



**Binding of the RNA chaperone Hfq to the type IV pilus base  
is crucial for its function in *Synechocystis* sp. PCC 6803**

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**1 Binding of the RNA chaperone Hfq to the type IV pilus base is crucial for its**  
**2 function in *Synechocystis* sp. PCC 6803**

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19

**20 Running Title**

21 Localization of Hfq in cyanobacteria

22

## 1    **Summary**

2    The bacterial RNA-binding protein Hfq functions in post-transcriptional regulation of  
3    gene expression. There is evidence in a range of bacteria for specific sub-cellular  
4    localization of Hfq, however the mechanism and role of Hfq localization remain  
5    unclear. Cyanobacteria harbour a subfamily of Hfq that is structurally conserved but  
6    exhibits divergent RNA-binding sites. Mutational analysis in the cyanobacterium  
7    *Synechocystis* sp. PCC 6803 revealed that several conserved amino acids on the  
8    proximal side of the Hfq hexamer are crucial not only for Hfq-dependent RNA  
9    accumulation but also for phototaxis, the latter of which depends on type IV pili. Co-  
10   immunoprecipitation and yeast two-hybrid analysis show that the secretion ATPase  
11   PilB1 (a component of the type IV pilus base) is an interaction partner of Hfq.  
12   Fluorescence microscopy revealed that Hfq is localized to the cytoplasmic membrane  
13   in a PilB1-dependent manner. Concomitantly, Hfq-dependent RNA accumulation is  
14   abrogated in a  $\Delta pilB1$  mutant, indicating that localization to the pilus base via  
15   interaction with PilB1 is essential for Hfq-function in cyanobacteria.

16

## 17   **Introduction**

18   The RNA chaperone Hfq is conserved in a wide range of bacterial phyla and plays an  
19   important role in post-transcriptional gene regulation (reviewed in Vogel and Luisi,  
20   2011). It belongs to the family of Sm/Sm-like proteins (Lsm), and is structurally and  
21   functionally related to the distant archaeal and eukaryotic homologs. The proximal  
22   and the distal faces of the characteristic hexameric torus structure of Hfq contribute  
23   to its RNA-binding capabilities, although both faces possess a distinct binding site

1 with divergent sequence specificities (Schumacher *et al.*, 2002; Link *et al.*, 2009).  
2 Additionally, charged residues on the lateral surface of the hexamer are involved in  
3 RNA binding (Sauer *et al.*, 2012). Hfq binds to *trans*-encoded sRNAs as well as their  
4 target mRNAs, thus facilitating base pairing between the two strands with limited  
5 sequence complementarity. Hfq-binding and/or duplex formation may subsequently  
6 regulate gene expression at the level of RNA stability or translation (Vogel and Luisi,  
7 2011).

8        Apart from binding RNA, Hfq is known to interact with protein partners many of  
9 which are involved in RNA metabolism. In *E. coli* Hfq recruits RNase E for the  
10 degradation of bound RNAs (Morita *et al.*, 2005) and regulates polyadenylation  
11 dependent RNA decay in a complex with polynucleotide phosphorylase and poly(A)  
12 polymerase (Mohanty *et al.*, 2004). Hfq mediates antitermination by forming a  
13 complex with Rho (Rabhi *et al.*, 2011) and there is evidence that Hfq associates with  
14 the RNA polymerase via the ribosomal protein S1 (Sukhodolets and Garges, 2003;  
15 Windbichler *et al.*, 2008) in *E. coli*. Argaman *et al.* (2012) demonstrated that RelA, the  
16 regulator of bacterial stringent response, enhances multimerization of Hfq monomers  
17 and RNA binding by an unknown process. Furthermore, a large scale analysis  
18 revealed that Hfq directly or indirectly interacts with over 30 proteins of diverse (multi-  
19 protein complexes) (Butland *et al.*, 2005). Different studies regarding the subcellular  
20 localization of Hfq in *E. coli* produced ambiguous data and confirmed localization in  
21 the cytoplasm and the nucleoid (Kajitani *et al.*, 1994; Azam *et al.*, 2000) or mainly to  
22 the cell membrane (Diestra *et al.*, 2009). However, it remains unclear which factors  
23 contribute to the specific subcellular and probably dynamic localization of Hfq in the  
24 bacterial cell.

