respectively. Unlabeled polyP-300 was used as standard. Labeling efficiency (0.1-0.2%) was quantified with AF647 fluorescence in the same samples. HfqS65C was labeled with Cy3 Maleimide Mono-Reactive Dye (Amersham) following the manufacturer's protocol. Briefly,

~1 mg of purified HfqS65C was reduced with 180 μ g Tris-(2-carboxyethyl)phosphine hydrochloride (TCEP) in 100 mM NaCl and 50 mM K_2HPO_4 pH 7.5. Dye was dissolved in 50 μ L of anhydrous dimethyl sulfoxide and added to reduced HfqS65C yielding a final reaction volume of 1 mL. The reaction was incubated for 2 hours at room temperature and then overnight at 4°C. Excess dye was removed by a PD-10 desalting column packed with Sephadex G-25 resin equilibrated with the same buffer. The labeled protein was eluted and concentrated using an Amicon Ultra centrifugal filter with 3-kDa MW cutoff. Protein concentration and labeling efficiency (~30%) were calculated using a molar extinction coefficient of 150,000 $M^{-1}cm^{-1}$ at 552 nm for Cy3 and a correction factor of 0.08 for dye absorbance at 280 nm.

2.4.15 Native gel electrophoresis and electrophoretic mobility shift assay

25 μ M Hfq was incubated with varying concentrations of FAM-rA₃₀ and/or AF647-polyP-300 in 200 mM NaCl and 25 mM Tris pH 7.5 at room temperature. Samples were mixed with glycerol and loading dye and run on a tris-borate EDTA (TBE) 4 to 20% gel (Invitrogen) at 100 V for 4 hours on ice using TBE buffer. RNA and polyP were visualized using a fluorescence gel imager. Hfq was visualized using Coomassie blue stain.

2.4.16 In vitro condensate reconstitution

Purified Hfq (supplemented with 4% Cy3-Hfq S65C) was prepared in 50 mM NaCl and 20 mM HEPES pH 7.0 in the presence or absence of FAM-rA₃₀ and/or AF647-polyP-300. To generate the phase diagram, purified Hfq was prepared in 50 mM NaCl and 20 mM HEPES pH 7.0 in the presence or absence of the indicated concentrations of polyP-300. Concentrations of all biomolecules were indicated in figure legends. 50 μ L sample was mixed and imaged in 16-well CultureWell slides (#112358, Grace Bio-Labs) on the SP8 confocal microscope. Slides were treated with 5% (w/v) pluronic acid (Thermo Fisher) overnight and washed with ddH₂O prior to sample preparation. Quantitative analysis of the phase diagram was conducted using an automated pipeline. The raw images were normalized using the `equalize_adapthist` function of the `python` `skimage` module with CLAHE limits imposed. The normalized image was then blurred with a Gaussian filter, and edges were detected using the `canny` method of the `skimage` feature module. A disk with a radius of 3 pixels was used to close features, and then all enclosed regions with an area of at least 150 px were considered to be candidate condensates. We further imposed a series of circularity constraints on each candidate condensate to filter potential noise: the circularity (ratio

of $4 * \pi * \text{area} / \text{perimeter}^2$) was required to be at least 0.4, the ratio of major to minor axis required to be less than 1.4, the eccentricity required to be less than 0.8, the solidity required to be greater than 0.9, and the extent to be between 0.55 and 0.95 (using skimage-calculated features. We collected both the number and the total area of the condensates in each field of view (with 3-5 imaging fields collected for each condition). We then applied the Otsu thresholding method to the distributions of log-scaled condensate counts and total condensate areas (at the level of ALL imaging fields) to identify thresholds for what constituted 'enough' condensates (according to either of those metrics) to indicate phase separation. For each condition, we then took the median of the condensate counts and areas across the imaging fields and indicated condensates to be present if EITHER (or both) of the count or total area thresholds were exceeded.

2.4.17 Fluorescence in situ hybridization

As described previously (89), N-24 WT and $\Delta ppk hfq::hfq-mCherry$ strains were directly fixed in culture by 4% formaldehyde (#18814, Polysciences) at room temperature for 15 minutes and on ice for 30 minutes. Cells were then washed three times with chilled PBS, and resuspended in GTE buffer (50 mM glucose, 10 mM EDTA pH 8.0, 20 mM Tris-HCl pH 7.5) containing lysozyme and RNase inhibitor. Samples were loaded on poly-L-lysine treated coverslips and briefly incubated, washed three times with chilled PBS, once with chilled 80% methanol and once with chilled acetone. After drying for 5 minutes at 37°C, coverslips were washed once with chilled 50% ethanol and dried again at 37°C. Samples were then washed twice with 2xSSCT (0.3 M NaCl, 30 mM Na citrate, 0.1% Triton X-100), incubated in 2xSSCT + 50% formamide at 37°C for 30 minutes. For each sample, 2 μ L of AF647-labeled probe (IDT) was added to 20 μ L hybridization buffer (3xSSC, 50% formamide, 10% dextran sulfate and RNase inhibitor) and loaded on coverslips. Samples were incubated at 94°C for 2 minutes and then at 55°C overnight under hydrated conditions. Coverslips were washed twice with 2xSSCT + 50% formamide for 30 minutes at 37°C, once with 2xSSCT + 25% formamide for 10 minutes at room temperature, three times with 2xSSCT for 10 minutes, and once with PBS for 10 minutes. Coverslips were treated again with 4% formaldehyde and DAPI for 30 min at room temperature. They were then washed with PBS, 80% methanol and three times again with PBS at room temperature. Coverslips were sealed and imaged as described in previous sections.

2.4.18 Rifampicin treatment, sequencing, and data analysis for RNA stability measurements

Growth and harvest - N-24 WT and Δppk *E. coli* were supplemented with 150 $\mu\text{g/mL}$ rifampicin (#R3501, Sigma-Aldrich) and incubated at 37°C with 200 rpm shaking. Samples were collected before rifampicin addition, or at 10, 30 and 60 min after treatment, and were immediately mixed with RNAProtect Bacteria Reagent (#1018380, Qiagen); for the post-treatment timepoints, a separate untreated control was collected after the same lag time. Cells were pelleted and stored at -80°C. Each sample was also plated for CFUs at the time of harvest in order to provide a normalization factor (used below). Three biological replicates were performed for each genotype/condition combination.

Lysis and RNA purification - On ice, each sample pellet was resuspended in 90 μl of 1X TE (10 mM Tris pH 7.0, 1 mM EDTA pH 8.0), briefly centrifuged to remove liquid from tube walls and transferred to 1.5 ml tube and then transferred to 1.5 ml microfuge tubes. Then 1 μl of Ready-Lyse Lysozyme Solution (LGC Biosearch Technologies) was added to each sample and mixed. Samples were incubated at 30°C for 15 min and returned to ice. Then, 10 μl of Proteinase K (Thermo-Fisher) was added to each sample and mixed. Samples were incubated at 23°C for 15 min, with resuspension every two minutes then returned to ice. ERCC RNA spike-in control mix (Ambion) was diluted 1:25 in water as one batch (8.6 μl of ERCC, 206.4 μl water). Then, 5 μl of the diluted mix was added to each sample, except for Δppk t=60 (no rifampicin) replicate 1, which received 1.6 μl instead of 5 μl . After vortexing the samples, they were clarified by centrifugation (12,000 r.c.f., 30 sec.) and up to 250 μl of clarified lysate was transferred to a new tube. The samples were cleaned using RNA Clean & Concentrator-96 (Zymo Research) per manufacturer's directions for total RNA clean-up and eluted with 25 μl of water per sample. Each sample was transferred to tubes that contained 60 μl of water, 10 μl of 10X Baseline Zero DNase buffer, 5 μl of Baseline Zero DNase (LGC Biosearch Technologies), and 2 μl of RNase Inhibitor, Murine (recombinant, NEB) in each tube. Samples were incubated at 37°C for 30 min. The samples were re-cleaned using RNA Clean & Concentrator-96 (Zymo Research) per manufacturer's directions for total RNA clean-up and eluted with 20 μl water per sample. The concentration of RNA was determined using 5 μl samples and Quantifluor RNA system (Promega) per manufacturer's directions for quantitating RNA in multiwell plates.

Library preparation and sequencing - Appropriate amounts of nuclease-free water were added to 0.1 μg of RNA per sample to achieve 11 μl of starting material for NEBNext rRNA Depletion Kit (Bacterial, NEB). The kit was used per manufacturer's directions to remove rRNA from the

samples. Library preparation was performed using NEBNext Ultra II Directional RNA Library Prep Kit for Illumina (NEB). Samples were prepared per kit directions except that 2.5 μ l of diluted (1:100) NEBNext Unique Dual Index UMI Adaptors DNA Set 1 was used instead of the NEBNext Adaptor for Illumina, and that Omega Mag-Bind TotalPure NGS Beads were used instead of NEBNext Sample beads for post second-strand synthesis, post adapter ligation, and post PCR clean-up steps. Pooled libraries were subjected to sequencing on a NextSeq 2000 instrument.

Data analysis - After initial read preprocessing, alignment and QC using the IPOD-HR processing pipeline (equivalent to that described above for the RNA-seq samples), overlaps of aligned reads to genes were quantified using the `summarizeOverlaps()` function of the R package `GenomicAlignments` (version 1.26.0) running under R 4.0.2, with options “`singleEnd = FALSE`, `inter.feature = FALSE`, `ignore.strand = TRUE`, `mode = "IntersectionStrict"`, `fragments = TRUE`”. A composite reference transcriptome including both all annotated MG1655 transcripts and the transcripts from the ERCC spike-in set was used. Read counts for each feature (gen) were then normalized by the total read count for that sample, and then sequencing depths of genomic features further rescaled by the median abundance of ERCC transcripts in that sample (considering only ERCC transcripts with a relative abundance greater than 5×10^{-4}) in order to provide a relative estimate of per-cell transcript abundance. For each gene, we then fitted an exponential decay equation ($y = a \cdot \exp(-1 \cdot t/b)$, where y is the normalized transcript abundance, a and b are coefficients to be fitted, and t is the time) to the data for each time course, using the `scipy.optimize.curve_fit` function from `scipy` 1.8.0, using the ‘dogbox’ optimizer with both parameters constrained to be non-negative, and a and b initialized to 0.001 and 20, respectively; a represents a baseline abundance (at $t=0$) for each transcript, and b is the exponential decay time constant (in minutes); we then considered for further analysis only those transcripts for which the q-value of the main decay constant was less than 0.1 (indicating a reasonably confident fit). We considered ‘measurable’ decay constants to be those between 5 minutes and 65 minutes.

It is important to note that the half-life distributions observed in our experiments are substantially longer than those that have been previously measured for late exponential/early stationary phase *E. coli* and *Salmonella* (e.g., (197, 198)). There are both biological and methodological reasons likely contributing to these differences. On the biological side, we are targeting an unusual stress condition (long term nitrogen starvation), which has previously not been heavily investigated, and in which cells must surely have become dormant to a much greater extent than the growth

conditions typically used in prior transcription shutdown experiments (and in particular, the cells used in classic experiments were often under periods of rapid physiological change during which rapid transcriptome turnover could well be a benefit to the cells). From a technical standpoint, we deliberately chose to take a more extended series of timepoints (15 minute increments instead of 2 minute increments) in part because we were studying a long-term stress response in truly non-dividing cells; this process may bias us toward confident measurements of long-lived transcript stabilities vs. short-lived transcript stabilities, or might cause us to miss short-timescale decay of transcripts and instead focus on longer term behavior. Nevertheless, we were able to obtain confidently fitted models ($R^2 > 0.5$ and $q < 0.1$) for the transcripts corresponding to more than half of the genes in *E. coli* (2,743) in the RIF-treated conditions, arguing for our ability to make confident and reliable measurements of the decay processes at work in cells under long term nitrogen starvation, and in particular the long-term stability of transcripts under those conditions.

2.4.19 Untemplated poly(A) tail length determination

WT and Δppk Hfq-mCherry strains were streaked on LB agar plates from cryogenic storage and grown at 37 °C. Colonies (four biological replicates per strain) were inoculated into N⁺ Gutnick medium and grown overnight at 37 °C with shaking at 200 rpm. Overnight culture was back-diluted to OD₆₀₀=0.01 in 250 mL N⁻ Gutnick medium. At OD₆₀₀=0.4~0.45 (N⁺) and 24 hours after nitrogen-starvation (N-24), cultures equivalent to 40 ml OD₆₀₀=1.0 were collected and immediately spun down for 3 min at 13,000×g at 4 °C in a fixed-angle rotor. Cell pellets were shock-frozen in a dry ice-ethanol bath, and stored at -80 °C.

Sequencing Library Preparation. RNA extraction and cleaning: To lyse cells, 700 µl of DNA/RNA Shield (Zymo Research, R1100-50) was added to each pellet, cells were resuspended, incubated at room temperature for 5 min, and then frozen solid at -80°C. Upon thawing, lysate was transferred to 2 mL and clarified via centrifugation (12000 r.c.f, 5 min). The soluble fraction was transferred to new tubes. Samples were cleaned using RNA Clean & Concentrator-96 (Zymo Research) per manufacturer's directions for RNA clean-up from samples in DNA/RNA Shield. The cleaned samples were digested with BaselineZero DNase (1X Baseline Zero buffer, 10 µL of Baseline Zero enzyme, 2 µL of Murine RNase Inhibitor (NEB, M0314) for 30 min at 37°C. Samples were cleaned using an RNA Clean & Concentrator-96 (Zymo Research) per manufacturer's directions for total RNA clean-up. The cleaned samples were digested a second time with BaselineZero DNase (1X Baseline Zero buffer, 10 µL Baseline Zero enzyme, 2 µL of

Murine RNase Inhibitor (NEB, M0314)) for 60 min at 37°C. RNA was cleaned by the addition of 1.8X volumes of RNAClean XP beads (Beckman Coulter), mixing, incubating on ice for 15 min, removing the unbound fraction, washing undisturbed beads with 200 µL of 80% ethanol twice, removing the ethanol and allowing evaporation of the residual liquid, followed by elution of RNA in 25 µL of water. Samples were stored at -80°C.

RNA ligation and conversion to dsDNA: For each sample, 1 µg of RNA and water to reach a 40 µL total volume were added to a PCR strip tube. The 5' phosphates were removed from the RNA by rSAP (1X rCutsmart buffer, 3 units of rSAP, (NEB, M0371)); 80 units of murine RNase Inhibitor (NEB, M0314) incubated for 2 hr at 37°C. RNA was cleaned by the addition of 1.8X volumes of RNAClean XP beads (Beckman Coulter), mixing, incubating on ice for 15 min, removing the unbound fraction, washing undisturbed beads with 200 µL of 80% ethanol twice, removing the ethanol and allowing evaporation of the residual liquid, followed by elution of RNA in 7 µL of water. The RNA was transferred to new PCR strip tubes. T4 RNA ligase I (30 units, NEB, M0437M) was used to ligate a 5' phosphorylated, 3'-dideoxy terminated, UMI containing adaptor oligo (100 pmol of 5Phos/TCAAGCAGTAGNNNNNNNNCAGCAGTTCGATAAGC-GG/3ddC, synthesized by IDT) to the 3' ends of the RNA in a buffered reaction (1X T4 RNA Ligase Reaction Buffer, 12.5 % PEG 8000, 1 mM ATP, 40 units of murine RNase Inhibitor, 1/10 volume DMSO) at 16°C for 12-14 hr. The ligation product was cleaned by the addition of 1.8X volumes of RNAClean XP beads, mixing, incubating on ice for 15 min, removing the unbound fraction, washing undisturbed beads with 200 µL of 80% ethanol twice, removing the ethanol and allowing evaporation of the residual liquid, followed by elution of RNA-DNA in 7 µL of water. The RNA-DNA eluate was then transferred to new PCR strip tubes. The RNA of the RNA-DNA was fragmented in buffer (4 µL of 5x Protoscript II buffer, M0368S, NEB; 1 µL of 10mM dNTPs) with a primer complementary to the adaptor oligo (5'CCGCTTATCGAACT-GCTG, 20 pmol per reaction) at 94°C for 6 min prior to reverse transcription. The RNA-DNA was reverse transcribed using ProtoScript II Reverse Transcriptase (200 units of M0368S, NEB) which was mixed into each reaction after the addition of DTT (10mM final) and murine RNase Inhibitor (8 units) to each sample. The reactions were incubated 25°C for 5 min, 42°C for 1 hr, 80°C for 15 minutes, and then 4°C. After samples were placed on ice, 8 µL of NEBNext Second Strand Synthesis Reaction Buffer with dUTP (E7426AA, NEB), 1 µL of random primers (E7422AA, NEB), and 47 µL of water were mixed well into each 20 µL RT reaction. Then 4 µL of NEBNext Second Strand

Synthesis Enzyme (E7425AA, NEB) was mixed into each reaction. Reactions were incubated at 16°C for 1 hr. The dsDNA was cleaned by the addition of 1.8X volumes of Mag-Bind TotalPure NGS beads (Omega Biotek), mixing, incubating 5 min RT, removing the unbound fraction, washing undisturbed beads with 200 µL of 80% ethanol twice, removing the ethanol and allowing evaporation of the residual liquid, followed by elution of the DNA in 50 µL of water.

Library Preparation and sequencing: The DNA was prepared for sequencing using NEBNext Ultra II DNA Library Prep Kit for Illumina (E7645L, NEB) with NEBNext Multiplex Oligos for Illumina Dual Index Primers Set 1 (E7600S, NEB) per manufacturer's protocol (with a 1 to 10 dilution of the standard adapter) except the blue capped USER enzyme (E7428AA, NEB) was used instead of red capped USER enzyme (E7602AVIAL, NEB), Mag-Bind TotalPure NGS beads (Omega Biotek) were used instead of NEBNext Sample Purification Beads, and 14 cycles of amplification were performed at the PCR stage. Pooled reactions were sequenced using NextSeq 1000 Sequencing System (Illumina) using a P2 100 cycle kit with 75 bp read 1, 25 bp read 2, 19 bp index 1, and 8 bp index 2 reads.

Data Analysis. Reads were initially pre-processed and aligned using the same procedures as those noted above for the standard RNA-seq datasets, with the crucial exception that reads were aligned in local, rather than end-to-end, mode. Apart from this, the number of 'T' bases in the read (which, due to the location/orientation of our sequencing adapter, correspond to 3'-end 'A' bases in the original RNA) was taken to be the number of terminal As on that read. For each gene, we then aggregated the distribution of 3'-terminal 'A' counts occurring on reads mapping (in the correct orientation) to any position from 50 bp before to 250 bp after the end of that gene, thus assigning reads occurring in that 3' window to the gene that they are close to the end of. We note that while we could instead have performed this analysis at the level of annotated transcripts/transcriptional units, it appeared likely to us that in many cases we might miss polyA tail assignments due to imperfect knowledge/enumeration of all transcripts produced under the conditions being studied, whereas the primary weakness with our current approach is that we might assign to a single gene a polyA state that also affects other genes in the same operon.

For each gene, we then fitted zero-inflated negative binomial regression models to the distributions of aggregated polyA tail length observations across the 16 experiments performed here (four biological replicates for each of four conditions). We incorporated coefficients contributing to the mean polyA tail length for the possible combinations of *ppk* genotype and nitrogen-starvation

status (four degrees of freedom) and fitted only a single zero inflation rate and overdispersion parameter for each gene. Fits were performed using the python statsmodels package, version 0.13.2, with default parameters except that we allowed a maximum of 500 iterations for coefficient fitting.

2.4.20 GO Term Enrichment Analysis

All GO term enrichment analysis was performed using the iPAGE 1.2b package (199) using default settings except as otherwise noted. The GO term annotation set used was derived from the Uniprot (200) MG1655 reference proteome indexed by b-number for any nucleic acid based analysis, and for all Uniprot proteins associated with *E. coli* K12 (taxon ID 83333) indexed by Uniprot identifier for the mass spectrometry analysis. For continuous inputs (e.g., log2 fold changes), inputs were split into equally populated bins for the mutual information analysis; for discrete inputs, bins were allocated as shown.

2.4.21 Hfq-mCherry immunoprecipitation

Modified protocol of the RFP-Trap Magnetic Agarose beads (#rtma, Proteintech) was used to perform anti-mCherry immunoprecipitation. Briefly, *E. coli* MG1655 *hfq::hfq-mCherry*, WT or *Δppk*, were grown in N- Gutnick medium to log phase or N-24. An mCherry-only control was used to assess non-specific binding. 10-15 ODmL of bacteria was collected by centrifugation for every replicate. The bacteria were lysed in RIPA buffer (10 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.5 mM EDTA, 0.1 % SDS, 1 % Triton X-100, 1 % deoxycholate) with DNase A and DNase A buffer (#E1011, Zymo Research,). 600 μL buffer was used for 10 OD of bacterial pellet. Bacteria were lysed in Omni International Bead Beater Elite bead mill homogenizer using 0.1 mm Zirconia beads. Beads were settled by centrifugation at 3000g for 1 min, and the supernatant was removed in a fresh tube. The lysate was centrifuged at 16,000 g for 15 min at 4°C to remove debris. Supernatant was saved, and BCA assay was performed for protein estimation and input normalization. RFP Trap magnetic agarose beads were prepared by washing 50 μl of beads in 1 mL Dilution buffer (10 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.5 mM EDTA). Buffer was washed away using magnetic separation, followed by the addition of 400 μL of lysate and 600 μL of dilution buffer. Beads were incubated overnight at 4 in a nutator. The next day, beads were washed twice in the wash buffer (10 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.05 % Nonidet P40, 0.5 mM EDTA) by using magnetic separation followed by a wash in the Wash buffer without NP40. 20%

of the beads were aliquoted in a different tube, eluted in 30 μ L 2x SDS buffer (120 mM Tris-HCl pH 6.8, 20 % glycerol, 4 % SDS, 0.04 % bromophenol blue, 10% β -mercaptoethanol). These samples were run on a Biorad 4-20% stain-free TGX gel. Stain-free images were collected after the gel run, and the gel was further processed for anti-mCherry western blotting. The rest of the beads (80%) were separated using magnetic separation and froze at -80 °C before sending them for Mass spectrometry.

2.4.22 Gel excision and mass spectrometric (MS) analysis

For MS analysis of native gel, samples were collected and lysed in the same manner as native western blot. Two 4-20% TBE gels (#EC6225Box, Invitrogen) were run in parallel at 100V for 1.5 hours. One gel was subjected to native western blotting to identify the location of Hfq-mCherry oligomers, while the other gel is stained in warmed Coomassie blue for 5 min, destained overnight, and washed with ddH₂O for 24 hours. Area corresponding to the Hfq-mCherry oligomers was cut out from the WT N-24 lane, while areas of the same migration distance on other lanes were also collected. For analysis of co-IP samples, samples were run on a 4-12% NuPAGE Bis-Tris gel (#NP0336BOX, Thermo Fisher) at 175V for 12 min and stained as above. Gels were diced and washed repeatedly in 25 mM triethylammonium bicarbonate (TEAB, #T7408, Sigma-Aldrich) in 50 v/v % acetonitrile (ACN, #34998, Sigma-Aldrich) followed by 100% ACN. Gel pieces were then incubated in 25 mM TEAB and 10 mM dithiothreitol (#D11000, Research Products International) at 57°C for 1 hour, followed by iodoacetamide (#I6125, Sigma-Aldrich) at room temperature for 45 min. Gel pieces were dehydrated stepwise with 25 mM TEAB, 25 mM TEAB in 50% ACN and 100% ACN. After overnight digestion at 37°C with trypsin (#V5111, Promega), reaction was quenched with formic acid, and supernatant was collected. The remaining gel pieces were washed with 25 mM TEAB followed by 100% ACN, with both supernatants collected and pooled together. Pooled supernatants were dried in speed vacuum with heating and dissolved in 100 mM TEAB supplemented with formaldehyde and sodium cyanoborohydride. For dimethyl labelling, different samples were either labeled with light (CH₂O, #252549, Sigma-Aldrich) or medium (CD₂O, #sc-228228, Santa Cruz Biotechnology) formaldehyde. Corresponding light- and medium-labeled samples were subjected to electrospray ionization mass spectrometry on an Orbitrap Fusion Lumos Mass Spectrometer (Thermo Fisher). Results were obtained by searching against the *E. coli* strain K12 peptide database (Supplementary dataset 1).

2.4.23 GO term comparison with P-bodies/stress granules

We identified human proteins that are components of P-bodies and/or stress granules based on “Gold standard” entries in the RNA granule database (<https://rnagranuledb.lunenfeld.ca/>). GO terms were assigned to the (*E. coli*) H-bodies, (human) P-body, and (human) stress granule proteins based on annotations from UniProt. We then used custom-written python code to identify the sets of GO terms present at the two-way and three-way interfaces of the GO term lists. We assessed significance via a permutation test in which we simulated the null distribution by randomly reassigning the set of *E. coli* K12 proteins assigned to Hfq/polyP foci and calculating the GO term overlaps with P-bodies and/or stress granules for each of 1000 permutations.

2.4.24 Mammalian cell culture, plasmids and transfection

HEK293 cells (ATCC CRL-1573, ATTC, Manassas, VA, USA), HeLa cells (ATCC CCL-2, ATTC, Manassas, VA, USA) and NIH3T3 cells (ATCC CRL-1648, ATTC, Manassas, VA, USA) were grown and maintained in DMEM (Gibco 11995065) supplemented with 10% w/v Fetal Bovine Serum (#F4135, Sigma-Aldrich) and 1% w/v Penicillin-Streptomycin (#SV30010, Cytiva). All cells were cultured in a 37°C incubator at 5% CO₂. EcPPK1 was a gift from Michael Downey (Addgene plasmid # 108850). Transfections were carried out on cells seeded on glass coverslips in a 24-well plate using Lipofectamine LTX Reagent with PLUS Reagent (#15338100, ThermoFisher Scientific) as per the protocol provided on the manufacturer’s website. Cells were transfected for 24 hours prior to treatment with sodium arsenite.

2.4.25 Generation of yPpx expressing stable cell lines

yPpx fused to a 3xFLAG tag on the N-terminus was cloned into the pLVX-pTuner Green vector (# 632176, Takara Bio). The vector contained the coding sequence for an additional N-terminal destabilization domain (DD), which causes the degradation of the protein in the absence of the small molecule Shield1 (#632189, Takara Bio). Lentiviruses were generated with this construct at The Vector Core (University of Michigan Medical School). NIH3T3 cells were transfected with the DD-3x-FLAG-yPpx lentivirus in the presence of polybrene (#TR-1003-G, EMD Millipore) at a concentration of 10µg/ml. Cells were transfected for 24 hours and allowed to recover for 24 hours after removal of transfection media. Positively transfected clones were sorted based on ZsGreen fluorescence with the Cytotflex SRT (Beckman Coulter) using the B525 channel. Sorted cells were grown in the presence or absence of 0.5µM Shield1. The expression of yPpx was

verified by western Blotting using a mouse anti-FLAG M2 antibody (#F3165, Sigma-Aldrich).