1 Cyanobacteria are organisms conducting a phototrophic lifestyle. They  
2 perform oxygenic photosynthesis and are evolutionarily related to chloroplasts. The  
3 sequences of cyanobacterial Hfq orthologues are highly conserved among all  
4 cyanobacteria but quite distinct from those of other bacteria. Nevertheless, crystal  
5 structures of cyanobacterial Hfq homologs revealed that they retain a perfect Sm-fold  
6 and form the typical homohexameric torus structure (Bøggild *et al.*, 2009). However,  
7 the electrochemical surface is very distinct and the Hfq homolog of *Synechocystis* sp.  
8 PCC 6803 (hereafter *Synechocystis*) binds known enterobacterial sRNAs with very  
9 low affinity and is not able to complement an *E. coli hfq* mutant (Bøggild *et al.*, 2009),  
10 which raises the question about conservation of the described functions and structure  
11 of Hfq in cyanobacteria. Compared with the wide-ranging effects of inactivation of *hfq*  
12 in other bacteria, genetic inactivation of the *hfq* gene in different cyanobacteria  
13 resulted in relatively restricted phenotypes. In the filamentous nitrogen-fixing  
14 cyanobacterium *Nostoc* sp. PCC 7120, Hfq was found to be important for nitrate and  
15 nitrite assimilation (Puerta-Fernández and Vioque, 2011). A *Synechocystis*  $\Delta hfq$   
16 mutant showed a defect in type IV pilus (T4P) biogenesis and a decreased  
17 accumulation of several mRNAs centred on alterations in transcripts that are under  
18 the control of a cAMP receptor protein (CRP)-like transcription factor (SyCRP1)  
19 (Dienst *et al.*, 2008) which is involved in T4P biogenesis (Yoshimura *et al.*, 2002).  
20 Using T4P, *Synechocystis* exhibits flagella-independent “twitching motility” and is  
21 capable of phototaxis i.e. directed movement along a light gradient (Bhaya *et al.*,  
22 1999). Mutation of *hfq* in *Synechocystis* consequently leads to a loss of motility and  
23 phototactic response (Dienst *et al.*, 2008). Though many sRNAs have been reported  
24 for *Synechocystis* (Georg *et al.*, 2009; Mitschke *et al.*, 2011) little is known about their

1 roles in cellular functions. Moreover, neither binding of these sRNAs to Hfq nor  
2 involvement of protein interaction partners in Hfq-function has been demonstrated for  
3 cyanobacterial Hfq proteins.

4       Considering the divergent properties of cyanobacterial Hfq homologs  
5 compared to other bacteria, we elucidated which single amino acid residues are  
6 important for Hfq-function in *Synechocystis*. These are very different from standard  
7 Hfq proteins and are located outside the non-conserved putative RNA binding  
8 pocket. As we suppose that the RNA-binding properties of the cyanobacterial Hfq are  
9 very weak, if existing at all, we focused on identification of protein interaction  
10 partners. Here, we found that Hfq is localized to the cytoplasmic membrane by  
11 binding to the pilus subunit PilB1. Most importantly, we conclude that function of Hfq  
12 depends on its correct localization.

## 14 **Results**

### 15 *Sequence conservation of cyanobacterial Hfq homologs*

16 Hfq homologs in cyanobacteria were originally discovered by BLAST search  
17 (Valentin-Hansen *et al.*, 2004) but show great divergence from other bacterial Hfq  
18 proteins. To better address distribution and conservation of cyanobacterial Hfq  
19 proteins, we did a new BLAST search including the novel CyanoGEBA dataset (Shih  
20 *et al.*, 2013). Sequence analysis revealed that 115 out of 132 cyanobacterial  
21 genomes harbour an *hfq* homolog (Fig. S1). Obligate symbionts and strains from the  
22 genus *Prochlorococcus* mainly lack a discernible homolog. Examining the amino acid  
23 conservation with ConSurf (Ashkenazy *et al.*, 2010), it is remarkable that all of the

1 most conserved residues contribute to the surface of the proximal site (Fig. 1A, B).  
2 Around the entire torus, residues from the N-terminal  $\alpha$ -helix and the ultraconserved  
3 WQD-motif form an area of conserved amino acids which encloses the proximal RNA  
4 binding site. We will refer to this area as the proximal outer ring. On the other hand,  
5 structurally conserved residues contributing to the proximal (Schumacher *et al.*, 2002;  
6 Bøggild *et al.*, 2009), distal (Link *et al.*, 2009) and lateral (Sauer *et al.*, 2012) RNA  
7 binding sites are generally not highly conserved among cyanobacterial Hfq proteins  
8 (Fig. 1A, B and Fig. S2).

9 *The classical RNA-binding pockets seem not to be essential for Hfq function in*  
10 *Synechocystis*

11 The lack of conservation on sequence level and an unfavourable surface charge  
12 (Bøggild *et al.*, 2009) make the known RNA-binding pockets of Hfq an unlikely  
13 candidate for RNA-binding in *Synechocystis*. To verify this assumption, we  
14 constructed different Hfq variants carrying single-amino-acid substitutions of residues  
15 potentially involved in RNA-binding mainly because of its conserved position in the  
16 hexamer as deduced from structural information (Bøggild *et al.*, 2009). These  
17 mutated Hfq variants were introduced into a  $\Delta hfq$  mutant strain and tested for their  
18 ability to complement the mutant phenotype. We used the conjugative shuttle vector  
19 pVZ321 to express the Hfq derivatives Q15R (conserved residue on the proximal  
20 face of cyanobacterial Hfq proteins), G31N (altering the distal binding site), T43D and  
21 D44K (altering the proximal binding site) (Fig. 1A) under the control of the native *hfq*  
22 promoter in a  $\Delta hfq$  strain. In contrast to the non-motile  $\Delta hfq$  strain, all four strains  
23 expressing mutant Hfq variants showed positive phototaxis towards unidirectional