2.4.26 Mammalian cell immunofluorescence staining and microscopy

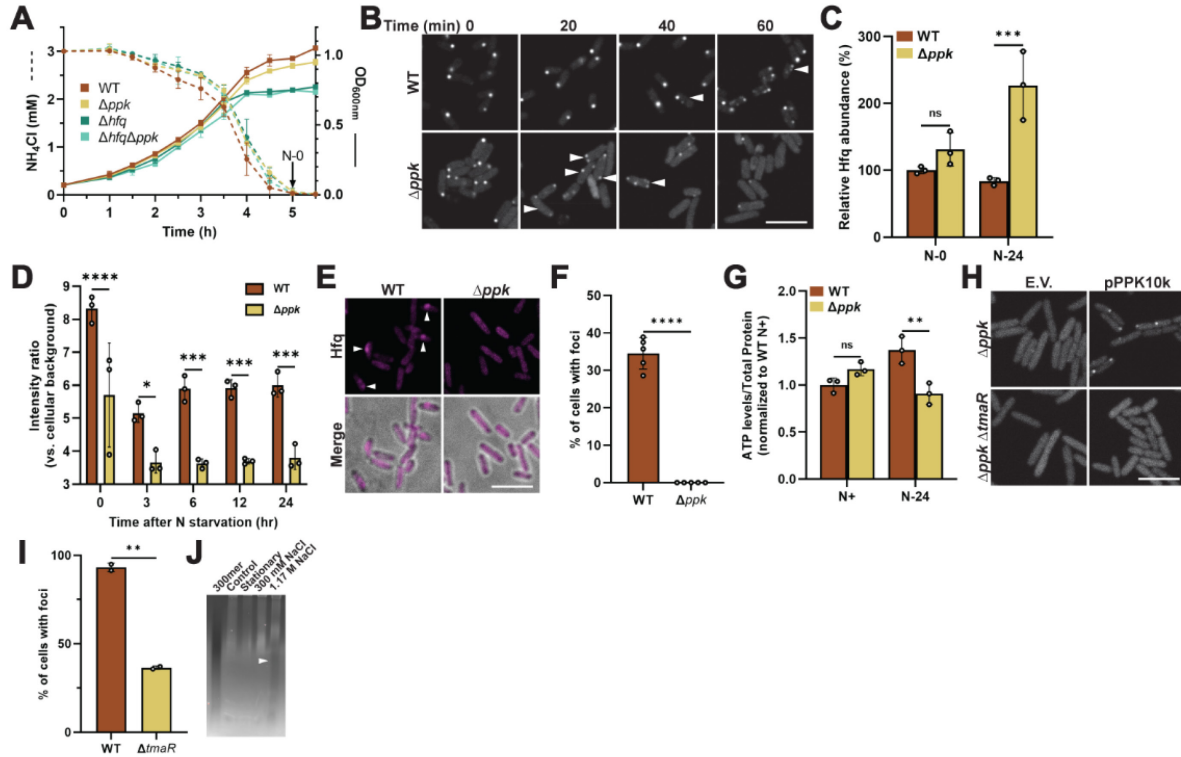
To visualize P-bodies and stress granules via fluorescence microscopy, transfected cells were detached using 0.25% w/v Trypsin-EDTA (#T4049, Sigma-Aldrich) and seeded onto 12 mm circular coverslips (#72230-01, Electron Microscopy Sciences) placed in a 24-well plate at a density of 50,000 cells/well. The cells were treated with buffer or 0.5 mM sodium arsenite (#S7400, Sigma-Aldrich) for 30 mins. Afterwards, the cells were fixed with freshly prepared 4% v/v paraformaldehyde (#1578100, Electron Microscopy Sciences) for 20 mins. Fixed cells were washed 3 times with Phosphate Buffered Saline (PBS) before permeabilization with 0.3% v/v Triton X-100 (#T8787, Sigma-Aldrich) for 1 hour. Triton X-100 was prepared in a solution of 1% w/v BSA (#A3059, Sigma-Aldrich) in PBS. After permeabilization, cells were washed with PBS and incubated in blocking solution (1% w/v BSA in PBS) for 1 hour. To visualize endogenous polyP, cells were incubated with PPXBD-mCherry at a concentration of 10 µg/ml prepared in blocking solution overnight at room temperature. To stain P-bodies, a mouse monoclonal antibody against EDC4 (#sc-374211, Santa Cruz Biotechnology) or a rabbit monoclonal antibody against DCP1A (# NBP2-59785, Novus Biologicals) were used at a concentration of 2 µg/ml. To stain stress granules, a rabbit polyclonal antibody against G3BP1 (#130572AP, Proteintech) was used at 1 µg/ml. Cells were incubated simultaneously with the PPXBD-mCherry probe and the respective primary antibodies. The next day, cells were washed with 1X PBS and incubated with respective secondary antibodies for 2 hours at room temperature protected from light. The secondary antibodies that we used were goat anti-mouse IgG-Alexa Fluor 647 (#A21235, Invitrogen) and goat anti-rabbit IgG-Alexa Fluor 488 (#A11008, Invitrogen). Cells were then washed with 1X PBS and incubated with DAPI (#D1306, Thermo Fisher Scientific) at a concentration of 1 µg/ml for 10 mins to stain the nucleus. Cells were washed 3 times before mounting them onto objective slides using Prolong Gold (#9071S, Cell Signaling Technology) as the mounting medium. Mounted coverslips were sealed with nail polish and imaged 24 hours post mounting. Mounted cells were visualized using a 63x oil objective on a Leica SP8 laser scanning confocal microscope (Leica GmbH, Mannheim Germany) on a DMI8 base using LAS X software.

2.4.27 Statistical analysis

Statistical analyses were performed by GraphPad Prism 10. Details of statistical tests can be found

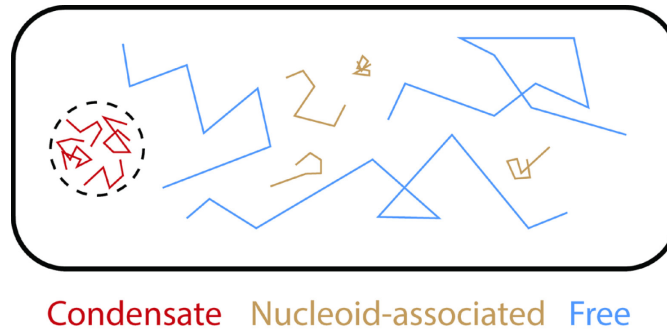
in associated figure legends. *n* stands for biological replicates number in WB/growth, stands for droplet/aggregate/foci number in FRAP data.

2.5 Supplemental information

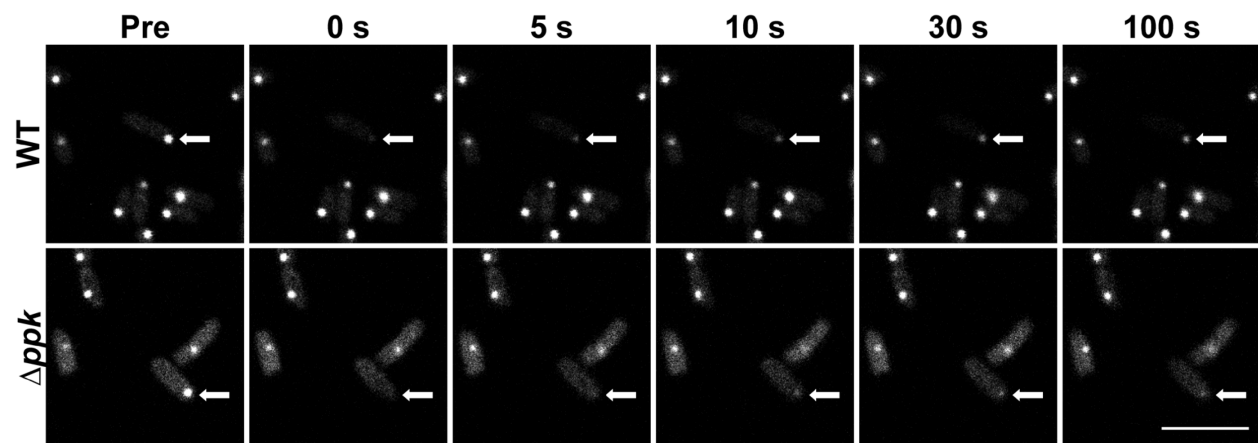


Supplementary Figure 2.1 PolyP-mediated Hfq foci formation in *Escherichia coli* (related to Fig. 2.1).

(A) Growth (solid lines) and nitrogen (N) levels (dashed lines) of WT, Δppk , Δhfq , and $\Delta hfq\Delta ppk$ *E. coli* MG1655 in Gutnick minimal medium supplemented with 3 mM NH_4Cl . Cells were grown at 37 °C with aeration. Entry of N starvation (N0) is indicated. Error bars indicate SD ($n=3$). (B) Subcellular localization of Hfq-mCherry in N24 MG1655 $hfq::hfq\text{-mCherry}$ WT or Δppk at indicated time points after 10% 1,6-hexanediol treatment. Bacteria containing multiple foci are highlighted by arrowheads. (C) Hfq-mCherry levels at N+ or N24 as determined by western blot using mCherry antibody. Total protein was used as loading control. Hfq-mCherry signal in N+ MG1655 $hfq::hfq\text{-mCherry}$ cells was set to 100%. Comparison was made by two-way ANOVA. Error bars indicate SD ($n=3$). *** $p < 0.001$. (D) Ratio of mean Hfq intensities in foci, versus non-focus pixels of the corresponding cells, for nitrogen-starved $hfq::hfq\text{-mCherry}$ cells in the indicated genotypes. Mean values are shown for four imaging fields in each of three biological replicates (points). (E) Immunofluorescence images of fixed N24 WT or Δppk *E. coli* MG1655 $hfq::hfq\text{-3xFLAG}$ using anti-FLAG antibodies for visualization. Hfq foci are indicated by arrowheads. (F) Quantification of bacteria containing Hfq-3xFLAG foci from same experiments as shown in (E). (G) Bacterial ATP levels normalized to total protein in indicated strains and conditions. ATP levels in WT N+ samples were set to 1. Comparisons were made with two-way ANOVA. Error bars indicate SD ($n=3$). ** $p < 0.01$. Comparison was made by unpaired *t* test. Error bars indicate SD ($n=5$). **** $p < 0.0001$. (H) Subcellular localization of Hfq-mCherry in exponentially growing Δppk or $\Delta ppk\Delta tmaR$ *E. coli* $hfq::hfq\text{-mCherry}$ carrying an empty plasmid or a plasmid expressing *ppk10k*. (I) Quantification of bacteria containing Hfq-mCherry foci in indicated strains after 4 h of osmotic stress as in Fig 1L. Scale bars: 5 μm . (J) PolyP extracted from *E. coli* MG1655 $hfq::hfq\text{-mCherry}$ after 4 h of mock treatment (control) or upon deep stationary phase (24h), osmotic stress treatment with 300 mM or 1.17 M NaCl in LB medium was visualized on TBE gel by DAPI staining and photobleaching. PolyP was shown as dark smears (arrowhead). Synthetic 300mer polyP was used as a reference.

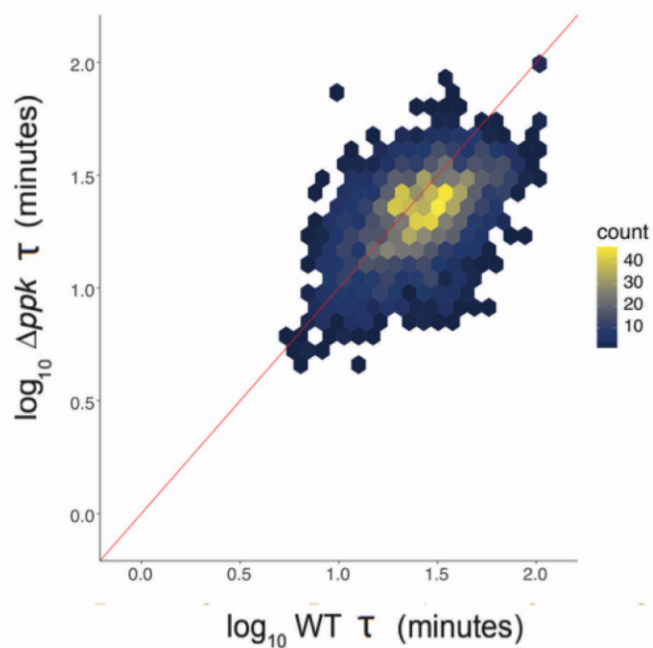


Supplementary Figure 2.2 Schematic of classification of Hfq-PAmCherry trajectories (related to Fig. 2.2A).
Trajectories are assigned into three states by their overlap with Hfq foci (black dashed circle): inclusion in a slow-diffusing condensate (red), a slow-moving nucleoid-associated state (yellow), and a fast-diffusing free state (blue).

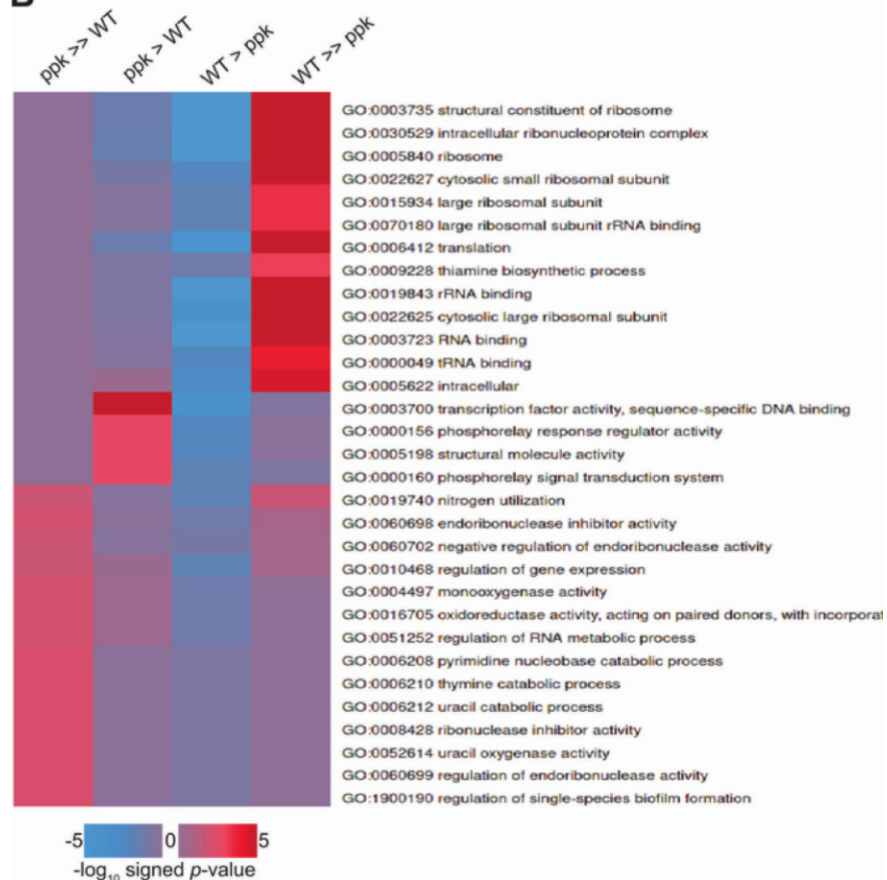


Supplementary Figure 2.3 Representative time course images showing the fluorescence recovery of Hfq-mCherry (related to Fig 2.3D).
Scale bar: 5 μm .

A

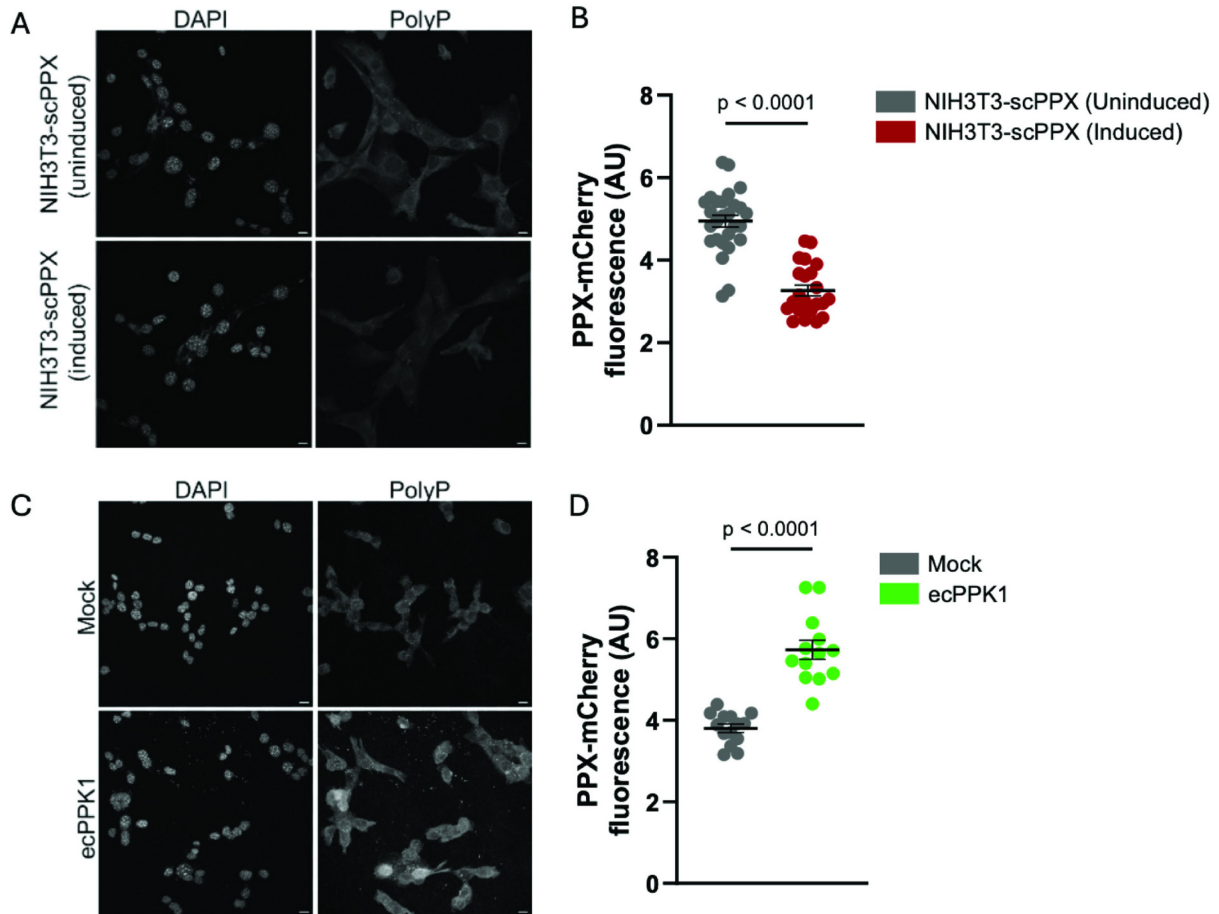


B



Supplementary Figure 2.4 Effects of polyP status on transcript stability in the N24 stress condition (related to Fig. 2.5).

(A) Comparisons of fitted half-lives for transcripts in the WT vs. *ppk* cell; a red line is shown along the main diagonal. (B) Full gene set enrichment analysis (performed using iPAGE software) on transcripts discretized into bins matching the categories in Fig. 2.5K.



Supplementary Figure 2.5 Manipulation of endogenous polyP levels in mammalian cells (related to Fig 2.6).

(A) A stable NIH3T3 cell line expressing a destabilization domain (DD) tagged yPPX was incubated in the absence (uninduced) and presence (induced) of small molecule, Shield1 (0.5 μ M) that allows for the expression of yPPX. The cells were fixed and stained for polyP with PPXBD-mCherry. (B) PolyP levels were quantified based on fluorescence intensity of the PPXBD-mCherry probe. Each data point represents the average mCherry fluorescence intensity per cell (after background correction) in an image of at least 20 cells. (C) NIH3T3 cells were transiently transfected with a bacterial polyP kinase (*EcPPK1*) and were fixed and stained for polyP with PPXBD-mCherry. (D) PolyP levels were quantified based on fluorescence intensity of the PPXBD-mCherry probe. Each data point represents the mCherry fluorescence intensity of one cell (after background correction). An unpaired *t* test was used to assess statistical significance (B, D). A representative set of images and quantification is shown ($n = 3$). Scale bars: 10 μ m.

Supplementary Table 2.1 Proteins enriched in Hfq-HMW complexes (related to Fig. 2.5).

Lysates were prepared from N24 *hfq::hfq*-mCherry WT or the *hfq::hfq*-mCherry Δppk strain and analyzed on native PAGE. In addition, N24 *hfq::hfq*-mCherry WT lysate was treated with either yPPX to degrade polyP or RNaseA to degrade RNA prior to the native PAGE. Corresponding regions of the gels were excised, proteins pairwise differentially labelled (WT v *ppk*; WT v WT + yPPX; WT v WT + RNaseA) and analyzed by MS/MS analysis. Proteins with a log2 fold change (l2fc) >1 in at least 3 out of 5 replicates in WT v Δppk , 2 out of 4 replicates in WT v WT + yPPX or 2 out of 2 in WT v WT + RNase A are shown.

Acc #	Gene	Description	Enriched in WT lysate versus Δppk + yPPX + RNaseA		
C4ZR49	Hfq	RNA-binding protein	X		
P0A7B1	Ppk	Polyphosphate kinase	X	X	
P0AG20	RelA	GTP pyrophosphokinase	X		
B1XAY9	HisS	Histidine--tRNA ligase	X	X	
B1XGY0	InfB	Translation initiation factor IF-2	X	X	X
C4ZYI0	InfC	Translation initiation factor IF-3	X	X	
C4ZZ48	RhlB	ATP-dependent RNA helicase	X	X	
P37765	RluB	Ribosomal large subunit pseudouridine synthase B	X	X	
P33643	RluD	Ribosomal large subunit pseudouridine synthase D	X	X	
C4ZQ93	RmlL	Ribosomal RNA large subunit methyltransferase I	X		
P21338	Rna	Ribonuclease I	X		
B1XDV3	RplL	50S ribosomal protein L9	X	X	
B1X6E6	RplQ	50S ribosomal protein L17	X	X	
C4ZUG4	RplX	50S ribosomal protein L24	X	X	
P0AG51	RpmD	50S ribosomal protein L30	X		
P0A7W1	RpsE	30S ribosomal protein S5	X	X	
P0ADZ4	RpsO	30S ribosomal protein S15	X		
P0A7T3	RpsP	30S ribosomal protein S16	X	X	
P0A7U3	RpsS	30S ribosomal protein S19	X	X	
P60390	RsmH	Ribosomal RNA small subunit methyltransferase H	X	X	
P14081	SelB	Selenocysteine-specific elongation factor	X		
B1XBU0	SmpB	SsrA-binding protein	X		
P00954	TrpS	Tryptophan--tRNA ligase	X	X	
B1XFU9	TyrS	Tyrosine--tRNA ligase	X	X	
P0AGJ5	YfiF	Uncharacterized tRNA/rRNA methyltransferase	X	X	
C4ZX86	Der	GTPase Der	X	X	
P0AEH5	ElaB	Protein ElaB	X		
B1XBZ0	RpoC	DNA-directed RNA polymerase subunit beta'	X		
B1X8V5	CbpA	Curved DNA-binding protein	X	X	
P0A972	CspE	Cold shock-like protein	X	X	
P0AES6	GyrB	DNA gyrase subunit B	X	X	
P0ACF0	HupA	DNA-binding protein HU-alpha	X	X	
P0ACF4	HupB	DNA-binding protein HU-beta	X	X	
C4ZQE5	RvuA	Holliday junction ATP-dependent DNA helicase	X	X	
P06612	TopA	DNA topoisomerase I	X	X	
P0A698	UvrA	UvrABC system protein A	X	X	
P0A6Y8	DnaK	Chaperone protein DnaK	X		
P0A9M0	Lon	Lon protease	X	X	

P0AEU7	Skp	Chaperone protein Skp	X	X	
B1X927	AccD	Acetyl-coenzyme A carboxyl transferase sub B	X		
B1XDI9	Eno	Enolase	X	X	
P0A991	FbaB	Fructose-bisphosphate aldolase class 1	X	X	
P0A9B2	GapA	Glyceraldehyde-3-phosphate dehydrogenase A	X	X	X
P15877	Gcd	Quinoprotein glucose dehydrogenase	X	X	
P0A6V1	GlgC	Glucose-1-phosphate adenylyltransferase	X	X	X
P21599	PykA	Pyruvate kinase II	X	X	
P0AD61	PykF	Pyruvate kinase I	X	X	X
P0ABQ0	CoaBC	Coenzyme A biosynthesis bifunctional protein	X		
P38038	CysJ	Sulfite reductase [NADPH] flavoprotein alpha-comp	X	X	X
P0AA89	DosC	Diguanylate cyclase	X		
P0A6P7	EngB	Probable GTP-binding protein	X	X	
P39180	Flu	Antigen 43	X	X	
P63235	GadC	Glutamate/gamma-aminobutyrate antiporter	X	X	
P0ACC7	GlmU	Bifunctional protein	X	X	
P09831	GltB	Glutamate synthase [NADPH] large chain	X	X	X
P0ADG7	GuaB	Inosine-5'-monophosphate dehydrogenase	X	X	
P06987	HisB	Histidine biosynthesis bifunctional protein HisB	X		
C4ZXA5	IscS	Cysteine desulfurase IscS	X	X	
C4ZRS3	LpxA	Acyl-UDP-N-acetylglucosamine O-acyltransferase	X	X	
P21645	LpxD	UDP-3-O-(3-hydroxymyristoyl)glucosamine N-acyltransferase	X	X	
P45955	CpoB	Cell division coordinator	X	X	
P0AEZ3	MinD	Septum site-determining protein	X	X	
C4ZXV5	MoaC	Cyclic pyranopterin monophosphate synthase	X	X	
P02931	OmpF	Outer membrane porin F	X	X	
P76002	PliG	Inhibitor of g-type lysozyme	X	X	
P23865	Prc	Tail-specific protease	X	X	
P0AGC3	Slt	Soluble lytic murein transglycosylase	X	X	
C4ZRQ6	YaeH	UPF0325 protein	X	X	
P75863	YbcbX	Uncharacterized protein	X	X	
P0A9K3	YbeZ	PhoH-like protein	X	X	
P30177	YbiB	Uncharacterized protein	X	X	
P76177	YdgH	Protein YdgH	X	X	
P0ACY1	YdjA	Putative NAD(P)H nitroreductase	X	X	
P46853	YhhX	Uncharacterized oxidoreductase	X	X	
P76116	YncE	Uncharacterized protein YncE	X	X	

Supplementary Table 2.2 GO-term enrichment analysis of Hfq-foci associated proteins (related to Fig. 2.6A).
Shown are the set of GO terms present in the three-way interface between Hfq-foci, human P-bodies, and human stress granules.

DNA-related
GO:0003677 DNA binding
GO:0003697 single-stranded DNA binding
GO:0006281 DNA repair
GO:0006355 regulation of transcription, DNA-templated
RNA-related
GO:0003723 RNA binding
GO:0003724 RNA helicase activity
GO:0006401 RNA catabolic process
GO:0019843 rRNA binding
GO:0008298 intracellular mRNA localization
GO:1990904 ribonucleoprotein complex
GO:0008143 poly(A) binding
GO:0003676 nucleic acid binding
Translation related
GO:0003743 translation initiation factor activity
GO:0005840 ribosome
GO:0006412 translation
GO:0006417 regulation of translation
GO:0043022 ribosome binding
Stress related
GO:0009410 response to xenobiotic stimulus
GO:0009636 response to toxic substance
GO:0034605 cellular response to heat
GO:0042594 response to starvation
Locations
GO:0005576 extracellular region
GO:0005694 chromosome
GO:0005737 cytoplasm
GO:0005829 cytosol
GO:0016020 membrane
GO:0005886 plasma membrane
Other
GO:0000287 magnesium ion binding
GO:0005524 ATP binding
GO:0007165 signal transduction
GO:0008270 zinc ion binding
GO:0010468 regulation of gene expression
GO:0015031 protein transport
GO:0016301 kinase activity
GO:0016887 ATPase activity
GO:0042802 identical protein binding
GO:0042803 protein homodimerization activity
GO:0046872 metal ion binding
GO:0065003 protein-containing complex assembly
GO:0032991 protein-containing complex

Supplementary Table 2.3 Bacterial strains, cell lines, plasmids and oligonucleotides used in this study.

Bacterial strain		Marker	Source
BL21 (DE3) (<i>F- ompT gal dcm lon hsdSB(rB- mB-) λ(DE3[lacI lacUV5-T7 gene 1 ind1 sam7 nin5]) ykgD:: cat+ </i>)			Lab stock
BL21(DE3) pET21a- <i>hfq</i>		Amp	(150)
BL21(DE3) pET21a- <i>hfqS65C</i>		Amp	(150)
MG1655 (<i>F-, λ-, rph-I ilvG- rfb-50</i>)			(201)
MG1655 Δ <i>ppk</i>			(150)
MG1655 Δ <i>hfq</i>			(150)
MG1655 Δ <i>hfq</i> Δ <i>ppk</i>			(150)
MG1655 Δ <i>ppx</i>			(111)
MG1655 Δ <i>ppk</i> pBAD18b		Amp	(150)
MG1655 Δ <i>ppk</i> pBAD18b <i>ppk</i>		Amp	(150)
MG1655 Δ <i>ppk</i> pBAD18b <i>hfq</i>		Amp	(150)
MG1655 Δ <i>hfq</i> pBAD18b		Amp	(150)
MG1655 Δ <i>hfq</i> pBAD18b <i>ppk</i>		Amp	(150)
MG1655 Δ <i>hfq</i> pBAD18b <i>hfq</i>		Amp	(150)
MG1655 <i>hfq::hfq-PAmcherry</i>			(150)
MG1655 <i>hfq::hfq-PAmcherry</i> Δ <i>ppk</i>			(150)
MG1655 <i>hfq::hfq-mCherry</i>			This study
MG1655 <i>hfq::hfq-mcherry</i> Δ <i>ppk</i>			This study
MG1655 <i>hfq::hfq-mcherry</i> Δ <i>ppx</i>			This study
MG1655 <i>hfq::hfq-3xFLAG</i>			
MG1655 <i>hfq::hfq-3xFLAG</i> Δ <i>ppk</i>			
MG1655 <i>hfq::hfq-mcherry</i> Δ <i>tmaR</i>			This study
MG1655 <i>hfq::hfq-mcherry</i> Δ <i>tmaR</i> Δ <i>ppk</i>			This study
MG1655 <i>hfq::hfq-mcherry</i> Δ <i>tmaR</i> pTmaR-mNeonGreen		Kan	This study
MG1655 <i>hfq::hfq-mcherry</i> Δ <i>tmaR</i> Δ <i>ppk</i> pTmaR-mNeonGreen		Kan	This study
MG1655 Δ <i>hfq</i> Δ <i>tmaR</i> pTmaR-mNeonGreen		Kan	This study
MG1655 Δ <i>hfq</i> Δ <i>ppk</i> Δ <i>tmaR</i> pTmaR-mNeonGreen		Kan	This study
MG1655 <i>hfq::hfq-mCherry rne::rne-mTurquoise2</i>			This study
MG1655 <i>hfq::hfq-mCherry rhlB::rhlB-mTurquoise2</i>			This study
MG1655 <i>hfq::hfq-mCherry pnp::pnp-mTurquoise2</i>			This study
MG1655 <i>hfq::hfq-mCherry eno::eno-mTurquoise2</i>			This study
MG1655 <i>hfq::hfq-mCherry rne::rne-mTurquoise2</i> Δ <i>ppk</i>			This study
MG1655 <i>hfq::hfq-mCherry rhlB::rhlB-mTurquoise2</i> Δ <i>ppk</i>			This study
MG1655 <i>hfq::hfq-mCherry pnp::pnp-mTurquoise2</i> Δ <i>ppk</i>			This study
MG1655 <i>hfq::hfq-mCherry eno::eno-mTurquoise2</i> Δ <i>ppk</i>			This study
MG1655 <i>hfq::hfq-mcherry</i> Δ <i>ppk</i> pBAD18b		Amp	This study
MG1655 <i>hfq::hfq-mcherry</i> Δ <i>ppk</i> pBAD18b <i>ppk</i>		Amp	This study
MG1655 <i>hfq::hfq-mcherry</i> Δ <i>ppk</i> pBAD18b <i>hfq</i>		Amp	This study
MG1655 <i>hfq::hfq-mCherry</i> Δ <i>ppk</i> pWSK129		Kan	This study
MG1655 <i>hfq::hfq-mCherry</i> Δ <i>ppk</i> pPPK		Kan	This study
MG1655 <i>hfq::hfq-mCherry</i> Δ <i>ppk</i> pPPK10k		Kan	This study
MG1655 <i>hfq::hfq-mCherry</i> Δ <i>ppk</i> Δ <i>tmaR</i> pWSK129		Kan	This study
MG1655 <i>hfq::hfq-mCherry</i> Δ <i>ppk</i> Δ <i>tmaR</i> pPPK10k		Kan	This study
Mammalian cell line		Catalog Number	
HEK293		ATCC® CRL-1573™	
HeLa		ATCC® CCL-2™	
NIH3T3		ATCC® CRL-1648™	
Plasmid	Description	Marker	Source
pKD4	Kanamycin resistance cassette donor	Kan	(189)

pKD46	λ red recombinase	Amp	(189)
pCP20	Flp recombinase	Amp	(189)
pET21a	IPTG inducible vector for protein purification	Amp	Novagen
pET21a- <i>hfq</i>	IPTG inducible vector for Hfq purification	Amp	(150)
pET21a- <i>hfqS65C</i>	IPTG inducible vector for Hfq ^{S65C} purification	Amp	(150)
pWSK129	Low copy number vector	Kan	(117)
pPPK	Expression vector for <i>ppk</i> by endogenous <i>ppk</i> promoter based on pWSK129	Kan	(117)
pPPK10k	Expression vector for <i>ppk10k</i> by endogenous <i>ppk</i> promoter based on pWSK129	Kan	(117)
pTmaR-mNeonGreen	Expression vector for <i>tmaR-mNeonGreen</i> by endogenous <i>tmaR</i> promoter based on pWSK129	Kan	This study
pBAD18b	Arabinose-inducible expression vector	Amp	(150)
pBAD18b <i>ppk</i>	Arabinose-inducible expression vector for <i>ppk</i>	Amp	(150)
pBAD18b <i>hfq</i>	Arabinose-inducible expression vector for <i>hfq</i>	Amp	(150)
pcDNA3.1(-)	Mammalian expression vector	Amp	(202)
<i>EcPPK1</i>	Mammalian expression vector for <i>E. coli</i> PPK	Amp	(202)
pLVX-pTuner Green	Mammalian expression vector for DD-tagged proteins	Amp	Takara
DD-3x-FLAG-scPPX	Mammalian expression vector for DD-tagged yeast PPX	Amp	This study
Oligonucleotide	Sequence		
FAM-rA ₃₀	FAM-(rA) ₃₀		
hfq-mFP-in-F	GCAACAGGACAGCGAAGAAACCGAATCGGCTGGCTCCGCTGCTGG		
hfq-mFP-in-R	CCGTGTAAAAAACAGCCCGAAACCCAAGCTTATGAATATCCTCCTTAGTTC		
hfq-mFP-out-F	GTAGCAGCGCGCAGAATACTTCCGCGCAACAGGACAGCGAAGAAACCGAA		
hfq-mFP-out-F	CGGGGAACGCAGGATCGCTGGCTCCCCGTGTAAAAAACAGCCCGAAAC		
hfq-FLAG-in-F	GCAACAGGACAGCGAAGAAACCGAAGACTACAAAGACCATGACGG		
hfq-FLAG-in-R	CCGTGTAAAAAACAGCCCGAAACCCCTCCTTAGTTCCTATTCCG		
hfq-seq-F	ATGGCTAAGGGGCAATCTTTACAAG		
rne-mFP-in-F	TCCTGCGCGTCCGCAACCTGTTGAGTCGGCTGGCTCCGCTGCTGG		
rne-mFP-in-R	CGGCAACACATCATGCCTCTGCCGCTCCTGCGCGTCCGCAAC		
rne-mFP-out-F	GGGCTTGATTACTTTGAGCTAATTACAAGCTTATGAATATCCTCCTTAGTTC		
rne-mFP-out-F	AATAAAAAAGCCCTGGCAGTTACCAGGGCTTGATTACTTTGAGCTAATTAC		
rne-seq-F	TTGAAACGGTAGCTGCGGTC		
rhlB-mFP-in-F	TCCGCGTAATCGTCGTCGTTTCAGGTTTCGGCTGGCTCCGCTGCTGG		
rhlB-mFP-in-R	TGATTTTGAGTATGACATTTTTTATCAAGCTTATGAATATCCTCCTTAGTTC		
rhlB-mFP-out-F	GCAATGGTCCGCGTCGTAAGTGGCGCTCCGCGTAATCGTCGTCGTTTC		
rhlB-mFP-out-F	TCTTGCCATCTTGATACAGTTTGAATGATTTTGAGTATGACATTTTTTATCAA GC		
rhlB-seq-F	ATTGGTCGTACAGGTCGCG		
eno-mFP-in-F	TCGTAAAGAGATCAAAGGCCAGGCATCGGCTGGCTCCGCTGCTGG		
eno-mFP-in-R	ATTTTAAATCAGATAAAGTCAGTCCAAGCTTATGAATATCCTCCTTAGTTC		
eno-mFP-out-F	TGGGCGAAAAAGCACCGTACAACGGTCGTAAAGAGATCAAAGGCCAGG		
eno-mFP-out-F	AAAAAATGCCAGCCCGGAGGCTGGCATTTTTTAAATCAGATAAAGTCAGTCCA AGC		
eno-seq-F	CTGCAATCAAGATGGCGAAAGATG		
mFP-seq-R	GCCCAGTAGCTGACATTCATC		
tmaR-BamHI-220-F	ACACACGATCCTCAGCTTGAGTTGCTATCGAATCTG		
tmaR-R	CTTCGCTTCGCCGTTTTTCATTTTCGC		
tmaR-mFP-in-F	CGAAATGAAAAACGGCGAAGCGAAGTCGGCTGGCTCCGCTGCTG		
mNG-stop-HindIII-R	ACACACAAGCTTTTACTTGTATAATTCGTCCATCCCCATC		
tmaR-mFP-in-R	CAACCCTCATTGAATAAAACGGGAACAAGCTTATGAATATCCTCCTTAGTTC		
tmaR-KO-F	TAAAAGCATTGTCATCTGCGG		
tmaR-KO-R	GCATACAGAAGAAGGCTGCC		

Unprocessed gel images can be obtained at <https://doi.org/10.1371/journal.pbio.3003775.s009>.

Mass spectrometry results can be obtained at <https://doi.org/10.1371/journal.pbio.3003775.s016>.

Sequencing data can be obtained at <https://doi.org/10.1371/journal.pbio.3003775.s018>.

Chapter 3 Intragenomic Homologs with Distinct Phase Behaviors Facilitate Bacterial Stress Response³

3.1 Introduction

A significant fraction of eukaryotic and bacterial genes have homologous sequences within the same genome (201, 203). These intragenomic sequences, commonly known as homologs, are typically acquired through either gene duplication or horizontal gene transfer (204). Their acquisition and maintenance represent evolutionary responses to environmental pressure and have important implications for virulence and antibiotic resistance of bacteria (205, 206). Yet, delineating the respective function of individual homologs is often challenging due to the lack of phenotypes upon the loss of single homologs. Thus, homologs are regularly overlooked as functionally redundant, potentially dismissing their contributions to organismal fitness in non-optimal environments (205).

The *Histone-like Nucleoid Structuring* (H-NS) protein family exemplifies the functional and structural diversity of intragenomic homologs in bacteria. Its prototypic member H-NS is an abundant, nucleoid-associated protein (NAP) widely present in Gram-negative bacteria (207). H-NS functions primarily as a transcriptional silencer by oligomerizing into extended filaments along AT-rich regions of the nucleoid enriched in horizontally acquired genes (48, 50, 208). Accordingly, deletion of *hns* leads to widespread transcriptional de-repression and profound changes in bacterial growth, metabolism and virulence (50, 55, 209, 210). Bacteria harboring H-NS often encode one or more chromosomal and/or plasmid-borne H-NS homologs, yet how they

³ Contents of this chapter are accepted for publication in *Molecular Cell* as Guan, J., Sahoo, B. R., Brant, T. S., Mahadevan, P., Brooks, A., Mosalaganti, S., Bardwell, J. C. A. & Jakob, U. (2026) Intragenomic homologs with distinct phase behaviors broaden the range of cellular stress response. The publisher permits the reproduction of articles by the author for the purpose of an academic thesis. A preprint is available at bioRxiv under the title “Paralogs with distinct phase behaviors broaden the *in vivo* stress response range of condensates.” I performed all experiments in this chapter except cryo-ET, TEM and MD simulation. B.R.S. performed MD simulation. T.S.B. performed cryo-ET analysis. P.M. acquired TEM images, A.B. helped with immunoblotting and strain construction. The study was supervised by S.M., J.C.A.B. and U.J. Recombinant proteins were expressed and purified by Ke Wan from the Bardwell lab. All authors contributed to the research design, data analysis, and writing.

functionally coordinate with H-NS is unclear (211). In *Enterobacteriaceae*, which includes the model bacterium *Escherichia coli*, the most common chromosomally encoded H-NS homolog is the Suppressor of mutant *td* phenotype A (StpA) protein (Fig. S3.1A-C) (212, 213). StpA and H-NS display overlapping DNA binding landscapes (214, 215), consistent with their ability to form heterodimers and mixed filaments *in vivo* and *in vitro* (216-218). Compared with filaments comprised solely of H-NS, mixed H-NS/StpA filaments are more thermostable and show an increased capacity to stall RNA polymerase (217), likely due either to StpA's higher thermal stability, DNA bridging ability and/or DNA binding affinity compared to H-NS (216, 219, 220). Nonetheless, under favorable conditions, cellular StpA levels are kept low through negative autoregulation and the repressive function of H-NS (217, 221). Deletion of *hns* transcriptionally upregulates *stpA*, allowing it to mildly repress part of the H-NS regulon (28, 222). In comparison, deletion of *stpA* has negligible impacts on H-NS genome binding, gene transcription, bacterial growth or virulence (28, 214, 223). For these reasons, StpA has long been considered to serve as a backup of H-NS (222, 224). However, the *hns* deletion mutant is too defective to persist in nature (213, 225), making it unlikely that bacteria would specifically maintain StpA as an evolutionary backup against complete loss of H-NS. Simple backup also fails to explain why StpA is targeted by Lon-mediated proteolysis in the absence of H-NS (218, 226). Rather, these findings suggest that StpA is disadvantageous unless compensated by the presence of H-NS. System-wide studies revealed that StpA's abundance increases dramatically in response to high temperature or hyperosmolarity (227), conditions where its deletion also starts to impair bacterial fitness (228). These results made us wonder whether StpA was evolutionarily selected for its physicochemical properties that confer H-NS-like functions under non-optimal growth conditions, even though StpA might be deleterious under standard growth conditions and hence require tight control. Here, we present *in vitro* and *in vivo* evidence that *E. coli* H-NS and StpA exhibit distinct phase behaviors. H-NS forms fluid, metastable condensates, while StpA precipitates into long, insoluble fibrils. When combined, H-NS and StpA create mixed droplets at a lower saturation concentration (c_{sat}) with enhanced stability against heat and osmotic stress as compared to homotypic H-NS condensates. We show that *E. coli* exposed to heat or osmotic stress increases their StpA to H-NS ratio, preserving the integrity of nucleoid-associated assemblies and repressing the H-NS regulon under stress. Structural investigations reveal differences in flexibility at the D2 oligomerization site accounting for these behaviors. Our findings support organisms modulating the stoichiometry

of homologs with differing phase behaviors to maintain gene regulation and fitness across varying environmental conditions.

3.2 Results

3.2.1 *H-NS and StpA form dynamic subcellular compartments in vivo*

To simultaneously characterize the *in vivo* properties of *E. coli* H-NS and its homolog StpA, we constructed a derivative of the wild type (WT) *E. coli* strain MG1655, expressing both H-NS tagged at the C-terminus with the monomeric fluorescent protein mNeonGreen (H-NS-mNG) as well as StpA tagged with mCherry (StpA-mCh) at their respective endogenous loci. We grew this strain, which showed WT-like growth behavior (Fig. S3.1D) in defined minimal medium, and conducted live cell imaging. Fluorescent signals of both tagged proteins were associated with each other as well as with the nucleoid (Fig. 3.1A and S3.1E, upper panels). However, we noted that in exponentially growing bacteria, H-NS-mNG and StpA-mCh signals were not evenly distributed along the nucleoid but found enriched in compartments $< 1 \mu\text{m}$ in spatial dimensions, which underwent dynamic structural rearrangements (Fig. S3.1F). Fluorescence recovery after photobleaching (FRAP) experiments on these H-NS/StpA-enriched domains revealed significant fluorescence recovery, suggesting that both proteins form a dynamically exchangeable sub-nucleoid compartment, indicative of *in vivo* biomolecular condensates (Fig. S3.1G-H). These results were in line with recent reports showing that H-NS phase-separates with DNA *in vitro* (229). In late stationary phase, H-NS-mNG and StpA-mCh form even more distinct and colocalized foci that align with the longitudinal axis of the bacteria (Fig. 3.1A and S3.1E, lower panels). Both NAPs were predominantly soluble in stationary bacteria (Fig. S3.1I), indicating that these foci are not aggregates. Consistent with the liquid-like nature of these foci, we observe fusion and fission events among foci formed during log phase and in stationary bacteria. (Fig. S3.1J). FRAP experiments supported the dynamic nature of these foci by showing that the fluorescence for both proteins recovered (Fig. 3.1B-C). Of note, the fluorescence of H-NS-mNG recovered to a significantly higher extent than the fluorescence of StpA-mCh, suggestive of a potentially homolog-specific difference in dynamic diffusivity. A swap of the fluorescent protein tags between the homologs did change in their respective dynamic behaviors, indicating that the differences are not tag-specific (Fig. S3.1K-L). Upon recovery from stationary phase, H-NS-mNG and StpA-mCh simultaneously dispersed from the foci, eventually revealing the diffuse pattern observed during

exponential growth (Fig. 3.1D). Distinct H-NS/StpA foci were also observed upon cultivation of the strains in rich medium (Fig. S3.1M) and detected when H-NS was fused to validated monomeric fluorescent proteins (e.g., mTurquoise2, mBaoJin (230-232)) (Fig. S3.1N) or the much smaller 3xFLAG tag (Fig. S3.1O). Stationary-phase strains expressing H-NS-mNG in concert with mCherry-tagged fusions of other known oligomerization-competent NAPs demonstrated that these unrelated fusion proteins either did not form distinct foci (i.e., HupA-mCh, IhfA-mCh, Lrp-mCh, Fis-mCh) or formed puncta that did not colocalize with H-NS-mNG (i.e., Dps-mCh, Hfq-mCh) (Fig. S3.1P). Collectively, our data provide evidence that *E. coli* homologs H-NS and StpA form co-condensate-like sub-nucleoid compartments, in which they display distinct dynamic properties.

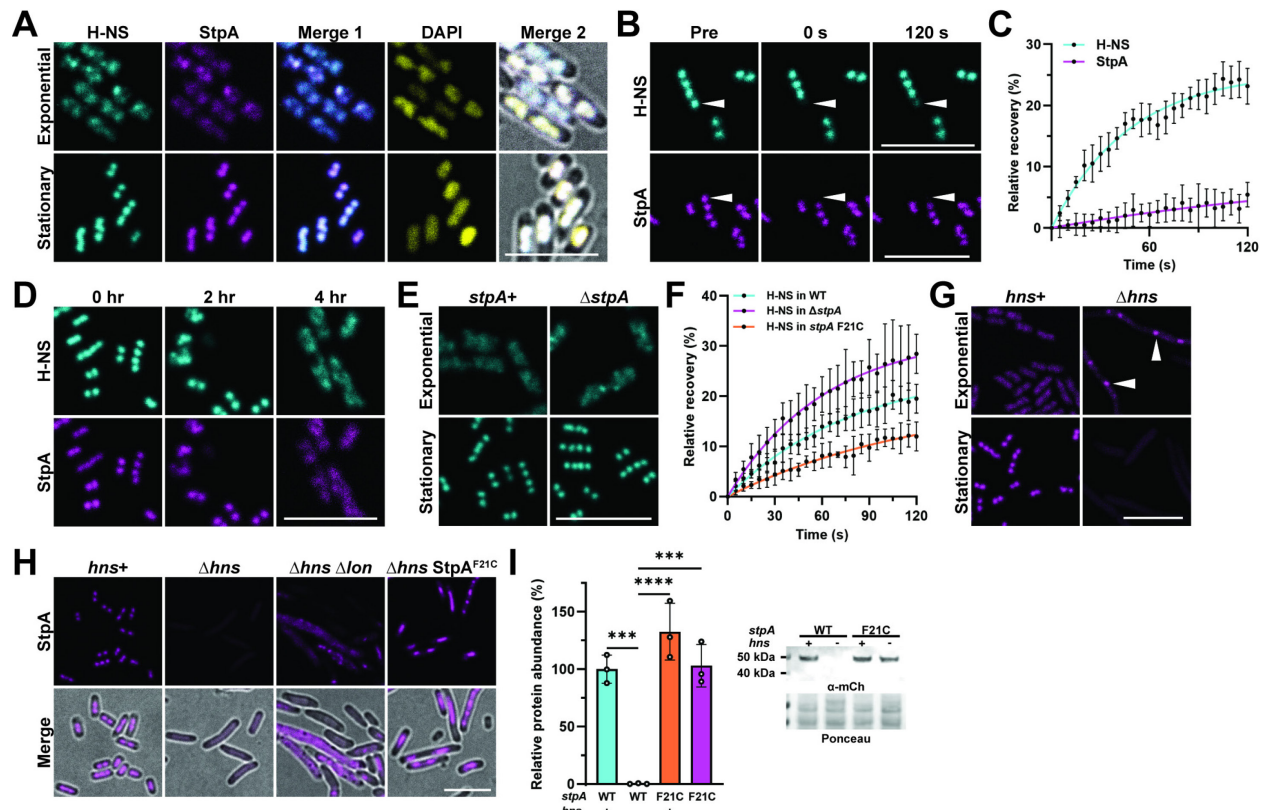


Figure 3.1 H-NS and StpA form dynamic compartments in *E. coli*.

E. coli MG1655 endogenously expressing tagged proteins were grown in Gutnick+ minimal medium at 37°C. **(A)** Fluorescence and brightfield (BF) images of the H-NS-mNG and StpA-mCh expressing *E. coli* in exponential or late stationary phase. Nucleoids are stained with DAPI. Merge 1: overlay of mNG/mCh channels. Merge 2: overlay of all channels. **(B)** Representative images of fluorescence recovery after photobleaching (FRAP) experiments on H-NS-mNG or StpA-mCh puncta in stationary phase cells. Frames depict puncta (arrowheads indicate photobleached area) before (pre) or immediately upon (0 s) or 120 s after photobleaching. **(C)** FRAP quantification of (B) (mean, SD, $n = 8$). **(D)** Fluorescence images of the same strains before, 2 hr or 4 hr after dilution into fresh medium. **(E)** Fluorescence images of *stpA*⁺ or Δ *stpA* *E. coli* expressing H-NS-mNG in exponential or stationary phase. **(F)** FRAP quantification of H-NS-mNG foci in *stpA*⁺, Δ *stpA* or *stpA*^{F21C} strains (mean, SD, $n = 6-8$). **(G)** Fluorescence images StpA-mCh in *hns*⁺ or Δ *hns* *E. coli* in exponential or stationary phase. StpA-mCh puncta are marked by arrowheads. **(H)** Fluorescence and BF images of StpA-mCh or StpA^{F21C}-mCh in indicated backgrounds during stationary phase. **(I)** Abundance of StpA-mCh or StpA^{F21C}-mCh in *hns*⁺ or Δ *hns* strains as quantified by western blotting using antibodies against

mCherry. Total protein was used as a loading control ($n = 3$, SD, one-way ANOVA, *** $p < 0.001$, **** $p < 0.0001$). Scale bars: 5 μm .

3.2.2 H-NS and StpA affect each other's dynamic *in vivo* behaviors

To investigate whether H-NS and StpA differ in their individual dynamic behavior *in vivo*, we endogenously expressed H-NS-mNG in an *E. coli* ΔstpA strain and, conversely, StpA-mCh in a Δhns strain. We did not detect major differences in the spatial distribution of H-NS-mNG in exponential or stationary phase ΔstpA strain as compared to the strain with wild-type StpA expression (Fig. 3.1E). However, similar-sized H-NS-mNG foci formed in stationary phase displayed increased relative FRAP recovery rate in the ΔstpA strain (Fig. 3.1F, compare cyan and magenta traces). These results suggested that while formation of *in vivo* H-NS-mNG foci occurs independently of StpA-mCh, co-presence of the homolog within the foci reduces the diffusivity of H-NS-mNG. In contrast to H-NS-mNG, the dynamic behavior of StpA-mCh was strongly dependent on H-NS. In exponentially growing Δhns bacteria, a large fraction of StpA-mCh was present as discrete foci that showed no significant recovery upon photobleaching (Fig. 3.1G and S3.1G-H). Solubility fractionation confirmed that most of StpA-mCh is indeed insoluble (Fig. S3.1Q). The same behavior was observed when we labeled StpA with mNG (Fig. S3.1R-S). In stationary Δhns cells, StpA-mCh was barely detectable and no focal enrichment of StpA-mCh was observed (Figs. 3.1G-I). Consistent with previous studies, we found that the StpA-mCh levels could be restored under stationary phase upon deleting Lon protease or introducing the F21C (StpA^{F21C}-mCh) mutation, which prevents Lon-mediated degradation of StpA (226) (Fig. 3.1H-I). Yet, despite restoration in protein abundance, StpA still failed to assemble into WT-like puncta in either strain (Fig. 3.1H). Together, our results suggested that H-NS is important for maintaining StpA's stability, dynamic behavior and subcellular localization.

In the presence of H-NS-mNG, the StpA^{F21C}-mCh level exceeded that of StpA-mCh in WT cells, and both proteins co-localized in WT-like foci (Fig. S3.1T). Yet, the diffusivity of H-NS-mNG in these foci decreased compared to WT bacteria (Fig. 3.1F, orange trace). The observation that the fluidity of H-NS-mNG decreases at higher StpA^{F21C} to H-NS ratios (Fig. S3.1U) and increases when StpA is absent (Fig. 3.1F) suggested that the cellular StpA to H-NS ratio regulates the dynamic diffusivity of H-NS. Together, these results demonstrated that despite their high sequence homology and structural similarity, H-NS and StpA differ in their *in vivo* phase behaviors and appear to reciprocally affect each other's *in vivo* properties. Whereas H-NS forms dynamic compartments on its own but is affected in its dynamic diffusivity by StpA, StpA appears poorly

soluble on its own and requires H-NS for stability, diffusivity and localization.

3.2.3 H-NS and StpA display highly distinct phase behaviors *in vitro*

To investigate why H-NS and StpA behave so differently *in vivo*, we purified both proteins and investigated their *in vitro* phase behaviors under physiologically relevant buffer conditions (153). For visualization and for FRAP experiments, we supplemented H-NS with 1-4% of Cy3-labeled H-NS and StpA with 1-4% of Cy5-labeled StpA^{F21C}. Because supplementation with the labeled proteins did not detectably affect the phase behavior of unlabeled proteins, we used labeled and unlabeled protein preparations interchangeably. We found that upon dilution from high salt storage buffers into phase separation buffer, H-NS readily assembles into spherical condensate-like droplets (Fig. 3.2A, upper panel) whereas StpA precipitates in form of seemingly amorphous aggregates (Fig. 3.2A, lower panel). Co-incubation of H-NS with FAM-labeled 150 bp DNA amplified from an endogenous H-NS binding site (48) resulted in H-NS/DNA co-condensates (Fig. 3.2B, upper panel) and a reduction in H-NS c_{sat} from 25 μM to 5 μM (Fig. 3.2C and S3.2A). Unexpectedly, we found that FAM-DNA also colocalizes with amorphous StpA aggregates (Fig. 3.2B, lower panel), suggesting that the precipitated StpA might retain DNA-binding ability. Unlike with H-NS, however, addition of DNA did not detectably affect StpA's aggregation concentration threshold (Fig. S3.2B). FRAP analysis of homotypic H-NS condensates revealed that Cy3-H-NS fluorescence rapidly recovered upon both partial-droplet (Fig. 3.2D) and full-droplet (Fig. S3.2C) photobleaching and seemed unaffected by DNA (Fig. 3.2E and S3.2D). No significant recovery was detected in the case of StpA, independent of DNA (Fig. 3.2D and F). To explore the structural properties of these assemblies in more detail, we performed cryo-electron tomography (cryo-ET) on H-NS condensates and the StpA aggregates. Initial medium-magnification montages of the frozen-hydrated H-NS and StpA samples revealed rounded, droplet-like morphology for the H-NS samples and a non-uniform, aggregate-like morphology for the pure StpA sample (Fig. S3.2E). Cryo-ET analysis revealed an interconnected mesh of densities in H-NS condensates and no obvious change in the coarse morphologies when DNA was added (Fig. 3.2G). In contrast, StpA aggregates revealed an entirely distinct cryo-ET morphology, with aggregates being composed of highly repetitive stacked densities (Fig. 3.2G). Transmission Electron Microscopy (TEM) analysis of negative stained StpA assemblies at a lower concentration revealed less packed linear structures, which, as was the case with H-NS, did not change visibly upon addition of DNA (Fig. 3.2H-I). Of note, no such fibrils were observed in the TEM for H-NS (Fig. S3.2F). Taken together, these *in*

in vitro data supported our *in vivo* findings, which indicated that on their own, the two closely related homologs H-NS and StpA differ drastically in their solubility, phase behavior and overall morphology.

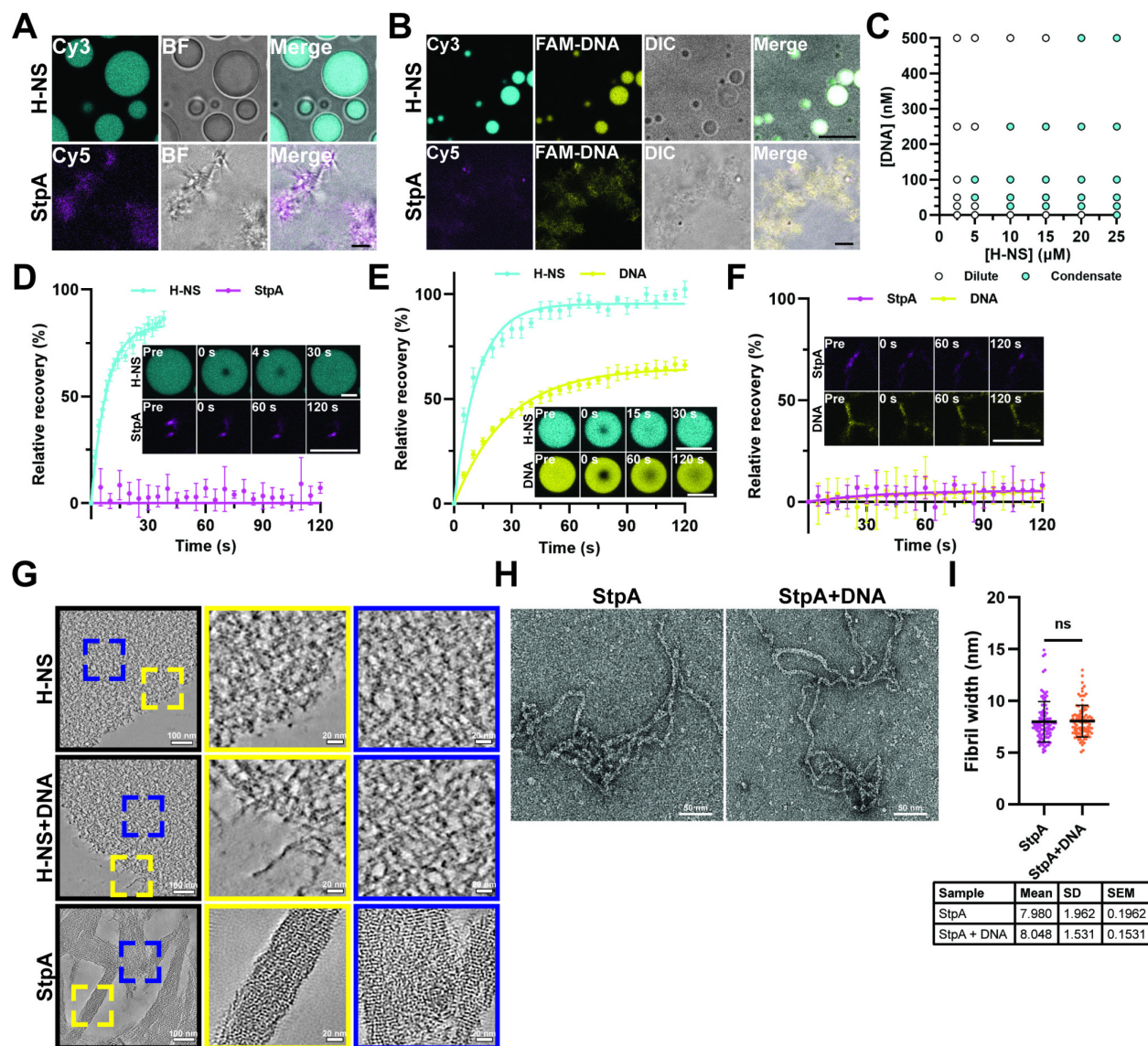


Figure 3.2 H-NS and StpA differ in their *in vitro* phase behavior.

(A) Fluorescence and BF images of 100 μM purified H-NS (+ 1% Cy3-H-NS) or StpA (+ 1% Cy5-StpA^{F21C}) upon dilution from high salt buffer into GMT-buffer (100 mM Na-Glu, 1 mM MgAc, 20 mM Tris-Ac, pH 7.5). Residual NaCl concentration was 30 mM (H-NS) or 50 mM (StpA), respectively. Images were taken after 20 minutes incubation at RT unless otherwise indicated. (B) Same as in (A) except that 25 μM H-NS (+ 4% Cy3-H-NS) or 25 μM StpA (+ 4% Cy5-StpA^{F21C}) in the presence of 100 nM FAM-labeled 150 bp DNA was used. (C) Phase diagram of H-NS and 150 bp DNA in GMT-buffer based on microscopy (see Fig. S2A). (D) Quantification of partial FRAP analysis of H-NS droplets or StpA precipitates shown in (A); mean, SD, $n = 4-6$. **Inset:** Representative images of FRAP time course. (E) and (F) Partial FRAP quantification of H-NS/DNA droplets (E) or StpA/DNA (F) precipitates shown in (B) (mean, SD, $n = 4$). **Inset:** Representative images of FRAP time course. (G) Slice through the reconstructed tomogram of condensates composed of 50 μM H-NS, 25 μM H-NS with 100 nM DNA or 50 μM StpA. Inset in the left panel are zoomed in on the right. (H) TEM image of insoluble 2.5 μM StpA precipitates in absence or presence of 5 nM DNA.

(I) Analysis of the mean width of StpA fibrils shown in (H) (t test, $n = 100$). Scale bars: 5 μm , unless otherwise indicated.

3.2.4 H-NS converts insoluble StpA fibrils into liquid-like droplets

Our *in vivo* results revealed strong dependence of StpA solubility on the presence of H-NS. To investigate whether H-NS affects also the *in vitro* solubility of StpA, we incubated pre-formed insoluble StpA fibrils with either H-NS only or DNA followed by H-NS. Surprisingly, in either case we observed immediate formation of mixed precipitates followed by slow conversion into droplets (Fig. 3.3A-B; S3.3A-B). Once the droplets formed, both H-NS and StpA displayed significant diffusivity (Figs. 3.3C and S3.3C), suggesting that the droplets formed are liquid-like. Moreover, and as shown *in vivo*, diffusivity of StpA remained significantly lower than H-NS's. The diffusivity of DNA in the condensates was similar to that of StpA (Figs. 3.3C, S3.3C). A phase diagram of H-NS *versus* StpA generated after 3 hours of incubation demonstrated that i) H-NS concentrations as low as 1.25 μM can convert 20 μM of insoluble StpA assemblies into liquid-like droplets and ii) co-incubation of 1.25 μM StpA and 1.25 μM H-NS is sufficient to form mixed droplets, effectively reducing the c_{sat} of H-NS by a factor of 20 (Fig. 3.3D). These results suggested that StpA, once in complex with H-NS, contributes features that promote phase separation. The phase transition of StpA fibrils was dependent on H-NS and independent of DNA (Fig. 3.3E), yet the rate at which these co-precipitates converted into droplets was sensitive to H-NS concentration (Fig. S3.3D) and the fastest when H-NS/DNA complexes were added to pre-formed StpA fibrils (Fig. 3.3E). When we added H-NS to pre-formed StpA fibrils at concentrations far above its own c_{sat} , H-NS immediately formed large droplets that engulfed StpA assemblies (Fig. 3.3F). StpA assemblies then dissolved inside these droplets within minutes, giving rise to homogeneous binary droplets. Hfq, an unrelated NAP with similar *in vitro* phase separating propensities as H-NS (150) was entirely ineffective in dissolving StpA precipitates (Fig. S3.3E). These results suggested that ability to trigger StpA phase transition is H-NS-specific. Conversion of mesoscale StpA precipitates into liquid-like droplets prompted us to investigate how H-NS, at the molecular level, modifies StpA. TEM analysis revealed that addition of H-NS dramatically decreased the length of pre-formed StpA fibrils, eventually causing their disappearance (Fig. 3.3G). Short StpA fibrils were even observed within protein-dense areas that presumably correspond to condensed droplets (Fig. 3.3G, asterisk). Cryo-ET analysis did not initially reveal obvious morphological differences between H-NS, H-NS/DNA, or H-NS/StpA/DNA droplets (Fig. 3.2G, 3.3H and S3.2E). However, when we compared the width of the densities in all three types of droplets from the central slice of

each tomogram we found that densities in the H-NS/StpA/DNA droplets are slightly but significantly wider than those in H-NS/DNA or H-NS condensates (Fig. 3.3I). In summary, these results confirm our *in vivo* observations and demonstrate that H-NS converts insoluble StpA fibrils into shorter fibrils that eventually co-phase separate with H-NS.

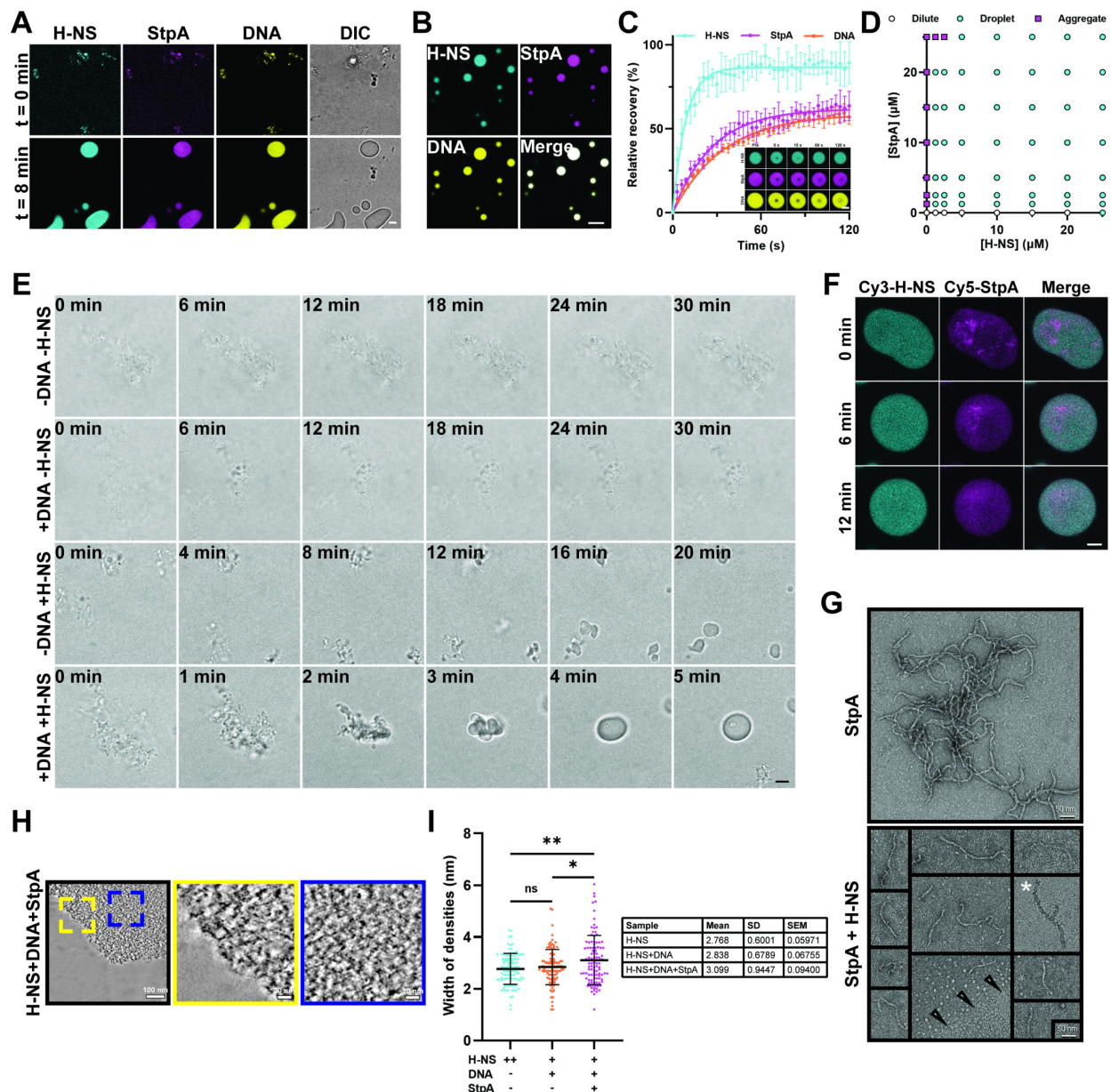


Figure 3.3 H-NS and StpA affect each other's phase behavior *in vitro*.

(A) Fluorescence and BF images of 25 μ M H-NS (+ 4% Cy3-H-NS), 10 μ M preformed StpA (+ 4% Cy5-StpA^{F21C}) fibrils and 100 nM FAM-DNA immediately upon and 8 min after mixture in GMT buffer (NaCl concentration 80 mM unless otherwise indicated). (B) Fluorescence images of the same samples after 30 min of incubation. (C) Quantification of partial FRAP of H-NS/StpA/DNA co-condensates shown in (B). Mean, SD, $n = 4-6$. **Inset:** Representative images of FRAP time course. (D) Phase diagram of H-NS and StpA acquired 3h after incubation in GMT-buffer. (E) BF images of 10 μ M StpA in GMT-buffer at indicated time points after addition of 25 μ M H-NS and/or 100 nM DNA. (F) Fluorescence images of 50 μ M H-NS (+ 2% Cy3-H-NS) and 10 μ M preformed StpA (+ 4%

Cy5-StpA^{F21C}) fibrils at indicated time points after mixing in GMT-buffer. **(G)** TEM images of 2.5 μ M StpA +/- 2.5 μ M H-NS after 30 min of incubation in GMT-buffer. Arrowheads indicate boundary of presumed droplets. Asterisk indicates fibril embedded within presumed droplets. **(H)** Slice through the reconstructed tomogram of condensates composed of 25 μ M H-NS, 10 μ M StpA and 100 nM DNA. Inset in the left panel are zoomed in on the right. **(I)** Quantification of density width inside droplets (width $n = 101$, one-way ANOVA, * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$). Scale bars: 5 μ m, unless otherwise indicated.

3.2.5 StpA to H-NS ratio defines fluidity, thermal stability and salt resistance of droplets

Based on these results, we wondered whether interacting homologs such as H-NS and StpA, through their distinct phase behaviors, might provide organisms with the capacity to adjust the dynamic properties and stability of liquid-like assemblies over a wider range of environmental conditions, an intriguing idea in light of previous studies demonstrating that StpA levels increase relative to H-NS at higher temperatures or osmolarities (217, 227). To test this hypothesis, we generated mixed H-NS/StpA/DNA droplets with constant total protein (25 μ M) and DNA (100 nM) concentrations, yet variable StpA to H-NS ratios. Droplets were clearly noticeable in all samples except the StpA alone sample, which formed precipitates. FRAP analysis revealed that as StpA to H-NS ratio increased from 1:10 to 10:1, the rates and yields of fluorescence recovery significantly decreased for all three droplet components (Fig. 3.4A-B). These results, in agreement with our *in vivo* observations (Fig. 3.1F and S3.1U), supported a model in which the ratio between StpA and H-NS defines the diffusivity of all components of the droplets. Diffusivity and stress resistance of condensates are regulated by the type and strength of multivalent interactions and often display a negative correlation (233-235). We thus tested the stability of our droplets upon elevated osmolarity or temperature. Whereas 100 mM NaCl dissolved homotypic H-NS droplets, mixtures of H-NS and StpA (with the latter present at 1/10 of the H-NS concentrations) were stable (Fig. S3.4A). NaCl titration revealed that by increasing StpA:H-NS ratio, the mixed droplets, both with (Fig. 3.4C) and without DNA (Fig. S3.4B), became increasingly more salt resistant, eventually reaching the salt resistance of pure StpA fibrils (i.e., 500 mM NaCl). Thermal stability of the droplets was equally dependent on the presence of StpA (Fig. 3.4D). Whereas H-NS/DNA droplets dissolved upon incubation at 45°C, presence of StpA maintained the droplet stability (Fig. 3.4D). StpA/DNA mixtures in the absence of H-NS failed to form droplets even at these elevated temperatures (Fig. 3.4D). These results suggest a model in which organisms utilize homologs such as the H-NS/StpA pair, which are apparently located at the opposite ends of the phase spectrum, to maintain condensate formation, fluidity, stability and likely function under a range of environmental stress conditions.

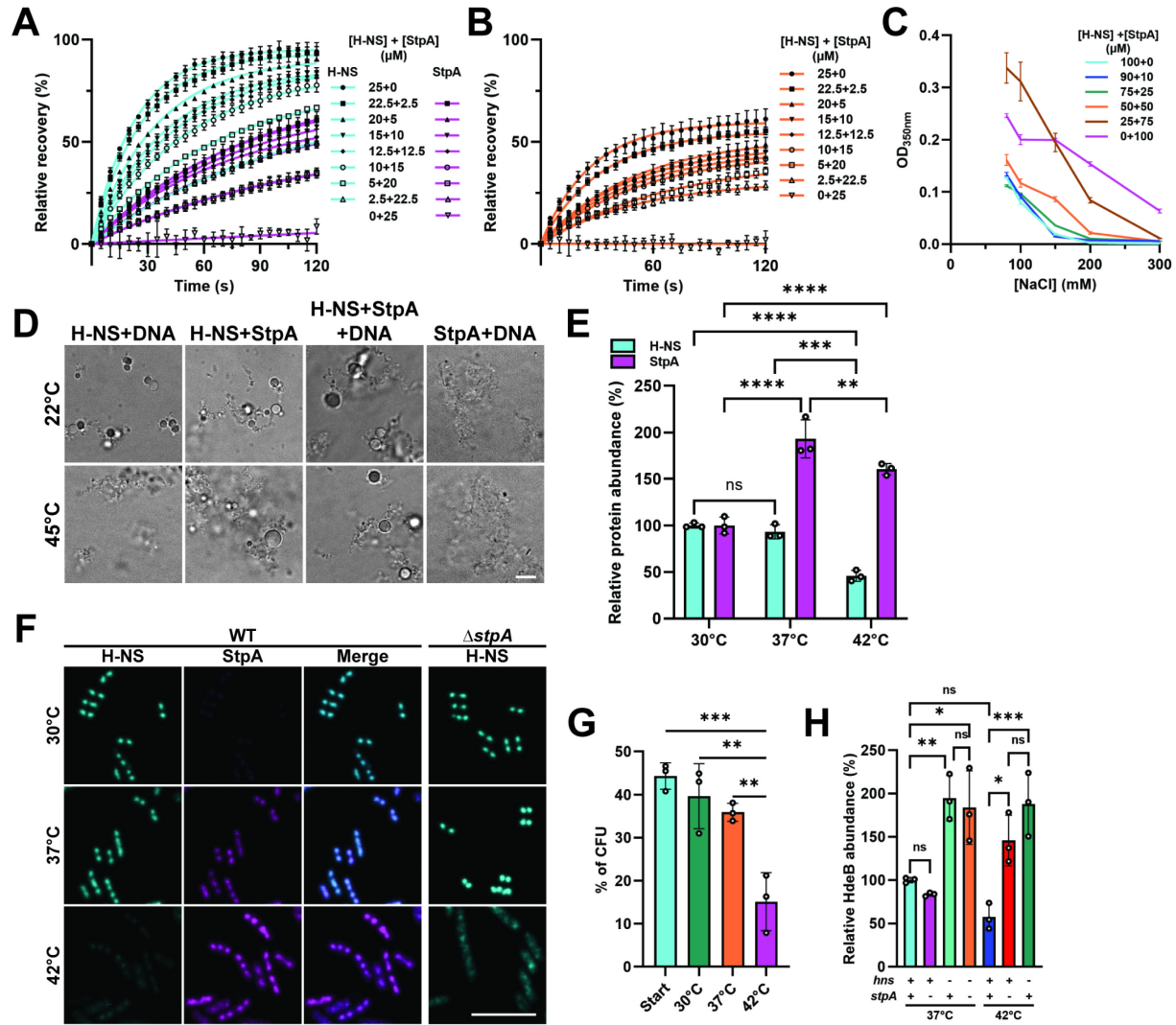


Figure 3.4 StpA to H-NS ratio defines co-assembly properties and bacterial fitness.

(A) and **(B)** Quantification of H-NS and StpA full FRAP (A) and DNA partial FRAP (B) in H-NS/StpA/DNA droplets using different ratios of H-NS:StpA (total protein 25 μ M) and fixed DNA (100 nM) in GMT-buffer. **(C)** Turbidity measurements of H-NS/StpA/DNA mixtures at different protein ratios and NaCl concentrations. DNA concentration was 100 nM. **(D)** BF images of 25 μ M H-NS and/or 10 μ M StpA with or without 100 nM DNA in GMT buffer. Samples were either incubated at 22°C for 40 min (upper panel) or at 22°C for 20 min followed by 20 min at 45°C for another 20 minutes (lower panel). Before imaging, samples were fixed with 4% formaldehyde. **(E)** Abundance of H-NS-mNG and StpA-mCh in stationary phase *E. coli* grown at the indicated temperatures. Quantification was performed by western blotting against mNG (H-NS) or mCh (StpA). Ponceau S staining of total protein was used for normalization; one-way ANOVA, $n = 3$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$). Average protein abundance at 30°C was set to 100%. **(F)** Fluorescence images of *E. coli* expressing H-NS-mNG and StpA-mCh, or H-NS-mNG in a $\Delta stpA$ background grown for 24h at indicated temperatures. **(G)** Percentage of *stpA::Kan* strain CFU over total CFU before and 24h after co-culture with WT *E. coli* at the indicated temperatures. Mean, SD, one-way ANOVA, $n = 3$, ** $p < 0.01$, *** $p < 0.001$. **(H)** Abundance of HdeB-3xFLAG in *E. coli* WT, Δhns , $\Delta stpA$ and $\Delta hns\Delta stpA$ strains, grown to mid-exponential phase at 37°C or 42°C. No growth for the $\Delta hns\Delta stpA$ strain was observed at 42°C (Fig. S3.4C). Quantification was performed by western blotting using antibodies against the 3xFLAG tag. Ponceau S staining of total protein was used for normalization (one-way ANOVA, $n = 3$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). NaCl concentration of all *in vitro* samples was adjusted to 80 mM unless indicated otherwise. Scale bars: 5 μ m.

3.2.6 Stress-induced changes in *StpA*:H-NS maintain condensate stability and function

To test this model *in vivo*, we monitored temperature-induced changes in relative StpA and H-NS levels in *E. coli* grown at different temperatures, using single copy H-NS-mNG and StpA-mCh fusion strains integrated at their normal locus under their native promoter. Western blot analysis using antibodies against the fluorescent tags revealed that the relative StpA:H-NS ratio increased about 4-fold as we increased the growth temperature from 30°C to 42°C (Fig. 3.4E). This change in ratio was also reflected in the H-NS-mNG to StpA-mCh fluorescent signal of the foci formed in stationary phase, which contained increasingly more StpA-mCh relative to H-NS-mNG as the growth temperature was raised (Fig. 3.4F). While H-NS foci formation was independent of StpA at 37°C (Fig. 3.1E), H-NS-mNG no longer formed discrete foci during stationary phase in the $\Delta stpA$ strain at 42°C (Fig. 3.4F). Analysis of growth rates revealed that an increase in growth temperature also causes an increasingly more severe growth defect in the $\Delta stpA$ strain (Fig. S3.4C). Moreover, when we co-cultured WT bacteria with equal colony forming units (CFUs) of the *stpA::Kan* strain for 24h, we observed drastically reduced CFU% of the *stpA* deletion strain at 42°C but not lower temperatures (Fig. 3.4G and S3.4D), indicating that StpA becomes increasingly more important for competitive fitness at higher temperatures. To link these defects with the endogenous functions of H-NS/StpA, we tested whether StpA expands the temperature range of transcriptional silencing. We tested bacterial strains endogenously expressing known gene targets of H-NS (*i.e.*, *dps*, *gadA*, *hdeB*) (236), epitope tagged to facilitate their detection via immunoblotting. This approach is superior to qPCR as it eases normalization and does not require identification of housekeeping genes unaffected by growth conditions and strain backgrounds. We found that the expression of all three tested targets was de-repressed upon loss of *stpA* at 42°C but not 37°C (Fig. 3.4H and S3.4E-F), suggesting that under high temperatures H-NS requires StpA to support gene silencing. We made similar observations when we exposed our bacteria strains to osmotic stress during growth. As NaCl levels increased in growth medium, H-NS-mNG expressing bacteria became increasingly dependent on StpA for foci formation, as well as maintenance of growth rates and competitive fitness (Fig. S3.4G-I). Together, our data indicate that StpA stabilizes supramolecular assemblies in the bacterial nucleoid and provides bacteria with improved resistance and fitness across a broad range of adverse growth conditions.

3.2.7 Structural differences in H-NS/StpA homo- and hetero-oligomers

The distinct behaviors of H-NS and StpA prompted us to search for potential structural differences

between the homologs. H-NS, and by homology StpA, harbor two interfaces in their N-terminal oligomerization domain: an antiparallel coiled-coil dimerization site 1 (D1), which mediates the formation of “head-to-head” dimers and a helix-turn-helix dimerization site 2 (D2), which engages in “tail-to-tail” polymerization that generate a linear chain of H-NS molecules (237, 238). Structural modeling of full-length *E. coli* H-NS/StpA dimers and tetramers using Chai discovery or AlphaFold3, also indicated the presence of these two interfaces (Fig. S3.5A-B) (237). Previous studies suggested that while the D1 dimerization interface in H-NS is heat and salt stable, the D2 interface is highly heat and salt sensitive, causing H-NS oligomers to dissociate into dimers (238-240). This property may explain the observed stress sensitivity of homotypic H-NS condensates. To better understand the structural differences contributing to their distinct phase behaviors, we performed all-atom molecular dynamics (MD) simulations on H-NS and StpA. We initially conducted MD simulations using the truncated H-NS (aa 1-82) and StpA (aa 1-83) homodimers. These simulations indicated that D1 interactions are stable under hyperosmotic conditions (500 mM KCl at 37°C) (238-240) (Fig. S3.5C). We then compared the simulated dynamics of full-length H-NS/StpA homo- and hetero-tetramers under the same conditions using their predicted model structures as input (Fig. S3.5C). Whereas backbone C α root-mean-square deviation (RMSD) of both H-NS and StpA homo-tetramers continued to drift even towards the end of simulation, indicating high conformational plasticity, H-NS/StpA hetero-tetramers adopted a low plateau RMSD, suggesting a more rigid geometry (Fig. S3.5D). In contrast to H-NS homo-tetramer, which retained a relatively linear organization (Fig. 3.5A), a more detailed structural analysis indicated the StpA homo-tetramer adopted a pronounced curvature (Fig. 3.5B). The hetero tetramer, in comparison, appeared more linear than StpA alone, consistent with its lower RMSD values and suggestive of dampened flexibility (Fig. 3.5C and S3.5D). Significant curvature in StpA homo-tetramers was rooted in the D2 interface, where the helices engage in symmetric interactions via four side chains (Arg54, Lys57, Trp61, Asp68). This interface was more flexible in H-NS homo-tetramers and uniformly more stable in StpA and StpA/H-NS tetramers as indicated by their lower root-mean-square fluctuation (RMSF) across the same residues (Figs. 3.5D-F). Unexpectedly, we observed that in both StpA and mixed tetramer, the DNA binding domain (DBD) of one dimer engaged in stable and symmetric interactions with the coiled-coil surface of the interface D1 in the neighboring dimer (Figs. 3.5B-C). In contrast, DBD in H-NS homo-oligomers flexibly radiated outward, only forming very transient interactions with its own D1

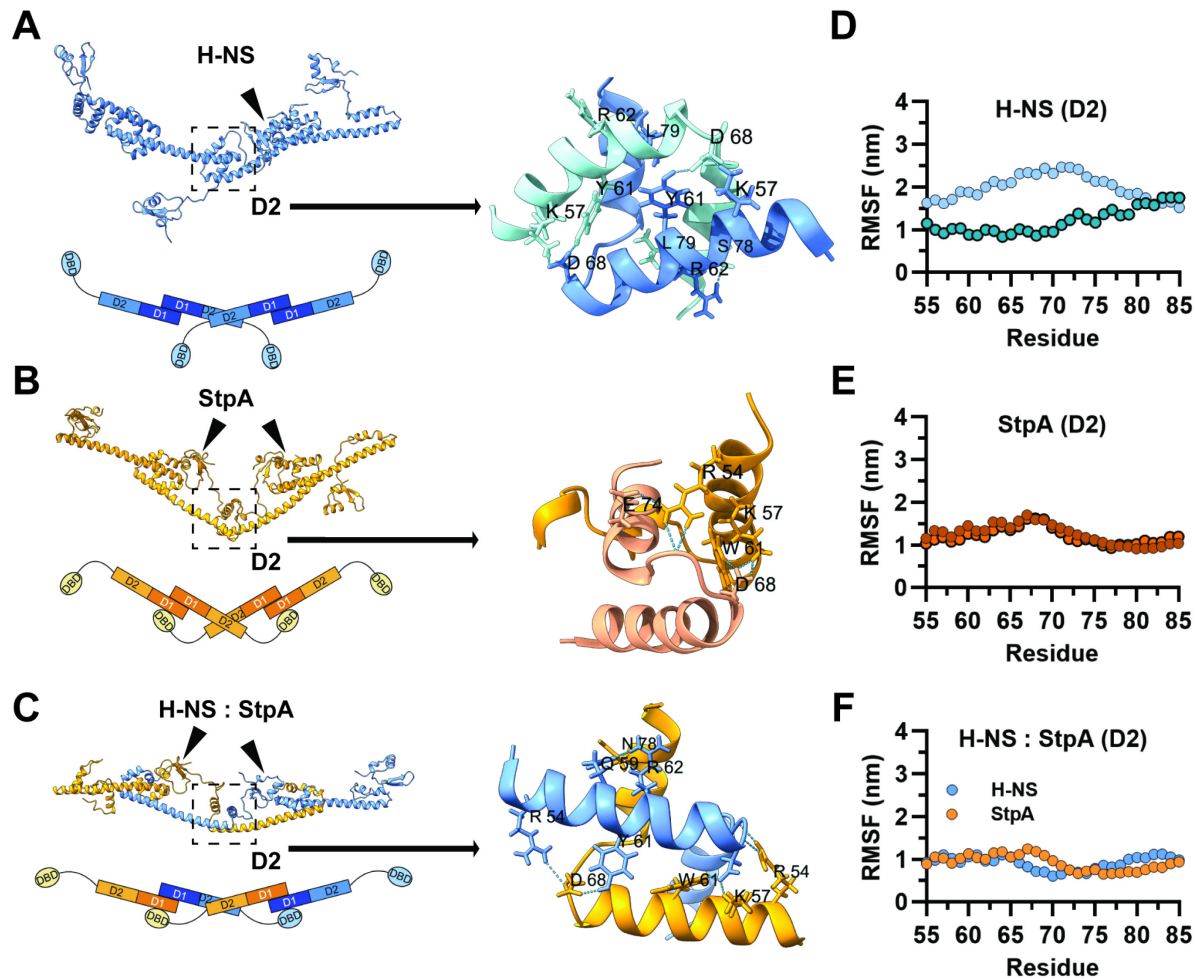


Figure 3.5 H-NS and StpA oligomers display distinct structural features.

(A)-(C) Final 200 ns snapshots of (A) H-NS tetramer, (B) StpA tetramer or (C) 2:2 H-NS:StpA tetramers modeled in 500 mM KCl at 37°C. Arrows highlight the DBD forming transient interactions with dimerization site 1 (D1). Zoom-in images highlight dimerization site 2 (D2) interface with residues involved in oligomerization. Different shades of color indicate individual protein chains. A diagram of oligomers highlighting structural domains is depicted below each predicted structure. H-NS and StpA molecules are colored as blue and gold, respectively. (D)-(F). Root-mean-square fluctuations (RMSF) of residues spanning the D2 interface in (D) H-NS tetramer, (E) StpA tetramer or (F) 2:2 H-NS:StpA tetramer.

domain (Fig. 3.5A). Earlier studies suggest that linear H-NS nucleoprotein filaments adopt a “hemi-sequestered” structure in which one DBD at each D2 contact directly binds DNA while the other DBD interacts with the N-terminal domain (241, 242). Combined with our simulation results, it is tempting to speculate that incorporation of StpA into these hemi-sequestered nucleoprotein filaments allows D1-DBD interactions between adjacent dimers, thereby enhancing filament stability. Together, these data suggested that while both homologs form dimers through D1, StpA engages in more stable homo- and hetero-oligomers through symmetric interactions not only at D2 but also between DBD and D1, effectively interlocking adjacent dimers.

3.2.8 Oligomerization domains of H-NS and StpA are critical for phase behavior and stability

Our computational results suggested that the major structural differences between H-NS and StpA reside in their N-terminal oligomerization domain. To experimentally test these results, we first swapped the oligomerization domains between the two homologs while leaving the linker and DBD unchanged. We named the chimeras H-S-S and S-H-H, according to their relative domain contributions where H indicates H-NS and S indicates StpA (Fig. 3.6A). *In vivo* analysis revealed that the S-H-H chimera displayed StpA-like features, i.e., stabilizing H-NS foci formation under high growth temperature (Fig. 3.6B) and forming dynamically arrested foci in a Δhns background (Fig. S3.6A-B). Conversely, the H-S-S chimera resembled H-NS, i.e. forming foci under stationary phase at 37°C, which dissipate at 42°C in the absence of StpA (Fig. 3.6B). These results supported our model that the N-terminal oligomerization domain is ultimately responsible for their different phase behaviors. *In vitro* analysis further revealed that purified H-S-S protein formed droplets at significantly lower c_{sat} (5 μ M) than H-NS and was not further reduced by DNA (Fig. 3.6C). It also facilitated the phase transition of purified StpA (Fig. S3.6C-D). Moreover, H-S-S condensates showed improved resistance towards osmolarity compared to H-NS condensates yet were still less stable than StpA-containing assemblies (Fig. S3.6E). The S-H-H variant, however, was insoluble when overexpressed, thus preventing its purification and *in vitro* characterization. This suggested that linker and DBD of H-NS are not sufficient to balance the insolubility of StpA's oligomerization domain. Collectively, these results implicated the N-terminal oligomerization domain as the main reason for the differences in phase behavior and stability between H-NS and StpA.

To investigate whether interface D2 is indeed responsible for differences in oligomer stability as suggested by simulation, we mutated the key residues identified as mediating D2 interactions in StpA (R54E, K57E, W61A, D68A) (Fig. 3.5B). We reasoned that this StpA variant, hereafter referred to as StpA^{D2-4M} to indicate the mutation (M) of four residues in D2, should be unable to form homo-complexes larger than a dimer and hence be unable to form aggregates at mesoscopic scale. Because StpA^{D2-4M} retains the D1 interface, we reasoned that this mutant should still be able to interact with H-NS, yet terminate H-NS oligomer formation upon incorporation, thus serving as a dominant negative factor in H-NS condensate formation. Finally, StpA^{D2-4M} should be unable to increase the stability of H-NS condensates under stress and therefore should no longer rescue the growth defects of the $\Delta stpA$ mutant. As predicted, we did not observe any dynamically arrested

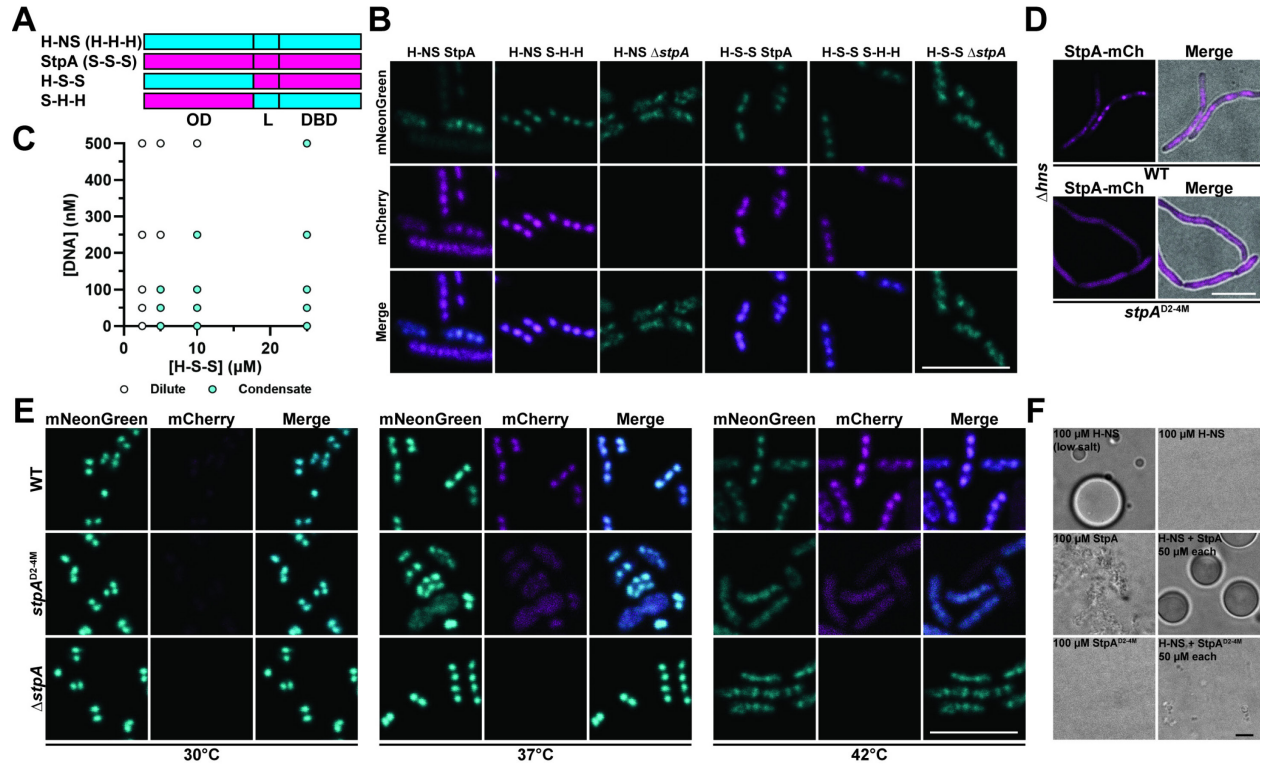


Figure 3.6 N-terminal domain defines phase behavior and stability of H-NS and StpA.

(A) Model depicting domain organization in H-NS, StpA and domain-swapped chimeras. (B) Fluorescence images of *E. coli* endogenously co-expressing H-NS-mNG, StpA-mCh or domain swapped chimeras grown for 24h at indicated temperatures. H-S-S was labeled with mNG and expressed from the *hns* locus. S-H-H was labeled with mCh and expressed from the *stpA* locus. (C) Phase diagram of H-S-S and 150 bp DNA in GMT buffer containing 80 mM NaCl. (D) Fluorescence images of *E. coli* expressing StpA-mCh or StpA^{D2-4M}-mCh in Δhns background during exponential phase. Overlay with BF channel was shown on the right. (E) Fluorescence images of *E. coli* endogenously expressing H-NS-mNG in StpA-mCh, StpA^{D2-4M}-mCh or $\Delta stpA$ backgrounds grown for 24h at indicated temperatures. (F) BF images of purified H-NS, StpA and/or StpA^{D2-4M} reconstituted at indicated concentrations in GMT buffer. The low salt sample contains 30 mM NaCl. All other samples contain 80 mM NaCl.

puncta when we endogenously expressed StpA^{D2-4M} in an exponentially growing Δhns strain (Fig. 3.6D). In fact, the StpA^{D2-4M}-mCh molecules displayed very rapid diffusivity (Fig. S3.6F). Moreover, whereas H-NS-mNG foci were readily observed at 30°C presumably due to low abundance of StpA^{D2-4M}-mCh (Fig. 3.6E), expression of StpA^{D2-4M} at 37°C led to partial dissolution of H-NS-mNG foci. The remaining foci displayed H-NS FRAP recovery rates that were similar to those observed in a $\Delta stpA$ strain (Fig. S3.6G). Upon further increase in growth temperature, we no longer observed distinct H-NS-mNG foci despite the presence of StpA^{D2-4M} (Fig. 3.6E). This lack of foci formation was also reflected phenotypically with cells endogenously expressing StpA^{D2-4M} showing impaired gene silencing and reduced competitive fitness at higher temperatures (Fig. S3.6H-I). *In vitro* reconstitution of purified proteins corroborated our *in vivo*

results and showed that StpA^{D2-4M} no longer formed insoluble aggregates (Fig. 3.6F). Addition of StpA^{D2-4M} to H-NS condensates resulted in small and sparse droplets (Fig. 3.6F). Not surprisingly, mutating the equivalent key residues in H-NS (R54E, K57E, Y61A, R62A, D68A) abrogated its ability to form foci *in vivo* and in droplets *in vitro* (Figs. S3.6J-K). In summary, these studies suggest that differences in the N-terminal oligomerization domain define the phase behavior of these homologs, causing H-NS to form metastable condensates and StpA to form insoluble, stress-resistant fibrils.

3.3 Discussion

3.3.1 A stress paradigm for condensate dynamics, stability and function

Stresses such as heat may disrupt multivalent interactions that are essential for condensates and other membraneless assemblies (233, 243), yet how organisms maintain the stability of these assemblies under environmental stresses remain poorly understood. In this study, we demonstrate that *E. coli* facilitates condensate-like nucleoid foci formation by adjusting the relative concentrations of two protein homologs with different phase behaviors. H-NS forms oligomers that assemble into highly fluidic and metastable condensate-like assemblies both *in vitro* and *in vivo*. The homolog StpA, in contrast, forms stable and insoluble fibrils. Upon mixing, StpA fibrils transition into stable H-NS/StpA droplets. By regulating StpA/H-NS ratio during stress, *E. coli* maintains nucleoid foci formation and function (Fig. 3.7). Under stress, *E. coli* lacking StpA fail to form proper H-NS foci, show increased H-NS target gene de-repression and significant growth defects. These findings suggest that stable condensate-like H-NS/StpA assemblies may contribute to gene silencing during stress. Chromatin capture assays are needed to ultimately determine whether H-NS/StpA phase separation is directly involved in gene silencing. However, a recent study revealed that in deep stationary, but not in exponential phase *E. coli*, H-NS mediates long-range looping of its binding sites, giving rise to a rosette-like nucleoid structure (244), suggesting that condensate formation might assist in bringing H-NS controlled genes into proximity. Further mechanistic studies are needed to clarify the role of phase separation in gene silencing.

3.3.2 Differences in H-NS and StpA structure contribute to distinct phase behaviors

Our findings clarify the structural basis for the distinct phase behaviors of H-NS and StpA. Oligomerization is crucial for both condensation and aggregation (245, 246), as larger oligomers

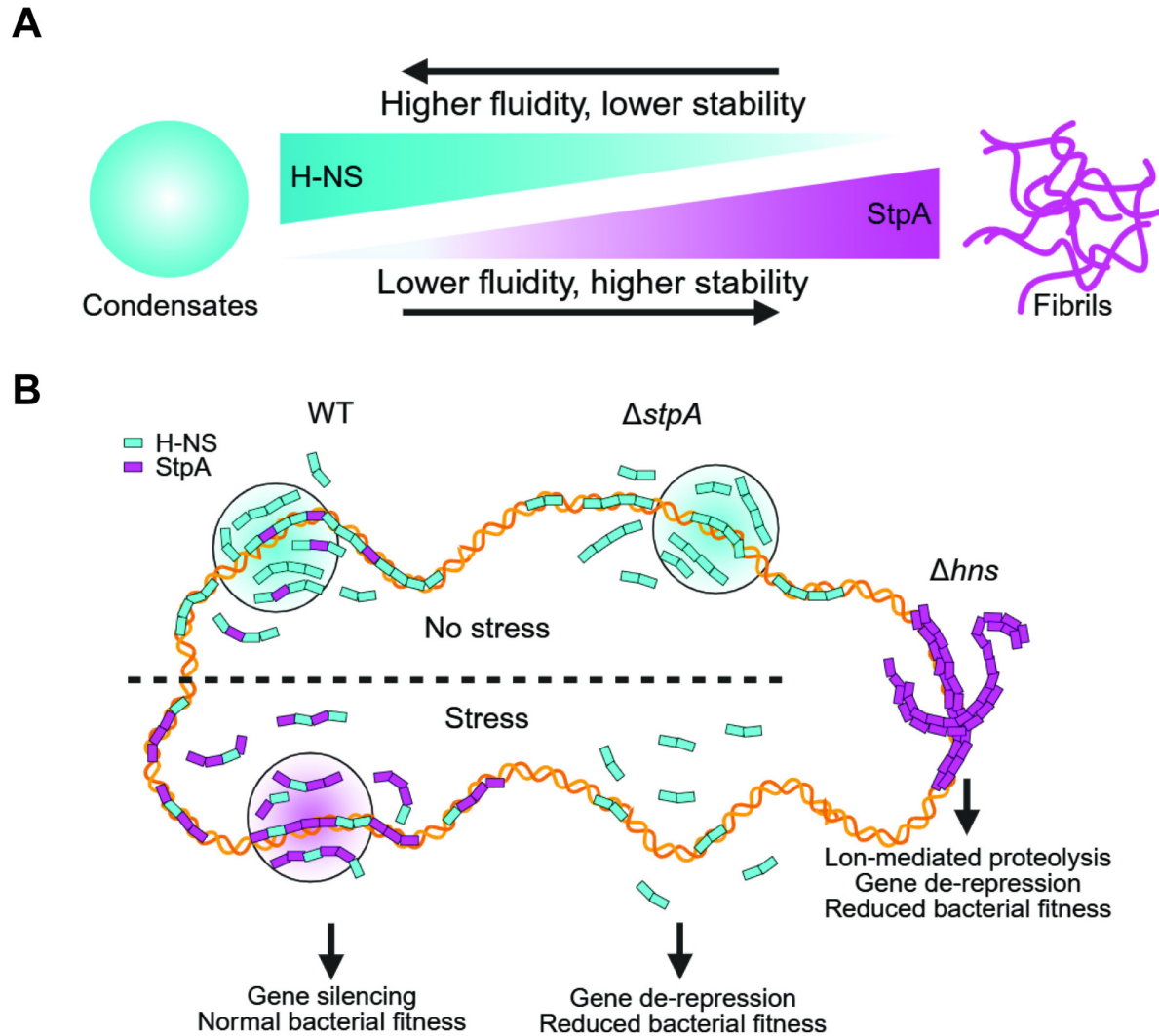


Figure 3.7 Model of bacterial heterochromatin modulation by interplay between NAP homologs.

(A) NAP homologs H-NS and StpA lie at different ends of the phase spectrum. H-NS forms biomolecular condensates while StpA forms insoluble fibrils. **(B)** Innate control of H-NS to StpA ratios under different growth conditions modulates the dynamic properties, stability and function of bacterial heterochromatin condensates.

reduce mixing entropy and increase multivalency, thereby enhancing supramolecular assembly (247-249). We show that mutations in the key residues of the D2 dimerization site impedes H-NS and StpA from forming condensates and aggregates, respectively (Figs. 3.6 and S3.6). The degree of oligomerization influences phase behavior, with larger oligomers tending to resist condensation and instead developing into aggregates (250, 251), implying that size differences in H-NS and StpA homo-oligomers may underlie the distinct solution behaviors of these proteins. Although the crowded conditions of H-NS condensates precluded the accurate quantification of H-NS oligomer

size, an earlier study suggested that even at high concentrations of 0.4 mM, H-NS only forms short ~40 nm oligomers composed of an average of eight monomers (237, 239). In contrast, StpA fibrils appear to be significantly longer, which is likely due to the increased stability at their D2 interfaces (Fig. 3.5). *In vitro* and *in vivo* functional and structural analysis of domain-swapped chimeras further supported the conclusion that the N-terminal oligomerization domains ultimately dictate the solution behavior of the homologs. Long-term passaged *Salmonella hns* deletions accumulate mutations in StpA's N-terminal domain, which caused the formation of smaller oligomers, likely enhancing StpA's solubility (225). Collectively, these findings indicate that the N-terminal domain's oligomerization propensities contribute to the distinct phase behaviors of H-NS and StpA.

3.3.3 Homologous proteins in biology – not simply backup systems?

The low solubility of StpA assemblies makes StpA a poor substitute for H-NS, challenging the previously proposed molecular backup model (224). Instead, our results indicate that StpA functions as a complementary system, regulating H-NS phase behavior in a concentration-dependent manner. This model aligns with findings that StpA mutants with weaker oligomerization compensate better for H-NS loss (225). It also explains the necessity of proteolytic removal of StpA in Δhns bacteria to prevent the accumulation of insoluble assemblies that potentially impair transcriptional regulation. StpA's function as a complementary support system might also apply to other H-NS homologs, such as Hfp in pathogenic *E. coli*, a protein which is induced by low temperature and whose combined deletion with *hns* leads to growth defects (252). These homologs may regulate H-NS phase behavior and function under various stresses, enabling precise control over gene expression and aiding organismal adaptation. A comparable system may also exist in eukaryotes. Eukaryotic heterochromatin protein 1 (HP1) establishes transcriptionally silent domains, organizing DNA into a compact yet accessible state (253-256). While HP1 homologs HP1 α and HP1 γ form condensates with DNA, HP1 β readily dissolves them (257-259). By controlling the relative abundance of HP1 homologs, heterochromatin can thus be formed, stabilized or dissolved in a tunable manner (260). In conclusion, we demonstrate that controlling the ratio of nucleoid protein homologs with distinct phase behaviors allows dynamic modulation of bacterial functional chromatin, thereby facilitating bacterial stress response and adaptation.

3.4 Materials and methods

3.4.1 Media and culture conditions

Bacteria were grown in either Luria-Bertani (LB) broth (rich media) or Gutnick medium (33.8 mM KH₂PO₄, 77.5 mM K₂HPO₄, 5.74 mM K₂SO₄, 0.41 mM MgSO₄) supplemented with 0.4% glucose, 10 mM NH₄Cl, 10 μM ferric citrate and MOPS micronutrients (261) (hereafter referred as Gutnick+ medium). Unless otherwise specified, the growth temperature was set to 37°C and shaking was set to 200 rpm. For imaging and sampling purposes, overnight cultures of bacteria were inoculated into fresh media to an OD₆₀₀ of 0.003. Exponentially growing cells were imaged and/or collected when OD₆₀₀ reached 0.2 (exponential growth) or 24h thereafter (late stationary phase) (48).

3.4.2 Bacterial strain construction

Bacterial strains and plasmid vectors used in this study are listed in Table S1. DNA amplifications were performed using Platinum Superfi II Polymerase (Thermo Fisher Scientific). Primers used in this study can be found in Table S2. Endogenous fluorescent protein fusion strains were constructed using λ-Red recombination (189). Briefly, linear DNA fragments flanked by at least 50 bp homologous sequences were electroporated into competent *E. coli* str. K-12 substr. MG1655 carrying the pKD46 plasmid. Recombination was selected by kanamycin resistance. Positive colonies were confirmed by PCR screening and Sanger sequencing (Eurofins Genomics). Successfully constructed strains express the protein of interest fused to a fluorescent protein at the C-terminus and linked by a 14-aa peptide (262). Gene knockouts were performed using P1 transduction with donor strains from the Keio collection (263), and positive colonies were validated by PCR screening. For removal of selection markers, pCP20 plasmids were electroporated into competent bacteria and expression was induced at 43°C (189). Kanamycin-sensitive cells were selected for further experiments.

3.4.3 Growth assays

Overnight bacterial cultures were inoculated into fresh Gutnick+ medium and grown at 37°C with shaking at 200 rpm. At mid-logarithmic phase, cultures were back-diluted to the same initial OD₆₀₀ and loaded into a transparent 96-well plate (Corning Costar) at a volume of 150 μl per well. Growth curves under the indicated conditions were generated by measuring OD₆₀₀ every 10 minutes on a

TECAN Spark® plate reader at the indicated temperatures using orbital shaking. Gutnick+ medium containing no bacteria was used for normalization.

3.4.4 Competition assays

Overnight bacterial cultures of competing strains were diluted and combined in fresh Gutnick+ medium so that OD₆₀₀ equals 0.01 for each strain. NaCl was added to the medium when needed. Cultures were grown at desired temperatures with shaking at 200 rpm. Immediately before and 24 hr after culture, a 10 µL sample was collected, diluted and plated on LB and LB + Kan agar plates, which were then incubated overnight at 37°C before CFU counting.

3.4.5 Live cell imaging and *in vivo* FRAP measurements

Bacterial cultures were loaded onto a glass coverslip and fixed with an agarose pad. Nucleoids were stained with 1 µg/ml 4',6-diamidino-2-phenylindole (DAPI) for 15 minutes before being washed once in spent medium. Fluorescence and differential interference contrast (DIC) imaging was performed on a Leica SP8 inverted confocal microscope controlled by Leica LAS X software with white light laser, 405 nm diode laser, PMT detector, HyD detector and 100x oil objective lens. DAPI was imaged using 405 nm excitation. mNeonGreen was imaged using 505 nm excitation. mCherry was imaged using 592 nm excitation. Super-resolution images were acquired from a Zeiss Lattice SIM5 microscope, using the GFP and RFP channels. *In vivo* FRAP was performed with LAS X's Zoom In mode. Circular regions of interest with diameter = 0.5 µm were photobleached with 505 nm (mNeonGreen) or 592 nm (mCherry) lasers, respectively. Three images were captured prior to photobleaching to obtain the initial fluorescence intensity, and cells were imaged every five seconds for two minutes to track fluorescence recovery. Fluorescence intensity change over time was background corrected, and data were fitted using Graphpad Prism to an exponential curve $F=A(1-e^{-t/\tau})$ to obtain the apparent recovery time τ .

3.4.6 Immunoblotting

For the quantification of steady-state protein abundance *in vivo*, cultures at the indicated growth conditions were collected, washed and resuspended in SDS-polyacrylamide gel loading buffer (6.5 mM Tris-HCl pH 7.0, 10% v/v glycerol, 2% w/v SDS, 0.05% w/v bromophenol blue, 2.5% v/v β -mercaptoethanol). Samples were incubated at 95°C for 10 minutes and loaded onto a 4-12% NuPAGE Bis-Tris gel (Invitrogen). After running at 175V for 45 minutes, the gel was transferred to a polyvinylidene difluoride membrane (Bio-Rad) and blocked with EveryBlot blocking buffer

(Bio-Rad) for 5 minutes with agitation. The membrane was then incubated at RT in blocking buffer containing 1:1000 diluted mouse anti-FLAG (Sigma), mouse anti-mNeonGreen (Chromotek) or rat anti-mCherry (Thermo Fisher Scientific) monoclonal antibody for one hour. After five washes with TBST (20 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.1% v/v Tween-20), the membrane was incubated at RT in blocking buffer containing 1:10,000 diluted anti-mouse or anti-rat secondary antibody labeled with IRDye 680RD or 800CW (LI-COR Biosciences) for one hour. Following multiple washes in TBST, the membrane was imaged using the LI-COR Odyssey CLx imager. Gel quantification was performed with ImageJ, and normalization was performed against total protein by either Coomassie staining of a parallel gel, or by Ponceau staining of the same blot prior to blocking. Three biological replicates were included in all experiments.

3.4.7 Fractionation of soluble and insoluble proteins

Following previously published protocols (196), cultures were collected and resuspended in 50 μ l chilled lysis buffer (10 mM potassium phosphate pH 6.5, 1 mM EDTA, 20% w/v sucrose, 1 mg/ml lysozyme, 50 U/ml benzonase). After incubating the samples for 30 minutes on ice, samples were frozen at - 80°C and then re-thawed. Upon addition of 360 μ l chilled buffer A (10 mM potassium phosphate pH 6.5, 1 mM EDTA) and protease inhibitor (ApexBio), the samples were transferred into 2 ml tubes containing 200 μ l 425-600 μ m glass beads (Sigma-Aldrich). The tubes were subjected to 30-minute shaking at 1,400 rpm at 4°C. Afterwards, 200 μ l of the lysate was collected and centrifuged at 16,000 rcf for 20 minutes. The supernatant containing all soluble proteins was collected and mixed with ¼ volume of 100% v/v trichloroacetic acid. After incubation of the samples for 10 minutes on ice, the precipitant was washed thrice with chilled acetone, heated at 37°C for less than one minute and resuspended in 100 μ l SDS-polyacrylamide gel loading buffer. The pellet containing the insoluble proteins was washed with buffer A, followed by buffer B (buffer A containing 2% v/v Nonidet P-40) and buffer A and also resuspended in 100 μ l SDS-polyacrylamide gel loading buffer. Samples were then incubated at 95°C and subjected to immunoblotting as described as above.

3.4.8 Protein purification

E. coli strain BL21 (DE3) carrying a pET-21a-H-NS-His6-SUMO plasmid was grown in LB-media (plus ampicillin) at 37°C until OD₆₀₀ of 0.5 was reached. To induce protein expression, 0.1 mM of IPTG was added to the culture. After overnight induction at room temperature, the cells

were collected by centrifugation (20 min, 5,000xg, 4°C) and resuspended in lysis buffer, consisting of 40 mM Tris-HCl pH 8.0, 10 mM NaPO₄ pH 8.0, 400 mM NaCl, 15 mM imidazole, 10% v/v glycerol 250 µM MgCl₂, 0.5 µg/ml DNase I (Invitrogen), 0.5 µg/ml RNase A (Thermo Fisher Scientific), and cOmplete protease inhibitor (Roche). After sonication for seven minutes, the lysate was centrifuged twice for 30 minutes at 30,000xg. The supernatant was loaded onto an equilibrated HisTrap HP column (Cytiva). The column was washed with lysis buffer followed by lysis buffer containing 30 mM imidazole, and H-NS was eluted with lysis buffer containing 500 mM imidazole. The eluate was supplemented with ULP1 SUMO protease (Thermo Fisher Scientific) and dialyzed overnight against 40 mM Tris-HCl pH 8.0, 300 mM NaCl. The dialyzed sample was passed through an equilibrated HisTrap HP column again, and the flowthrough containing the cleaved protein was mixed 1:3 with 25 mM Tris-HCl pH 8.5 and loaded onto a HiTrap Q column (Cytiva). H-NS was eluted with a gradient from 9 to 80% ÄKTA pure buffer B (1 M NaCl, 50 mM NaPO₄ pH 8.0). The purified sample was dialyzed against 25 mM Tris-HCl pH 7.5 and 300 mM NaCl, concentrated and stored at -80°C. H-S-S and H-NS^{D2-5M} were purified similarly. For purification of StpA and StpA F21C, *E. coli* strains BL21 (DE3) carrying pET-21a-StpA or StpAF21C-GST were grown at 37°C in LB (plus ampicillin) until OD₆₀₀ of 0.5 as reached. Then, 0.1 mM of IPTG was added and protein production was induced at room temperature for 4 hours. The cells were pelleted by centrifugation (20 min, 5,000xg, 4°C) and lysed by sonication for 8 minutes in buffer containing 40 mM Tris-HCl pH 8.0, 10 mM NaPO₄ pH 8.0, 500 mM NaCl, 5 mM DTT, 10% v/v glycerol, 250 µM MgCl₂, 0.5 µg/ml DNase I (Invitrogen), 0.5 µg/ml RNase A (Thermo Fisher Scientific) and cOmplete protease inhibitor (Roche). The lysates were centrifuged twice for 30 minutes at 37,500xg. The supernatant was loaded onto an equilibrated 5 ml GSTrap FF column (Cytiva). The column was washed with lysis buffer and StpA was eluted with 500 mM imidazole in lysis buffer. The eluate was digested with HRV 3C protease (Thermo Fisher Scientific) and dialyzed against 40 mM Tris-HCl pH 7.5 and 500 mM NaCl overnight at 4°C. The dialyzed sample was passed through an equilibrated 5 ml GSTrap FF column (Cytiva). The flowthrough was mixed 3:25 with 50 mM NaPO₄ pH 6.0 and passed through a HiTrap SP HP column (Cytiva). StpA and StpA F21C were eluted with a gradient from 9 to 80% ÄKTA pure buffer B. The purified sample was dialyzed against 25 mM Tris-HCl pH 7.5 and 500 mM NaCl, concentrated and stored at -80°C. StpA^{D2-4M} was purified similarly as StpA.

3.4.9 *In vitro* protein and DNA labeling

To fluorescently label purified proteins, 1 mg of purified protein was reduced at room temperature for 10 minutes with 180 μ g Tris-(2-carboxyethyl) phosphine (TCEP) hydrochloride (Thermo Fisher Scientific) in buffer containing 50 mM K_2HPO_4 and 100 mM (for H-NS) or 500 mM (for StpA-F21C) NaCl. A pack of Cy3 (for H-NS) or Cy5 (for StpA-F21C) Maleimide Mono-Reactive Dye (Amersham) dissolved in 50 μ l DMSO was supplemented to the reduced protein solution to a final volume of 1 ml. The solution was incubated for two hours at room temperature followed by an overnight incubation at 4°C. For removal of any excess dye, PD-10 desalting columns packed with Sephadex G-25 resin (Cytiva) were used following the manufacturer's instructions. The protein eluates were concentrated with Amicon Ultra Centrifugal Filters of 3 kDa molecular weight cutoff. The protein concentration and labeling efficiency were calculated using a molecular extinction coefficient of 150,000 $M^{-1}cm^{-1}$ at 552 nm for Cy3 and at 650 nm for Cy5, with a correction factor at 280 nm of 0.08 for Cy3 and 0.05 for Cy5 following manufacturer's guidelines. To label DNA, primers with 5'-FAM labels (IDT) were used to amplify DNA fragments of defined lengths (150 bp, 400 bp, 1 kbp, 2.5 kbp) from the *waa* operon (48) by colony PCR. The product size was validated by agarose gel electrophoresis and labeled DNA products were purified by phenol-chloroform precipitation.

3.4.10 In vitro condensate formation and FRAP analysis

Purified H-NS and/or StpA proteins were diluted to the indicated concentrations in 100 mM monosodium L-glutamate, 1 mM magnesium acetate and 20 mM Tris-acetate pH 7.5 (GMT-buffer). Due to the composition of storage buffers, homotypic H-NS condensates contain 30 mM NaCl. H-NS/DNA condensates in Figs. 2 and S2 also contain 30 mM NaCl, but similar results were observed in the presence of 50 and 80 mM NaCl. StpA aggregates in Figs. 2 and S2 contain 50 mM NaCl, but similar results were observed in the presence of 80 mM NaCl. All samples for experiments performed in Figs. 3, 4, 6, S3, S4 and S6 contained 80 mM NaCl unless indicated otherwise. To test condensate stability, NaCl was added at the indicated concentrations. Samples were prepared in CS16-CultureWell removeable chambered coverglass (Grace Bio-Labs) treated overnight with 5% w/v pluronic acid (Thermo Fisher Scientific) and washed extensively with ddH₂O. Imaging was performed on the same SP8 confocal microscope as described above. FAM, Cy3 and Cy5 were visualized using 495, 532 and 647 nm lasers, respectively. Phase transition was visualized with timelapse imaging. FRAP samples were incubated at room temperature for 30 minutes prior to imaging. For photobleaching of entire condensates, droplets with a diameter of ~

5 μm were selected. For partial photobleaching, regions with a diameter of $\sim 0.5 \mu\text{m}$ were selected among droplets with diameter of $> 10 \mu\text{m}$. FRAP settings were the same as described above.

3.4.11 Preparation of condensates for turbidity measurements

H-NS or StpA at the indicated concentrations were prepared at room temperature as described above and supplemented with the indicated concentrations of DNA and/or NaCl. 100 μl of each sample was loaded into a transparent 96-well plate (Corning Costar) and absorbance at 350 nm was measured with a TECAN Spark® plate reader. Buffer containing no protein was used for normalization. All measurements were conducted in triplicates.

3.4.12 Gene repression studies

E. coli MG1655 encoding select 3xFLAG or mCherry tagged H-NS-silenced genes (236) at their endogenous loci were grown in Gutnick+ medium at 37°C or 42°C to mid-exponential phase, collected by centrifugation at 4000g, and subjected to immunoblotting as described above. Neither H-NS nor StpA was tagged in these strains.

3.4.13 Cryo-electron tomography of condensates

Plunge-freezing condensates - 200 mesh copper grids with R2/1 holey carbon film (Quantifoil GmbH) were glow discharged for 30 s at 5 mA on a Pelco EasiGlow system. 3 μL of either H-NS alone, H-NS+DNA, or H-NS+DNA+StpA condensate-containing mixture was applied to grids in the environmental chamber of a Vitrobot Mark IV (Thermo Fisher Scientific) maintained at 24 °C and 90% humidity. The grids were back-side blotted for 2.5 s with a blot force of 0 and plunge-frozen in liquid ethane. Frozen grids were clipped into Autogrids (Thermo Fisher Scientific) and stored in liquid N₂ until further use.

Tilt-series acquisition & reconstruction - Grids containing condensates were imaged using a Titan Krios G4i transmission electron microscope (Thermo Fisher Scientific) operated at 300 kV, equipped with a K3 direct electron detector (Gatan Inc.) and a Bioquantum imaging filter (Gatan Inc.). Tilt series were acquired by a dose-symmetric scheme (264) from +51° and -51° starting at 0° with 3° increments. Tilt series were acquired at a nominal magnification of 53,000x, equating to a pixel size of 1.681 Å/pixel and a filter slit width of 20 eV, using SerialEM software (265) and scripts from PACETomo (266). Total dose per tilt series was $\sim 160 \text{ e}^-/\text{\AA}^2$. The tilt series were aligned using patch tracking in IMOD (267). All other reconstruction steps were performed using

WarpTools software (<https://doi.org/10.5281/zenodo.13982246>). Reconstructed tomograms were denoised using cryoCARE (268).

Tomogram analysis- Fibril widths were measured manually using the measure feature in 3dmod from the IMOD software package (267). 101 measurements were performed per sample/condition. Fibril width jitter plots were generated using the ggplot2 system within RStudio (version 2025.5.1.513).

3.4.14 Negative stain transmission electron microscopy

Samples were stained with 0.75% (w/v) uranyl formate onto formavar coated 400 mesh copper grids stabilized with evaporated carbon film (Electron Microscopy Sciences). Briefly, 5 μ L of sample was loaded onto glow discharged grids and were wicked away with Whatman paper after 45 seconds. Grids were washed twice with ddH₂O and once with uranyl formate solution before incubating in uranyl formate for 45 seconds. Excess stain was wicked away with Whatman paper and grids were imaged at room temperature using FEI Tecnai T12 microscope operating at 120kV. Images were captured at 50,000x with a sampling of 2.1 Å/pixel.

3.4.15 Molecular dynamics (MD) simulations of H-NS and StpA assemblies

Structural models of dimeric and tetrameric assemblies of *E. coli* H-NS and StpA proteins were generated using the Chai Discovery platform and the AlphaFold 3 server (269, 270). The linear end-to-end truncated dimers were constructed from residues 1-82 of H-NS and 1-83 of StpA, forming the canonical dimerization site 1 and site 2 interfaces as reported previously (237, 239, 240). Higher-order oligomers, including the homo-tetramers of H-NS (residues 1-137) and StpA (residues 1-134), as well as a hetero-tetrameric complex comprising two subunits of H-NS and two of StpA, were built using the same approaches. All initial models adopted extended conformations consistent with experimentally inferred linear arrangements of H-NS and StpA oligomerization domains. All-atom MD simulations were prepared with CHARMM-GUI (271) using the CHARMM36m force field (272) and TIP3P water model. A rectangular box of $\sim 25 \times 15 \times 15$ nm was used for tetrameric complexes, and $\sim 13 \times 13 \times 13$ nm was used for the truncated dimers. Each system was neutralized with 0.5 M KCl, a concentration chosen to probe the effect of elevated salt on complex stability. MD simulations were carried out using GROMACS version 2025.2 (273). After steepest-descent energy minimization, restrained equilibration was performed for 2.5 ns under constant particle number, pressure, and temperature (NPT ensemble). Production

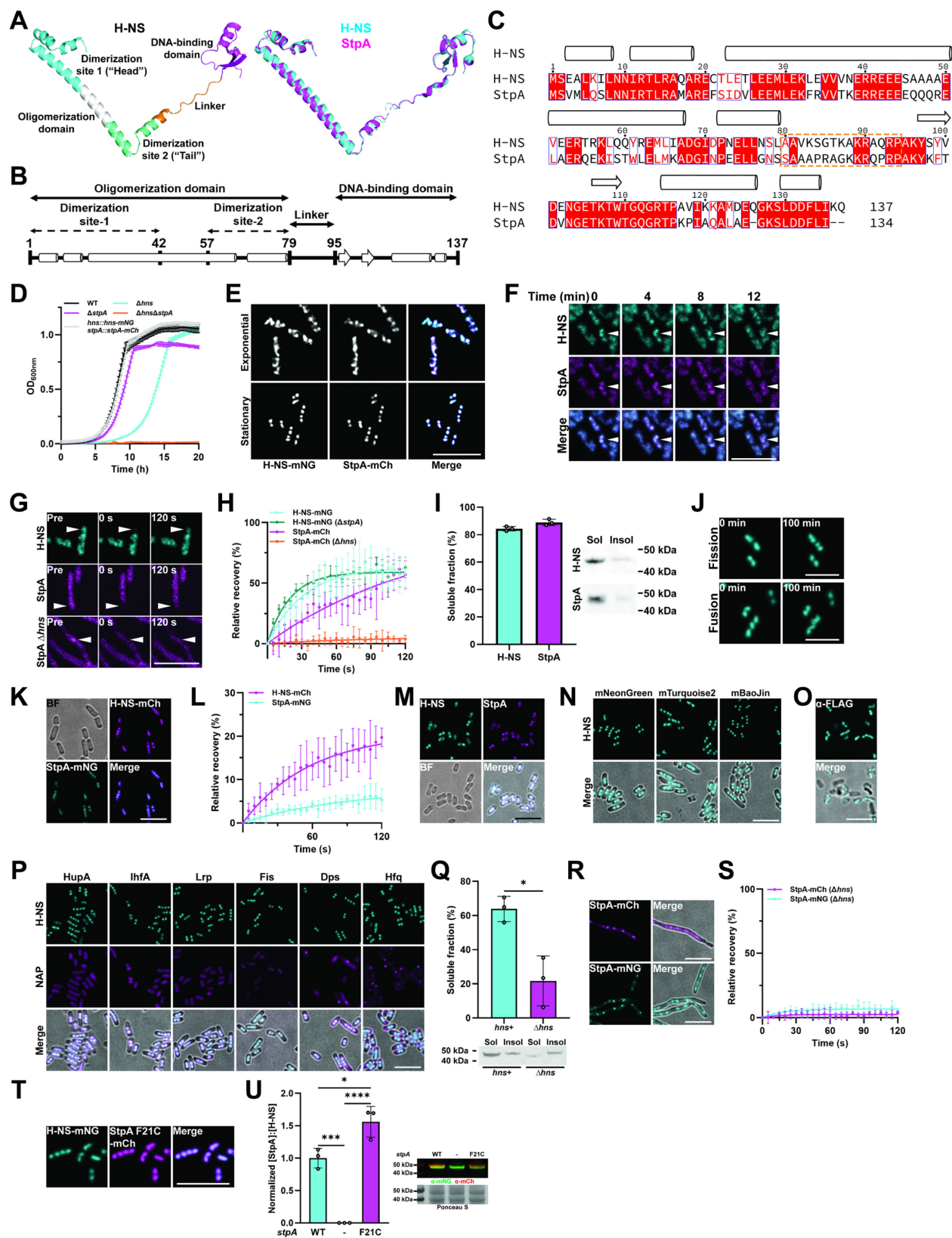
simulations were run for 200 ns at 310.15 K with a 2-fs integration time step and periodic boundary conditions. Temperature was controlled using the Nosé-Hoover thermostat, and pressure was maintained with the Parrinello-Rahman barostat. Long-range electrostatics were treated with the particle mesh Ewald (PME) method, with a real-space cutoff of 1.2 nm. Van der Waals interactions were smoothly switched off between 1.0 and 1.2 nm. All covalent bonds involving hydrogens were constrained with the linear constraint solver (LINCS) algorithm (274). Trajectory analyses were performed with GROMACS utilities, including root mean square deviation (RMSD) and root mean square fluctuation (RMSF). Structural ensembles and conformational transitions were further examined using UCSF ChimeraX (275) and Discovery Studio Visualizer studio (276). Twenty snapshots from each MD trajectory were extracted at 10 ns intervals for structural comparison, and representative final MD structures at 200 ns were selected for figure preparation.

3.4.16 Statistical analysis

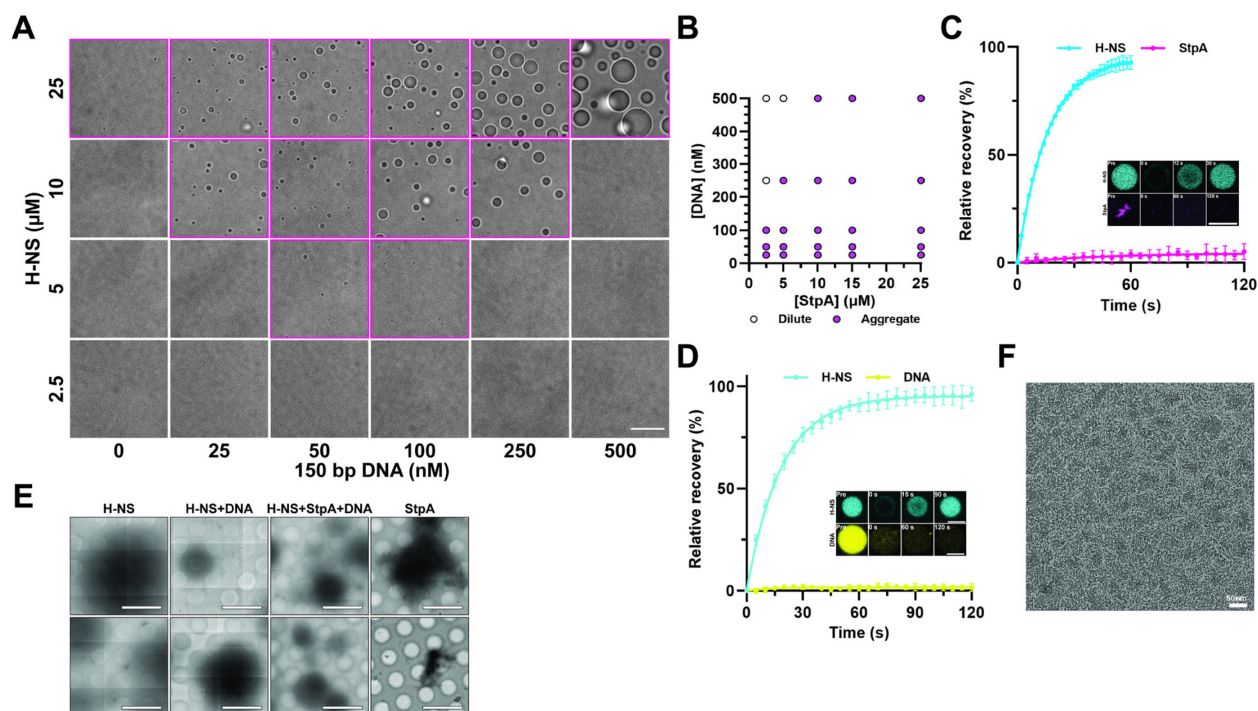
All statistical analyses were performed by GraphPad Prism 10. Details of statistical tests can be found in associated figure legends. *n* stands for number of biological replicates in WB/growth data, stands for droplet/aggregate/foci number in FRAP data.

3.5 Supplemental information

Supplementary Figure 3.1 *In vivo* phase behavior of the *E. coli* homologs H-NS and StpA (related to Fig. 3.1). (A) **Left:** Structure of *E. coli* H-NS as predicted by AlphaFold 3 with domains highlighted. **Right:** Alignment of H-NS and StpA based on AlphaFold predicted structures using jFATCAT (flexible). (B) Domain organization and functional delineation of H-NS and StpA (StpA DBD spans residues 95-134). (C) Sequence alignment of *E. coli* H-NS and StpA. Red boxes with white letters indicate identical residues, white boxes with red letters indicate residues with similar properties, dashed orange box indicates disordered linker. (D) Growth curves of *E. coli* MG1655 WT, Δhns , $\Delta stpA$ or $\Delta hns/stpA$ or MG1655 co-expressing H-NS-mNG and StpA-mCh. (E) Representative super-resolution fluorescence images of exponential and stationary phase *E. coli* co-expressing H-NS-mNG and StpA-mCh. (F) Live cell time course images of exponential H-NS-mNG and StpA-mCh expressing bacteria grown at 37°C. Spatial reorganization of sub-nucleoid compartments are highlighted by arrowheads. (G) FRAP images of H-NS-mNG or StpA-mCh microdomains in exponentially grown WT *E. coli* (upper and middle panel) or StpA-mCh in the Δhns strain (lower panel). Frames depict puncta before (pre), immediately after (0s) and 120 sec after photobleaching. Arrowheads depict the photobleached areas. (H) Quantification of H-NS-mNG and StpA-mCh FRAP signals of bacteria shown in (G). Mean, SD, *n* = 4-5. (I) Solubility fractionation of H-NS-mNG and StpA-mCh in stationary phase *E. coli*. A representative western blot image depicting soluble (Sol) and insoluble (Insol) fractions is shown. (J) Fluorescence time course images of H-NS-mNG in *E. coli* grown to stationary phase. (K) Fluorescence and BF images of stationary *E. coli* co-expressing H-NS-mCh and StpA-mNG. (L) FRAP quantification of bacteria shown in (K). Mean, SD, *n* = 4. (M) Fluorescence and BF images of stationary *E. coli* expressing H-NS-mNG and StpA-mCh grown in LB medium. (N) Fluorescence and merged BF images of *E. coli* expressing H-NS C-terminally tagged with indicated fluorescent proteins. (O) Immunofluorescence and merged BF images of stationary phase *E. coli* expressing H-NS-3xFLAG. Cells were fixed and immunofluorescent detection was conducted using anti-FLAG antibodies. (P) Fluorescence and merged BF images of stationary phase *E. coli* expressing H-NS-mNG (cyan) and select mCh-tagged *E. coli* NAPs (magenta). Lower panel depicts the merged overlay of mNG, mCh and BF channels. (Q) Solubility fractionation of StpA-mCh in *hns*⁺ or Δhns background during exponential growth in LB medium (*t*-test, SD, *n* = 3, * *p* < 0.05). A representative western blot image depicting soluble (Sol) and insoluble (Insol) fractions is shown. (R)

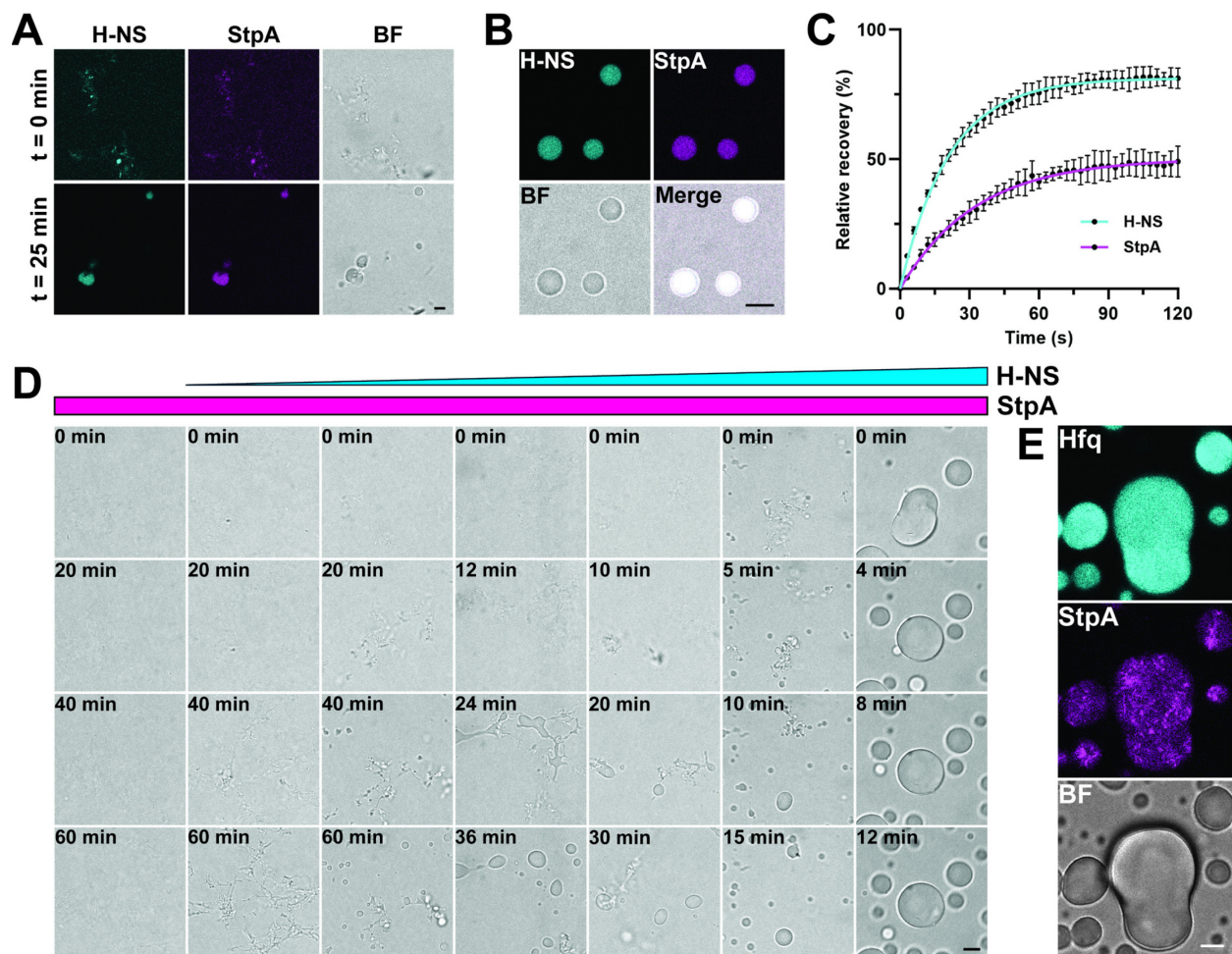


Fluorescence and merged BF images of exponential phase Δhns *E. coli* expressing StpA-mCh or StpA-mNG. **(S)** Quantification of FRAP signals of bacteria shown in (R). Mean, SD, $n = 4$. **(T)** Fluorescence images of stationary *E. coli* expressing H-NS-mNG and StpA^{F21C}-mCh. **(U)** Normalized StpA:H-NS ratios in WT, $\Delta stpA$ and $stpA^{F21C}$ strains. Quantification was performed by western blotting against mNG (H-NS) and mCh (StpA). Ponceau S stain against total protein was used for normalization. One-way ANOVA, mean, SD, $n = 3$, * $p < 0.05$, *** $p < 0.001$, **** $p < 0.0001$. A representative western blot image was shown. Unless indicated otherwise, all bacteria were grown in Gutnick+ minimal medium at 37°C with 200 rpm shaking and were imaged live. Scale bars: 5 μ m.



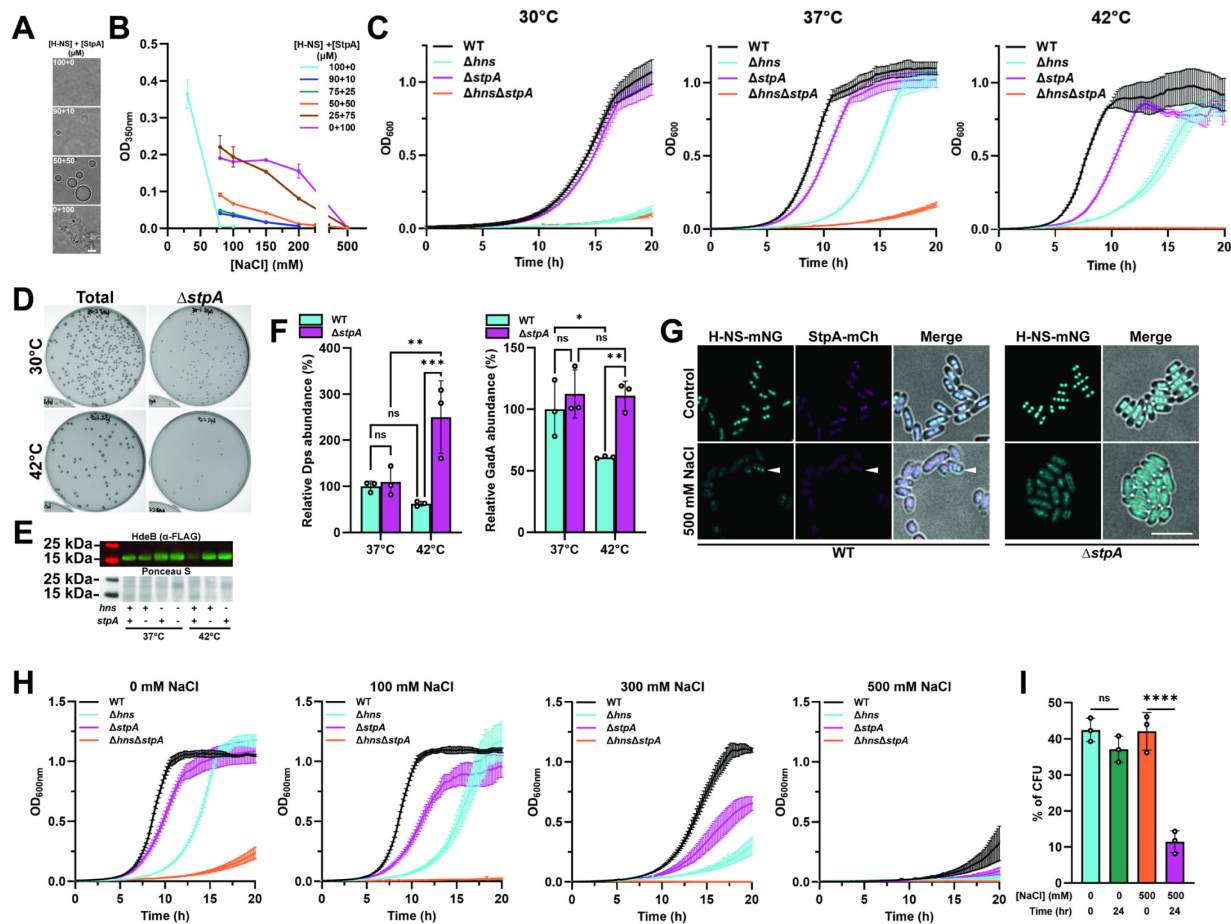
Supplementary Figure 3.2 H-NS and StpA form co-condensates (related to Fig. 3.2).

(A) BF images of droplets formed with various concentrations of H-NS and 150 bp DNA in GMT buffer to generate phase diagram shown in Fig. 2C. Panels containing droplets are highlighted in magenta. (B) Phase diagram of StpA and 150 bp DNA in GMT buffer containing 50 mM NaCl. (C) FRAP quantification of 100 μ M H-NS (+ 1% Cy3-labeled) droplets or 100 μ M StpA (+1 % Cy5-labeled StpA F21C) precipitates after bleaching the entire droplet (i.e., full FRAP); mean, SD, $n = 4$. **Inset:** Representative images of H-NS (above) or StpA (below), depicting before, immediately upon and indicated time points after photobleaching. (D) Full FRAP quantification of 25 μ M H-NS (+ 4% Cy3-labeled) mixed with 100 nM DNA (FAM-labeled) in co-condensates; mean, SD, $n = 4$. **Inset:** Representative images of H-NS and DNA depicting before, immediately upon and indicated time points after photobleaching. (E) Cryo-TEM Medium Magnification Montages demonstrating representative morphology of indicated droplets and aggregates. (F) TEM analysis of 2.5 μ M purified H-NS prepared in GMT buffer. Scale bar: 50 nm. Scale bars: 5 μ m, unless otherwise indicated.



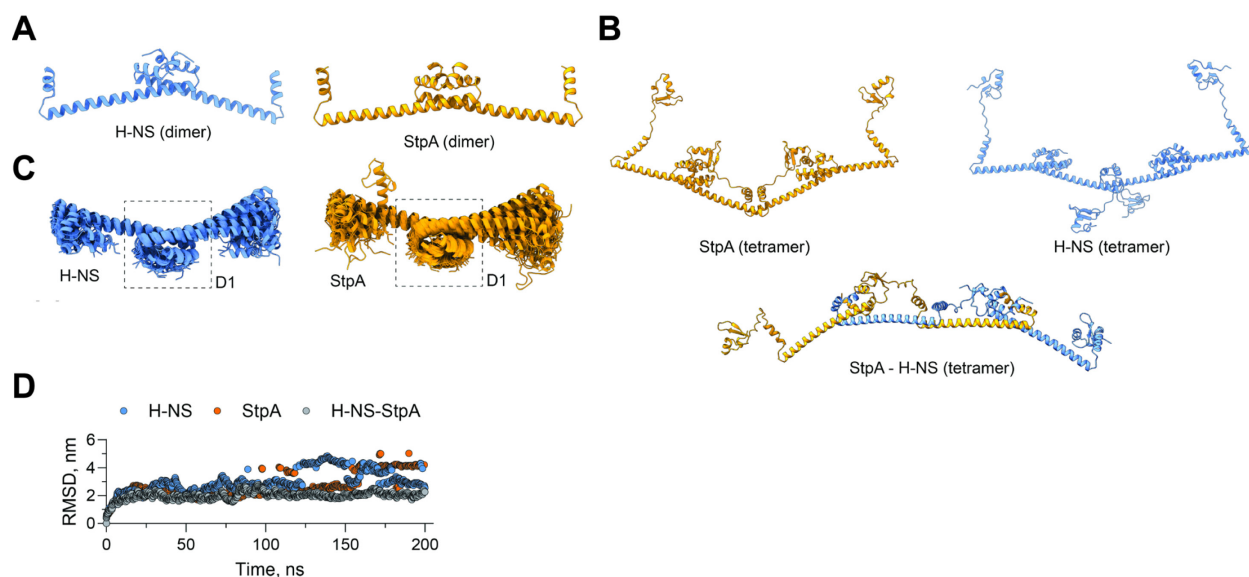
Supplementary Figure 3.3 H-NS and StpA form co-condensates (related to Fig. 3.3).

(A) Fluorescence and BF images of 25 μ M H-NS (+ 4% Cy3-H-NS) and 10 μ M preformed StpA (+ 4% Cy5-StpA^{F21C}) fibrils immediately upon and 25 min after mixture in GMT buffer. (B) Fluorescence images of the same samples as in (A) after 40 min of incubation. (C) Quantification of partial FRAP of H-NS/StpA co-condensates shown in (B). Color code as before (mean, SD, $n = 4-6$). (D) BF time course images of 10 μ M StpA mixed with 0, 1, 2.5, 5, 10, 25 and 50 μ M H-NS in GMT buffer. (E) Fluorescence images of 100 μ M Hfq (+ 1% Cy3-Hfq^{S65C}) mixed with 10 μ M preformed StpA fibrils in GMT buffer and incubated for 40 minutes. Scale bars: 5 μ m.



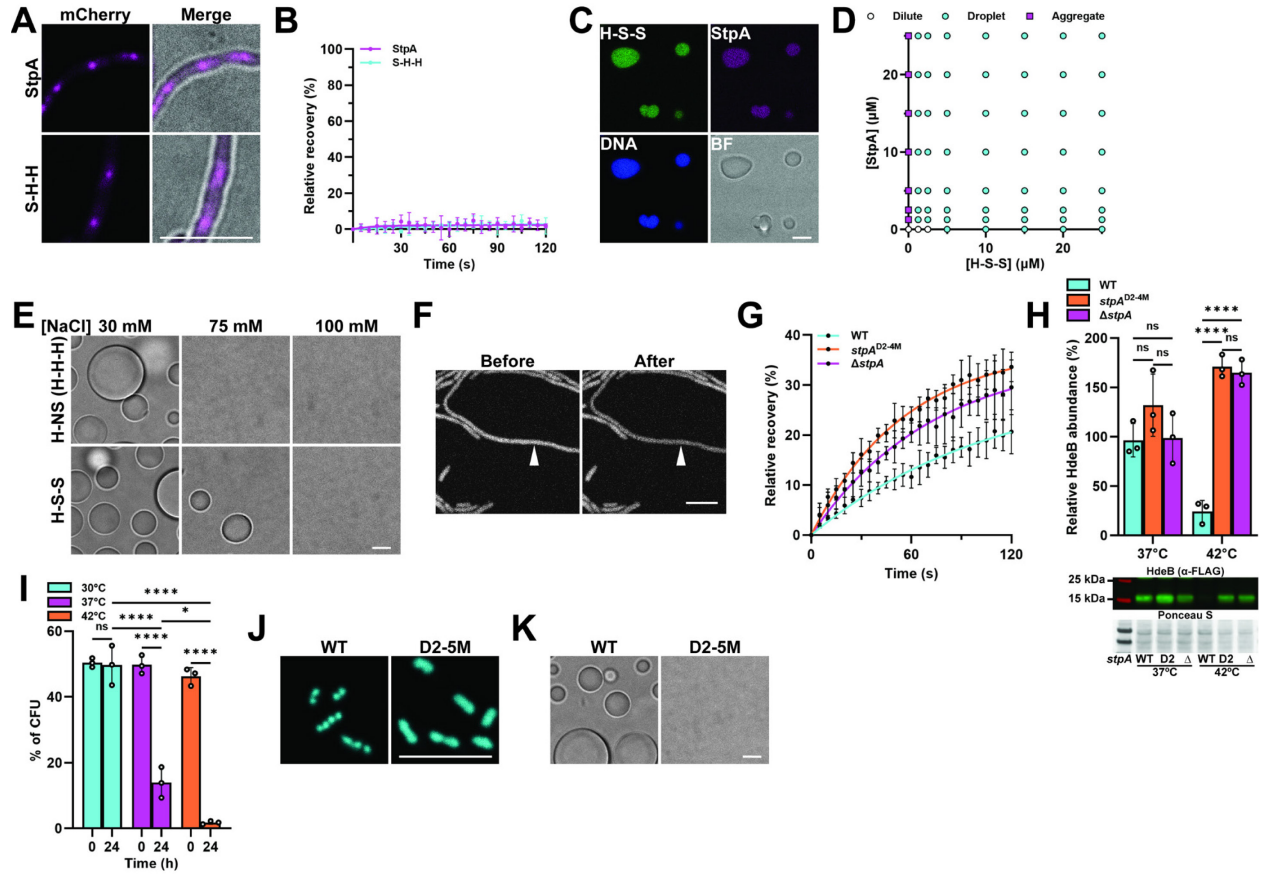
Supplementary Figure 3.4 StpA is required for bacterial fitness under stress (related to Fig. 3.4).

(A) BF images of H-NS and StpA mixed in GMT buffer at indicated concentrations in the presence of 100 mM NaCl. Scale bar: 5 μ m. (B) Turbidity of H-NS and StpA mixed at the indicated concentrations in GMT buffer containing different amounts of NaCl. (C) Growth curves of *E. coli* MG1655 WT, Δ hns, Δ stpA and Δ hns Δ stpA strains at the indicated temperatures. (D) Representative images of *E. coli* *stpA::Kan* strain co-cultured with WT strain at 30°C or 42°C for 24h. Total CFU was estimated by plating serially diluted culture on LB agar, *stpA::Kan* CFU was estimated by plating on LB+Kan agar. Related to Fig. 4G. (E) Representative Western blot image related to Fig. 4H. (F) Abundance of endogenously expressed Dps-mCh or GadA-3xFLAG in *E. coli* MG1655 WT or Δ stpA strains, grown to mid-exponential phase at 37°C or 42°C. Quantification was performed by Western blotting against 3xFLAG (GadA) or mCh (Dps). Ponceau S staining of total protein was used for normalization; two-way ANOVA, $n = 3$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. (G) Fluorescence and merged BF images of *E. coli* H-NS-mNG StpA-mCh or H-NS-mNG Δ stpA strains grown to stationary phase in Gutnick+ minimal medium containing 0 or 500 mM NaCl. Arrowhead highlights cells containing distinct foci. Scale bar: 5 μ m. (H) Growth curves of *E. coli* MG1655 WT, Δ hns, Δ stpA and Δ hns Δ stpA strains grown in Gutnick+ minimal medium containing indicated amounts of NaCl. (I) Percentage of *stpA::Kan* strain CFU over total CFU before and 24h after co-culture with WT *E. coli* in Gutnick+ minimal medium containing indicated amounts of NaCl. Mean, SD, one-way ANOVA, $n = 3$, **** $p < 0.0001$.



Supplementary Figure 3.5 MD simulation of H-NS and StpA (related to Fig. 3.5).

(A) Structure of N-terminally truncated H-NS (aa1-82) or StpA (aa1-83) dimers as predicted by AlphaFold 3. **(B)** Structure of full-length H-NS homo-tetramer, StpA homo-tetramer or 2:2 H-NS:StpA hetero-tetramer as predicted by AlphaFold 3 or Chai. **(C)** Ensembles of 20 superimposed snapshots (every 10 ns across a 200-ns run) for the N-terminally truncated dimers of H-NS and StpA at 500 mM KCl and 37°C. **(D)** Time evolution of root-mean-square deviation (RMSD) for full-length tetramers simulated under same conditions.



Supplementary Figure 3.6 Oligomerization domain determines protein phase behavior and stability (related to Fig. 3.6).

(A) Fluorescence and overlay BF images of exponential phase *Δhns E. coli* expressing StpA-mCh or S-H-H-mCh. (B) Quantification of full FRAP of StpA (magenta) or S-H-H (cyan) puncta shown in (B) (mean, SD, $n = 4-6$). (C) Fluorescence and BF images of 25 μM H-S-S (+ 4% Cy3-H-S-S) and 10 μM preformed StpA (+ 4% Cy5-StpA^{F21C}) fibrils 30 min after mixture in GMT buffer. (D) Phase diagram of H-S-S and StpA acquired 3h after incubation in GMT buffer. (E) BF images of 100 μM H-NS or H-S-S in GMT buffer containing indicated amount of NaCl. (F) Fluorescence images of exponential phase *Δhns E. coli* expressing StpA^{D2-4M}-mCh before and immediately after photobleaching, demonstrating fluorescence reduction throughout the cell. Arrowhead indicates point of photobleaching. (G) Quantification of full FRAP of H-NS-mNG foci in indicated strains under conditions shown in middle panel of Fig. 6E (mean, SD, $n = 6$). (H) Abundance of endogenously expressed HdeB-3xFLAG in *E. coli* MG1655 strains of indicated backgrounds, grown to mid-exponential phase at 37°C or 42°C. Quantification was performed by western blotting against 3xFLAG. Ponceau S staining of total protein was used for normalization; two-way ANOVA, $n = 3$, **** $p < 0.0001$). A representative blot is shown on the right. (I) Percentage of *stpA::stpA*^{D2-4M} strain CFU over total CFU before and 24h after co-culture with WT *E. coli* at indicated temperatures. Mean, SD, one-way ANOVA, $n = 3$, * $p < 0.05$, **** $p < 0.0001$. (J) Fluorescence images of stationary phase *E. coli* expressing H-NS-mNG or H-NS^{D2-5M}-mNG. (K) BF images of 100 μM H-NS or H-NS^{D2-5M} in GMT buffer containing 30 mM NaCl. Scale bars: 5 μm .

Supplementary Table 3.1 Bacterial strains, plasmid vectors and DNA oligos used in this study.

Bacterial strain		Marker	Source/Reference
<i>E. coli</i> str. K-12 substr. MG1655 (wildtype)			Lab strain collection
MG1655 Δ <i>hns</i>			(150)
MG1655 <i>stpA::Kan</i>		Kan	(150)
MG1655 Δ <i>stpA</i>			(150)
MG1655 Δ <i>hns</i> Δ <i>stpA</i>			This study
MG1655 <i>hns::hns-mNeonGreen</i>			This study
MG1655 <i>hns::hns-mNeonGreen stpA::stpA-mCherry</i>			This study
MG1655 <i>hns::hns-mNeonGreen</i> Δ <i>stpA</i>			This study
MG1655 <i>hns::hns-mNeonGreen hupA::hupA-mCherry</i>		Kan	This study
MG1655 <i>hns::hns-mNeonGreen dps::dps-mCherry</i>		Kan	This study
MG1655 <i>hns::hns-mNeonGreen ihfA::ihfA-mCherry</i>		Kan	This study
MG1655 <i>hns::hns-mNeonGreen lrp::lrp-mCherry</i>		Kan	This study
MG1655 <i>hns::hns-mNeonGreen fis::fis-mCherry</i>		Kan	This study
MG1655 <i>hns::hns-mNeonGreen hfq::hfq-mCherry</i>		Kan	This study
MG1655 <i>stpA::stpA-mCherry</i>			This study
MG1655 <i>stpA::stpA-mCherry</i> Δ <i>hns</i>			This study
MG1655 <i>hns::hns-mCherry stpA::stpA-mNeonGreen</i>		Kan	
MG1655 <i>stpA::stpA-mNeonGreen</i> Δ <i>hns</i>		Kan	
MG1655 <i>hns::hns-mNeonGreen stpA::stpA-F21C-mCherry</i>			This study
MG1655 <i>hns::hns-mNeonGreen stpA::stpA-F21C</i>			This study
MG1655 <i>stpA::stpA-F21C-mCherry</i> Δ <i>hns</i>			This study
MG1655 <i>stpA::stpA-mCherry</i> Δ <i>hns</i> Δ <i>lon</i>			This study
MG1655 <i>hns::hns-mTurquoise2</i>		Kan	This study
MG1655 <i>hns::hns-mBaoJin</i>		Kan	This study
MG1655 <i>hns::hns-3xFLAG</i>		Kan	This study
MG1655 <i>dps::dps-mCherry</i>		Kan	This study
MG1655 <i>dps::dps-mCherry</i> Δ <i>stpA</i>		Kan	This study
MG1655 <i>hdeB::hdeB-3xFLAG</i>		Kan	This study
MG1655 <i>hdeB::hdeB-3xFLAG</i> Δ <i>hns</i>		Kan	This study
MG1655 <i>hdeB::hdeB-3xFLAG</i> Δ <i>stpA</i>		Kan	This study
MG1655 <i>hdeB::hdeB-3xFLAG</i> Δ <i>hns</i> Δ <i>stpA</i>		Kan	This study
MG1655 <i>hdeB::hdeB-3xFLAG stpA::stpA R54E/K57E/W61A/D68A</i>		Kan	This study
MG1655 <i>gadA::gadA-3xFLAG</i>		Kan	This study
MG1655 <i>gadA::gadA-3xFLAG</i> Δ <i>stpA</i>		Kan	This study
MG1655 <i>hns::hns-mNeonGreen stpA::S-H-H-mCherry</i>		Kan	This study
MG1655 <i>hns::H-S-S-mNeonGreen stpA::S-H-H-mCherry</i>		Kan	This study
MG1655 <i>hns::H-S-S-mNeonGreen stpA::stpA-mCherry</i>		Kan	This study
MG1655 <i>hns::H-S-S-mNeonGreen</i> Δ <i>stpA</i>		Kan	This study
MG1655 <i>stpA::S-H-H-mCherry</i> Δ <i>hns</i>		Kan	This study
MG1655 <i>hns::hns-mNeonGreen stpA::stpA R54E/K57E/W61A/D68A-mCherry</i>		Kan	This study
MG1655 <i>stpA::stpA R54E/K57E/W61A/D68A-mCherry</i> Δ <i>hns</i>		Kan	This study
MG1655 <i>stpA::stpA R54E/K57E/W61A/D68A</i>		Kan	This study
MG1655 <i>hns::hns R54E/K57E/Y61A/R62A/D68A-mNeonGreen</i>		Kan	This study
BL21 (DE3)		Cam	New England Biolabs
Plasmid	Description	Marker	Source/Reference
pKD4	Kanamycin resistance marker donor	Kan	(189)
pKD46	λ -red recombinase expression plasmid	Amp	(189)
pCP20	Flp recombinase expression plasmid	Amp	(189)

pSUB11	FLAG tag donor	Amp	(277)
pTRC99a	Cloning vector for protein tagging	Amp	(278)
pET21a	Expression vector for protein purification	Amp	Novagen
pET21a-His-SUMO-HNS	Expression vector for H-NS purification	Amp	This study
pET21a-His-SUMO-StpA	Expression vector for StpA purification	Amp	This study
pET21a-His-SUMO-StpAF21C	Expression vector for StpA F21C purification	Amp	This study
pET21a-His-SUMO-HSS	Expression vector for H-S-S purification	Amp	This study
pET21a-His-SUMO-SHH	Expression vector for S-H-H purification	Amp	This study
pET21a-His-SUMO-HNS D2-5M	Expression vector for H-NS R54E/K57E/Y61A/R62A/D68A purification	Amp	This study
pET21a-His-SUMO-StpA D2-4M	Expression vector for StpA R54E/K57E/W61A/D68A purification	Amp	This study
PCR primers	Sequence		
hns-KO-seq-F	CTCACGTGCTGCGAAATCATC		
hns-KO-seq-R	AAGCCTGGCTTGAAGAAGAG		
stpA-KO-seq-F	TAATCTCGCGCAGGACTG		
stpA-KO-seq-R	ATTAATCGGCGTTGTACTGG		
hns-mFP-in-F	CCTCGACGATTTCTGATCAAGCAATCGGCTGGCTCCGCTGCTGG		
hns-mFP-in-R	TAAGCAAGTGCAATCTACAAAAGACAAGCTTATGAATATCCTCCTTAGTTC		
hns-mFP-out-F	AAGCAATGGATGAGCAAGGTAAATCCCTCGACGATTTCTGATCAAG		
hns-mFP-out-R	AAAAAATCCCGCCGCTGGCGGGATTTTAAGCAAGTGCAATCTACAAAAG		
hns-seq-F	CCGAACGAAGTCTGAATAGC		
stpA-mFP-in-F	GGTAAATCTCTCGACGATTTCTGATCTCGGCTGGCTCCGCTGCTGG		
stpA-mFP-in-R	TAGCAGCGACATCCGGCCTCAGTAACAAGCTTATGAATATCCTCCTTAGTTC		
stpA-mFP-out-F	CAATTGCTCAGGCGCTGGCAGAAGGTAAATCTCTCGACGATTTCTGATC		
stpA-mFP-out-R	TTGAGAAGCGACGCCGACGCGCCCTAGCAGCGACATCCGGCCTC		
stpA-seq-F	GAAAGCTGACGGAATTAACCCG		
pTRC99a-seq-R	GCCCAGTAGCTGACATTCATC		
lon-KO-seq-F	GTGAAGCACAGTCGTGTCATCTG		
lon-KO-seq-R	CAGTTTCAGCATCTGCAATATAGAG		
hupA-mFP-in-F	CAAGGCACTGAAAGACGCGAGTTAAGTCGGCTGGCTCCGCTGCTGG		
hupA-mFP-in-R	TAAAACTGTTCACTGCCACGCAATCCAAGCTTATGAATATCCTCCTTAGTTC		
hupA-mFP-out-F	CTAACGTACCGGCATTTGTTTCTGGCAAGGCACTGAAAGACGCGAGTTAAG		
hupA-mFP-out-R	AAAAGGGGTGAAACCACCCCTTCGTTAAAACTGTTCACTGCCACGCAATC		
hupA-seq-F	CAAAACCCAGGCTAAAGCTGC		
dps-mFP-in-F	GTGGTTTATCGAGTCTAACATCGAATCGGCTGGCTCCGCTGCTGG		
dps-mFP-in-R	AAGTTCTGCACCATCAGCGATGGATCAAGCTTATGAATATCCTCCTTAGTTC		
dps-mFP-out-F	CGTCTCGCGACCTGGATAAATTCCTGTGGTTTATCGAGTCTAACATCGAATC		
dps-mFP-out-R	AGGAAGCCGCTTTTATCGGGTACTAAAGTTCTGCACCATCAGCGATG		
dps-seq-F	GGGACCACTCAAGTTATCAACAG		
ihfA-mFP-in-F	CGAAAACGCTTCGCCCCAAAGACGAGTCGGCTGGCTCCGCTGCTGG		
ihfA-mFP-in-R	GAGCGGCCTTTTTAGTTAGATCAGACAAGCTTATGAATATCCTCCTTAGTTC		
ihfA-mFP-out-F	CCGGGCAGAAGTTAAAAAGCCGGGTCGAAAACGCTTCGCCCCAAAGAC		
ihfA-mFP-out-R	GACAGTGAAAAGAAAAAAGGCCGCGAGAGCGGCCTTTTTAGTTAGATCAGA		
ihfA-seq-F	GGATGCCAAAGAAGTGGTTGAAC		
lrp-mFP-in-F	TAATCGTCTGGTTATTAAGACGCGCTCGGCTGGCTCCGCTGCTGG		
lrp-mFP-in-R	ACGCCGATTTTGCACCTGTTCCGTGCAAGCTTATGAATATCCTCCTTAGTTC		
lrp-mFP-out-F	TTGTTATGGAAGAAGTCAAGCAGAGTAATCGTCTGGTTATTAAGACGCGC		
lrp-mFP-out-R	GGATTAACAGGAGTGAATCAAAATACGCCGATTTTGCACCTGTTC		
lrp-seq-F	GTTGAGATTACTCTGAATCGTGGC		
fis-mFP-in-F	AAAATTGAAAAAATACGGCATGAACTCGGCTGGCTCCGCTGCTGG		
fis-mFP-in-R	TAATCAAGCATTTAGCTAACCTGAACAAGCTTATGAATATCCTCCTTAGTTC		
fis-mFP-out-F	GCATCAACCGTGGTACGCTGCGTAAAAAATTGAAAAAATACGGCATGAACTC		

fis-mFP-out-R	TTCCCCATGCCGAGTAGCGCCTTTTAAATCAAGCATTTAGCTAACCTGAAC
fis-seq-F	ACTCGGTAAACAGGCACTG
hfq-mFP-in-F	GCAACAGGACAGCGAAGAAACCGAATCGGCTGGCTCCGCTGCTGG
hfq-mFP-in-R	CCGTGTAAAAAACAGCCCGAAACCCAAGCTTATGAATATCCTCCTTAGTTC
hfq-mFP-out-F	GTAGCAGCGCGCAGAATACTTCCGCGCAACAGGACAGCGAAGAAACCGAA
hfq-mFP-out-R	CGGGGAACGCAGGATCGCTGGCTCCCCGTGTAAAAAACAGCCCGAAACC
hfq-seq-F	ATGGCTAAGGGGCAATCTTTACAAG
stpA-F21C-2-F	ATGGCTCGCGAATGCTCCATTGACGTTCTTGAAG
stpA-1-F	ATGGCTCGCGAATTCTCCATTGACGTTCTTG
stpA-F21C-1-R	CTTCAAGAACGTCAATGGAGCATTTCGCGAGCCATCGCACGGAG
stpA-no-tag-F	AGGTAAATCTCTCGACGATTTCTGATCTAAGTGTAGGCTGGAGCTGCTTC
hns-1-F	ATGAGCGAAGCACTTAAATTTCTG
hns-no-tag-F	CCTCGACGATTTCTGATCAAGCAATAAGTGTAGGCTGGAGCTGCTTC
stpA-R	GATCAGGAAATCGTCGAGAGATTTAC
hns-R	TTGCTTGATCAGGAAATCGTCGAG
hns-3xFLAG-in-F	CCTCGACGATTTCTGATCAAGCAAGACTACAAAGACCATGACGG
hns-3xFLAG-in-R	TAAGCAAGTGCAATCTACAAAAGACCTCCTTAGTTCCTATTCCG
FAM-waaj-F	5'FAM-TTGTGGACTCATTTCCTGCCATAG
waaj-150bp-R	GACAATTGATGTGATGGAAACACC
hdeB-FLAG-in-F	TCAAGCATCTAATGACTTGCCGAATGACTACAAAGACCATGACGG
hdeB-FLAG-in-R	GCTACTCCTTTTTACTTGCACCTCACCTCCTTAGTTCCTATTCCG
hdeB-FLAG-out-F	AGAAAAATTTGTATACCTTCAAAAATCAAGCATCTAATGACTTGC
hdeB-FLAG-out-R	AAGGAGCAGCAAGATGGCTCAACTTGCTACTCCTTTTTACTTGCACCTC
hdeB-seq-F	TGGATGCTGCATGAAGAAACAG
gadA-FLAG-in-F	CCAGCAGAACAGCTTTAAACACACCGACTACAAAGACCATGACGG
gadA-FLAG-in-R	ATTCGGTTTTTACAACGTTATGTTACCTCCTTAGTTCCTATTCCG
gadA-FLAG-out-F	ATCACCCGAAACTGCAGGGTATTGCCAGCAGAACAGCTTTAAACACAC
gadA-FLAG-out-R	CCAGCTGTTTTTTTTAAAGGCTGGGCATTTCGGTTTTTACAACGTTATGTTAC
gadA-seq-F	CAAACGTAAAGATGGTGAAGATC
dsDNA	Sequence
H-S-S	ATGAGCGAAGCACTTAAATTTCTGAACAACATCCGTA CTCTTCGTGCGCAGGC AAGAGAATGTACACTTGAAACGCTGGAAGAAATGCTGGAAAAATTAGAAGTT GTTGTTAACGAACGTCGCGAAGAAGAAAGCGCGGCTGCTGCTGAAGTTGAAG AGCGCACTCGTAAACTGCAGCAATATCGCGAAATGCTGATCGCTGACGGTATT GACCCGAACGAAGTCTGAATAGCCTTTCTGCTGCTGCACCACGCGCTGGTAA AAAACGCCAGCCGCGTCCGGCGAAATATAAATTCACCGATGTTAACGGTGAA ACTAAACCTGGACCGGTCAGGGCCGTACACCGAAGCCAATTGCTCAGGCGC TGGCAGAAGGTAAATCTCTCGACGATTTCTGATCTAA
S-H-H	ATGTCCGTAATGTTACAAAGTTTAAATAACATTTCGCACCCTCCGTGCGATGGC TCGCGAATTCTCCATTGACGTTCTTGAAGAAATGCTCGAAAAATTCAGGGTTG TCACTAAAGAAAGACGTGAAGAAGAAGAACAGCAGCAGCGTGAAGTGGCAG AGCGCCAGGAAAAAATTAGCACCTGGCTGGAGCTGATGAAAGCTGACGGAAT TAACCCGGAAGAGTTATTGGGTAATAGCGCTGCCGTTAAATCTGGCACCAAA GCTAAACGTGCTCAGCGTCCGGCAAAATATAGCTACGTTGACGAAAACGGCG AACTAAACCTGGACTGGCCAAGGCCGTACTCCAGCTGTAATCAAAAAAGC AATGGATGAGCAAGGTAAATCCCTCGACGATTTCTGATCAAGCAATAA
H-NS D2-5M	TCTATTATTACCTCAACAAACCACCCCAATATAAGTTTGAGATTACTACAATG AGCGAAGCACTTAAATTTCTGAACAACATCCGTA CTCTTCGTGCGCAGGCAA GAGAATGTACACTTGAAACGCTGGAAGAAATGCTGGAAAAATTAGAAGTTGT TGTTAACGAACGTCGCGAAGAAGAAAGCGCGGCTGCTGCTGAAGTTGAAGAG GAAACTCGTGAAGTGCAGCAAGCGCGGAAATGCTGATCGTGCAGGTTATTG ACCCGAACGAAGTCTGAATAGCCTTGCTGCCGTTAAATCTGGCACCAAAAGCT AAACGTGCTCAGCGTCCGGCAAAATATAGCTACGTTGACGAAAACGGCGAAA

	CTAAAACCTGGACTGGCCAAGGCCGTACTCCAGCTGTAATCAAAAAAGCAAT GGATGAGCAAGGTAAATCCCTCGACGATTTCTGATCAAGCAA
StpA D2-4M	TACGCGACGAAATACTTTTTTTGTTTTGGCGTTAAAAGGTTTTCTTTATTATGT CCGTAATGTTACAAAGTTTAAATAACATTTCGCACCCTCCGTGCGATGGCTCGC GAATTCTCCATTGACGTTCTTGAAGAAATGCTCGAAAAATTACAGGGTTGTCAC TAAAGAAAGACGTGAAGAAGAAGAACAGCAGCAGCGTGAACCTGGCAGAGGA ACAGGAAGAAATTAGCACCGCGCTGGAGCTGATGAAAGCTGCGGGAATTAAC CCGGAAGAGTTATTGGGTAATAGCTCTGCTGCTGCACCACGCGCTGGTAAAA AACGCCAGCCGCGTCCGGCGAAATATAAATTCACCGATGTTAACGGTGAAC TAAACCTGGACCGGTACAGGGCCGTACACCGAAGCCAATTGCTCAGGCGCTG GCAGAAGGTAAATCTCTCGACGATTTCTGATC
mTurquoise2	ATGGTGTCCAAAGGGGAAGAATTATTCACGGGCGTCGTCCCAATCCTGGTAG AGCTCGATGGAGACGTGAATGGGCATAAATTCAGTGTAAAGCGGAGAGGGCGA AGGCGACGCAACATACGGAAAGCTGACCCTCAAGTTCATTTGCACAACGGGC AAATTGCCTGTGCCATGGCCGACATTGGTCACAACACTGAGTTGGGGTGTTCA ATGCTTCGCCCCGGTATCCCGATCACATGAAGCAACATGACTTTTTTAAAGTCGG CTATGCCGGAAGGATATGTACAAGAACGTACTATTTTTTTAAAGACGATGGC AACTATAAAACACGGGCTGAGGTAAAATTCGAAGGTGACACGCTCGTGAAC GTATTGAATTAAAAGGCATCGACTTTAAGGAGGATGGAACATTCTGGGCCA TAAGCTGGAGTATAACTATTTCTCGGATAACGTGTATATTACCGCAGATAAGC AAAAAAATGGTATCAAGGCAAATTTTAAAATCCGGCATAACATCGAGGACGG GGGGGTGCAGTTGGCTGACCACTACCAGCAGAACACGCCTATTGGGGATGGC CCTGTATTGCTGCCAGACAACCATTATCTCAGCACGCAGTCCAAGCTGAGCAA AGATCCAAACGAAAAACGTGACCACATGGTTTTATTGGAGTTCGTACAGCC GCTGGCATTACTCTGGGCATGGATGAGTTATACAAATAA
mBaoJin	ATGGTTAGTAAAGGTGAAGAAGAAAACATGGCCTCCACGCCGTTTAAATTCC AGCTGAAAGGCACCATCAACGGCAAAAGCTTTACCGTTGAAGGTGAAGGTGA AGGCAACAGCCATGAAGGCTCTCACAAAGGCAAATATGTCTGCACCAGCGGC AAACTGCCGATGAGCTGGGCGGGCGCTGGGTACAACCTTTGGCTATGGCATGA AATATTACACCAAATATCCGTCAGGTCTGAAAAACTGGTTCCGTGAAGTGATG CCGGGTGGTTTTACCTATGACCGCCACATCCAGTATAAAGGCGATGGCAGCAT CCATGCGAAACACCAGCACTTTATGAAAAACGGCACCTACCACAACATTGTT GAGTTCACCGGTCAGGACTTCAAAGAGAACTCTCCGGTGCTGACCGGTGATAT GAACGTCTCGCTGCCGAATGAAGTGCCGCAGATCCCGCGTGATGATGGTGTA GAATGCCCGGTCACGCTGCTGTATCCGCTGCTGTCTGACAAAAGCAAATATGT TGAAGCGCACCAAGTATACCATCTGCAAAACCGCTGCATAACCAGCCGGCGCCG GATGTGCCGTATCACTGGATCCGCAACAGTACACCCAGAGCAAAGATGATG CGGAAGAGCGCGACCACATCTGCCAGAGCGAAACGCTGGAAGCGCACCTGAA AGGCATGGATGAGCTGTACAAA

Chapter 4 Future Directions

The overarching goal of my thesis is to investigate the role of biomolecular condensates in bacterial stress response. I focused on two small DNA/RNA binding proteins whose condensation allows bacteria to adapt to and survive from stress such as starvation and heat. The first part of my thesis explored the formation, composition and function of a stress-induced bacterial RNP condensate. I showed that polyP scaffolds the RNA chaperone Hfq into high-order oligomers, which then phase separates into condensates known as H-bodies. PolyP directs H-bodies to specifically enrich and stabilize polyadenylated RNAs, thereby improving bacterial survival and recovery from starvation. In the second part of my thesis, I investigated the interplay between bacterial DNA-binding protein homologs H-NS and StpA. I revealed that while StpA forms fibrillar aggregates on its own, it can be dissolved by H-NS and stabilize the latter under unfavorable conditions. By tuning the relative abundance of H-NS and StpA, bacteria dynamically balance chromosomal fluidity and stability. Here, I will discuss the limitations and potential future directions based on my thesis work.

4.1 General limitations of the study

While my work demonstrated the involvement of biomolecular condensates in bacterial response, it remains challenging to definitively establish a causative, rather than correlative, relation between condensate formation and enhanced fitness upon stress. This is largely due to the inherent difficulties in specifying condensate-specific functions from the functions of its constituent molecules. Apart from a few examples (279), mutations that disrupt condensate formation will inevitably affect other essential properties such as RNA binding, oligomerization and substrate recognition. In my work described in Chapter 2, while loss of polyP impairs H-body formation, it may potentially affect Hfq's RNA interactome due to the physicochemical similarities between polyP and RNA. The flexible and charge-dense nature of polyP also makes it extremely challenging to exclude interactions between polyP and other proteins, which might involve in enhancing bacterial fitness as well. Future studies may benefit from identifying Hfq mutants that is still capable of binding polyP/RNA but do not form condensates. While a $\Delta tmaR$ mutant may

partially fulfil such requirements, any phenotypic consequences should be interpreted with caution since TmaR is also involved in carbohydrate metabolism (280). In the work described in Chapter 3, it remains to be seen whether functions related to H-NS/StpA condensates can be teased apart from those related to H-NS/StpA nucleoprotein filaments. While my work suggests that StpA can stabilize H-NS condensates upon stress, earlier studies have shown that under non-condensing conditions, StpA can enhance the *in vitro* transcriptional silencing ability of H-NS filaments upon high temperature (217). Therefore, it remains to be seen whether the stress-associated defects of a $\Delta stpA$ strain observed in our study is truly due to condensate dissipation or intrinsic instability of H-NS filaments. A potential approach to answering this question is to introduce mutants in H-NS's linker domain. Since several positively charged residues in this domain participate in DNA binding and filament assembly (281, 282), linker mutants should be designed with caution to minimize the impact on these properties.

Another limitation in my work is that for live cell imaging, I have to rely on fluorescent protein tags that are much larger than the proteins of interest. While immunofluorescence imaging with FLAG-tagged protein have excluded the possibility of tag-induced artifacts, fusing a small protein with a large tag will inevitably affect its diffusion and thereby alter the biophysical properties of the *in vivo* condensates. Future studies may benefit from *in vivo* protein labelling via incorporation of fluorescent unnatural amino acids (283), which minimizes size perturbation on small proteins.

4.2 Molecular basis of polyP-protein interactions

The key to understanding how polyP affects H-body formation and function is to obtain high-resolution structure of Hfq-polyP complexes. Identifying critical residues that directly mediate Hfq-polyP interactions would also help to design Hfq mutants that are defective in polyP, but not nucleic acids binding. However, obtaining high-resolution structures of Hfq-protein complexes using conventional structural approaches including X-ray crystallography and cryo-electron microscopy is challenged by the length heterogeneity of commercially available long-chain polyP, disordered nature of Hfq's C-terminal domain (CTD) (79, 284), as well as Hfq hexamer's propensity to form highly heterogeneous higher oligomers at high concentrations (285, 286). Nuclear magnetic resonance (NMR) spectroscopy serves as a more ideal tool to access the dynamic structures of Hfq-polyP complexes. NMR, especially ^{15}N and ^{13}C dual isotope labelling NMR can be tailored to resolve dynamic structures in solution state to atomic level (287). It has been used to probe for interactions between Hfq and RNAs (288, 289) as well as between the core and C-

terminal domains (284). It would be fruitful to use NMR to understand the key residues that directly mediate Hfq-polyP interactions. More importantly, Hfq form well-defined “monomers of hexamers” (~66 kDa) in the presence of short chain polyP (~60-mer) (150), which falls within the size limit of current protein NMR techniques (290). Additionally, Hfq-polyP interactions can be computationally studied using MD simulation or molecular docking, which has been successfully demonstrated in probing for the atomistic interactions between polyP and α -synuclein fibrils (291). Although more experiments are needed, my biochemical assays have already provided important insights into the structural details of Hfq-polyP binding. While DNA, which binds to Hfq’s proximal face, is competed off from Hfq by polyP, polyadenylated RNA, which binds to Hfq’s distal face, forms stable tertiary complexes with Hfq and polyP (Fig. 2.4D and (150)). This suggests that polyP might preferentially interact with Hfq’s proximal face, therefore only RNAs with poly(A) tracts can remain stably associated with Hfq under high levels of polyP. These results can potentially explain how polyP promotes the stabilization of polyadenylated transcripts (Fig. 2.5L). Earlier studies suggest that Hfq binding to polyadenylated tails could stabilize RNAs by blocking access of RNase E and PNPase to their cleavage sites (85, 292). Therefore, under polyP-accumulating conditions, polyadenylated transcripts remain Hfq-bound and protected from RNA degradosome activity, while other transcripts are fully exposed to enzymatic cleavage and decay. Techniques that allow probing of RNA motifs directly interacting with Hfq, such as CLIP-seq, may provide more insight into this scenario. Moreover, since all sRNAs contain a poly(U) tract that binds to Hfq’s proximal face, sRNA-mediated mRNA decay may be globally suppressed under polyP-accumulating conditions. β -galactosidase assays that measures sRNA-mRNA pairing activity (293) should be performed in N-starved *E. coli* in the future to test this hypothesis.

4.3 RNase E activity in H-bodies

An apparently paradoxical finding in Chapter 2 is the presence of RNA degradosome proteins and the proposed RNA-stabilizing function of H-bodies. While Hfq’s protective effect on RNAs with poly(A) tails (discussed above) may provide a partial explanation, it could not explain the benefit of recruiting RNA decay machineries into an RNA storage compartment. An outstanding question regarding this paradox is whether RNase E (and other enzymes) are active inside the H-body. A recent study in *C. crescentus* shows that accumulation of ribosome-depleted RNAs in stationary phase causes BR-bodies to rigidify from a liquid-like condensate into a solid-like state, thereby converting their function from mRNA decay to storage. Upon nutrient replenishment, BR-bodies

disassemble and release stored RNAs, allowing rapid translation to support recovery growth (75). While directly measuring enzymatic activity in live cells is challenging, quantitative assessment of RNA degradosome activity can be assisted by using MS2 reporter system (294). Fluorescently labelled MS2 coat proteins (FP-MCPs) are co-expressed with RNAs of interest, which contain inserts of repeat MS2 low-affinity binding sites (294). To maximally reduce background signal from free FP-MCPs, MCPs are engineered to be highly unstable unless in association with cognate RNA ligands (295). Tracking fluorescence decay over time following transcriptional shutoff allows real-time measurement of RNA stability. This approach has been successfully demonstrated in *C. crescentus* and yeast (75, 294), and may also be applied to *E. coli* H-bodies in the future.

In vitro assays using reconstituted condensates may also provide insight into RNase E activity in H-bodies. Sequestration of enzymes and substrates into condensates can greatly increase enzymatic activity (1). This is shown by enhanced exonuclease activity in reconstituted *C. crescentus* PNPase/RNase E-CTD condensates (296). On the other hand, condensate aging may negatively affect enzymatic activity (1), as shown by reduced endonuclease activity in aged *C. crescentus* RNase E condensates (75). Therefore, reconstituting *E. coli* Hfq-RNase E condensates in the presence of varying levels of polyP may reveal how recruitment of RNase E into H-bodies affects its enzymatic activity.

Finally, earlier studies reveal an imperfect colocalization between H-body and RNase E (88). This is likely because Hfq is a cytoplasmic protein while *E. coli* RNase E is typically membrane-anchored (297), which may result in an RNase E gradient within H-bodies with higher RNase E abundance near the cell membrane. Future experiments employing single-molecule tracking on both Hfq and RNase E may be helpful to test this hypothesis.

4.4 Potential overlap between polyP granules and bacterial RNP condensates

While my work show that polyP is present in *E. coli* H-bodies, it should be noted that these bodies are distinct from electron-dense, DAPI-sensitive polyP granules observed in many other organisms such as *Pseudomonas aeruginosa* and *C. crescentus* (122, 131). Interestingly, it is recently reported that polyP granules in *C. crescentus* partially colocalize with BR-bodies (298). It would therefore be intriguing to investigate how such interactions reciprocally affect the material properties and function of these two types of condensates. As the heterogeneous colocalization between RNase E and polyP resembles that between Hfq and RNase E (73, 298), it is possible that Hfq might serve as the bridge between types of condensates in *C. crescentus*. Furthermore, it would be important

to understand why *E. coli* does not typically produce large polyP granules despite the ability to accumulate high levels of polyP. It was shown that upon ectopic expression in *E. coli*, *C. crescentus* PPK forms distinct foci, yet no polyP granules are observed (131). In contrast, expressing *E. coli* PPK in *C. crescentus* results in polyP granules similar to those observed in wild-type cells (131). These results suggest that the ability to form polyP granules is likely due to factor(s) present in the cytoplasm, rather than intrinsic properties of the PPK enzyme.

4.5 Possible role of polyP in eukaryotic RNP body formation and function

Work in Chapter 2 reveals that polyP is enriched specifically in mammalian P-bodies, and reducing polyP levels leads to decrease in P-body numbers (Fig. 2.6). These findings suggest a fruitful direction for future investigations. Flow particle sorting-based approaches allows quantitative identification of P-body components (97), and it would be intriguing to perform similar analyses in mammalian cells ectopically expressing PPK and PPX. This may help to identify proteins and RNAs that are recruited into or excluded from P-bodies in a polyP-specific manner. It is also interesting to test whether the LSM1-7 complex, which is essential for P-body formation and evolutionarily conserved with Hfq (95, 299), directly interacts with polyP. This can be tested using native western blot as described in Chapter 2, or EMSA with purified mammalian LSM proteins. Yeast is a nice model organism for studying eukaryotic polyP functions due to its known polyP synthesis machinery and high genetic tractability (300, 301). Yeast contain P-bodies and a *vtc4* deletion strain, which is unable to produce polyP, can be used to study the role of polyP in P-body formation. Most importantly, it is much easier to perform endogenous protein tagging in yeast than in mammalian cells, thus allowing efficient assessment of live-cell P-body dynamics in presence and absence of polyP.

4.6 Structural nature of StpA fibril formation and dissolution

A key observation in Chapter 3 is the formation of StpA aggregates and their conversion into condensates. While our cryo-ET and TEM data provide insights into their fibrillar nature and tight packing at high concentrations, the exact molecular basis of these behaviors are unclear due to the lack of high-resolution structures. However, combining current experimental and computational data, I propose the following hypothesis: StpA fibrils can be dissolved by very high levels of salt or even sub-stoichiometric amounts of H-NS, suggesting that they likely represent a class of “dissociable aggregates” (302) that is distinct from the highly static and hardly dissociable amyloid

fibrils (303). Therefore, it is possible that StpA fibrils may undergo rare but non-trivial dissociation events that could not be captured by our observations, thus allowing slow exchange between monomers and small oligomers within and outside the fibrils. Previous studies suggest that H-NS and StpA preferentially form heterodimers in each other's presence (216). Upon addition to pre-formed StpA fibrils, H-NS, which mainly exists as short oligomers (237), slowly integrate into StpA oligomers due to the preference for forming heterotypic D1 contacts. This introduces one or more homotypic D2 contacts formed solely by H-NS. These contacts serve as weak points within oligomers that are prone to dissociation, thus causing the shortening in oligomer size. Reduction of oligomer size beyond a certain threshold eventually triggers the transition from aggregate-like assemblies to condensates or other soluble states. In the future, this hypothesis can be tested by single molecule tracking combined with total internal reflection fluorescence (TIRF) microscopy. Fluorescently labeled H-NS is added at very low concentrations to single, fluorescently labeled StpA fibrils tethered to glass coverslips. Their incorporation into fibrils can be observed with time. Small angle X-ray scattering can be used under high salt to measure the length distribution of H-NS/StpA homo- and hetero-oligomers. It is expected that even under non-aggregating conditions, incorporation of H-NS monomers will reduce the length of StpA fibrils. These experiments can be performed in parallel with MD simulation of pre-constructed StpA fibrils and free H-NS tetramers. Finally, preparing less intertwined StpA fibrils at lower concentrations may allow structural analysis by cryo-EM, thereby providing high-resolution data that may eventually reveal the structural nature of these assemblies.

4.7 Exploring the diversity of H-NS-like homologs

Gram negative bacteria, including *E. coli*, often encode multiple homologs of H-NS. As an extreme example, the β -proteobacterium *Burkholderia vietnamiensis* harbors no less than eighteen H-NS homologs (304). However, the function and biophysical properties of these proteins have received little attention. Among the very few studies, it is reported that Sfx, a H-NS homolog encoded by the R6K plasmid, forms condensates with R6K that excludes H-NS both *in vivo* and *in vitro* (305). A combination of cryo-ET and MD simulation may provide structural insights into these distinct phase behaviors. Similar NAP homologs also exist beyond the H-NS family. *Pseudomonas aeruginosa* encode MvaT/MvaU homologs that are distantly related to H-NS (306), while functional analogs of H-NS are also present in Gram positive bacteria in the form of intragenomic homologs, such as Rok/sRok in *Bacillus subtilis*, Lsr/LsrL in *Streptomyces venezuelae* and

Lsr/MSMEG_4727 in *Mycobacterium smegmatis* (307-309). Whether these homologs pairs also display distinct phase behavior and/or functional divergence would be a fruitful topic of research.

4.8 Genomic binding landscape of H-NS and StpA under different conditions

As DNA binding proteins, it is important to understand how the genomic binding landscapes of H-NS and StpA are regulated across different growth conditions and StpA:H-NS ratios. While it is widely accepted that both homologs have highly overlapping genomic binding sites (214, 215), it is unclear whether the two proteins are evenly distributed across a mixed nucleoprotein filament. Since StpA is sensitive to Lon-mediated proteolysis when not protected by H-NS, it is likely that long tracts of StpA nucleoprotein filaments will not exist *in vivo*, unless stabilized by Lon deletion or F21C mutation. Furthermore, H-NS is known to nucleate at AT-rich high-affinity binding sites and polymerize and spread to neighboring regions (310). As StpA stabilizes H-NS filaments, it is possible that higher StpA:H-NS ratio might result in an increase in H-NS nucleoprotein filament length. These questions can be addressed in the future by chromatin immunoprecipitation sequencing (ChIP-seq) or ChIP exonuclease (ChIP-exo), which allows access to protein-DNA binding sites to nucleotide resolution (311). Altering StpA:H-NS ratios can be achieved by growing bacterial strains at different temperatures or, more ideally, modifying the respective ribosome binding site and promoter strengths of *hns* and *stpA*.

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