1 white light comparable to the wild type (Fig. 1C). As a control we also studied  
2 accumulation of the sRNA SyR14 in the RNA-binding pocket mutants. This sRNA  
3 identified in dRNA-seq (Mitschke *et al.*, 2011) shows altered transcript accumulation  
4 in cells lacking Hfq due to an unknown mechanism. A supposed processing product  
5 of SyR14, which accumulates in the wild type, was not detected in the  $\Delta hfq$  mutant  
6 and overall transcript abundance was reduced (Fig. 1D). In contrast to the altered  
7 SyR14 accumulation in  $\Delta hfq$ , it is clearly seen that the putative RNA-binding pocket  
8 mutants accumulated wild-type levels of SyR14 and of its processing product (Fig.  
9 1D). In addition, we purified a 3xFLAG tagged version of Hfq from cell extracts in  
10 order to identify RNAs that are bound to Hfq (Fig. S4B). However, neither SyR14 nor  
11 other known Hfq-dependent sRNAs were found to be enriched in Hfq purifications.  
12 Taken together, our results as well as surface charge distribution imply that the  
13 putative RNA-binding pockets are not crucial for the function of Hfq in *Synechocystis*  
14 and most likely do not bind RNA.

#### 15 *Residues on the proximal outer ring are important for Hfq function*

16 In order to identify amino acid residues that are important for Hfq function, we  
17 performed random mutagenesis of the *hfq* gene sequence by error-prone PCR,  
18 followed by screening for motility. We used the cyanobacterial expression vector pUR  
19 (Wiegard *et al.*, 2013) containing an inducible promoter and a sequence encoding a  
20 3xFLAG-tag for detection of the protein. We had to use this mobilisable vector  
21 because the  $\Delta hfq$  mutant lost its natural competence for transformation due to the  
22 loss of T4P (Dienst *et al.*, 2008). The pUR-*hfq* derivatives harbouring the  
23 mutagenised *hfq* sequences were then transferred to  $\Delta hfq$  mutant cells via



1 conjugation and exconjugants were screened on low percentage agar plates under  
2 inducing conditions for motility. Most colonies showed the wild-type motile phenotype,  
3 meaning that either the introduced *hfq* sequence was not mutated in these clones or  
4 the mutation did not lead to a non-functional protein. In summary 116 non-motile  
5 clones that did not complement the  $\Delta hfq$  phenotype were subjected to Western Blot  
6 analysis using an antibody directed against the 3xFLAG tag that was fused to Hfq. In  
7 protein extracts of 38 of these clones we detected the Hfq protein in immunoblot  
8 analysis (Fig. S5), verifying the stability of the mutated protein variants. Sequence  
9 analysis revealed that 8 clones had single mutations leading to one amino acid  
10 residue change. In the other 30 clones that were not further analyzed *hfq* was  
11 mutated at more than one site, leading to two or more amino acid changes. Mutations  
12 led to following single amino acid replacements: R39H, Q15H, D42N, R3S, P9T,  
13 W40R, I18F, V58E (Fig. 1A, B). For seven mutants complete loss of motility was  
14 validated by phototaxis assays (Fig. 1E) and additionally, these mutants showed a  
15 defect in the accumulation and processing of SyR14 (Fig. 1F). Contrary to the initial  
16 screening the R3S mutant could partially complement the  $\Delta hfq$  phenotype showing  
17 reduced motility in the phototaxis assay and a faint signal for the SyR14 processing  
18 product, albeit the overall transcript accumulation was comparable to the non-  
19 functional mutants. Interestingly, Q15H could not complement the  $\Delta hfq$  phenotype,  
20 while the above mentioned Q15R mutant was indiscernible from the wild type. Most  
21 of the critical residues found in this approach are highly conserved among  
22 cyanobacteria and all (except V58) are congruent with the proximal outer ring while  
23 none of the critical residues contribute to the distal surface of Hfq, highlighting the  
24 functional importance of the proximal surface area (Fig. 1B).

# 1 *Hfq interacts with the T4P secretion ATPase PilB1*

2 Given the fact that binding of RNA to cyanobacterial Hfq proteins with high affinity  
3 could not be shown so far (Bøggild *et al.*, 2009), and our observations that Hfq-  
4 dependent RNAs were either not detected or enriched in Hfq purifications (Fig. S3B)  
5 and that the proximal and distal RNA-binding pockets most probably are not required  
6 for Hfq function, we hypothesized that Hfq may function differently from canonical  
7 bacterial Hfq proteins. We reasoned that the critical residues contributing to a large,  
8 highly conserved surface area on the proximal side of Hfq constitute an interface for  
9 protein interaction. To obtain a better understanding of the mode of action of Hfq, we  
10 carried out co-immunoprecipitation experiments in order to find protein interaction  
11 partners which may be important for Hfq function. Therefore we generated a  $\Delta hfq$   
12 strain expressing an N-terminally 3xFLAG tagged Hfq variant (Hfq-3xFLAG) under  
13 control of the *petJ* promoter from a neutral site in the chromosome (Kuchmina *et al.*,  
14 2012). The fusion protein was shown to be functional by Northern blot analysis and  
15 phototaxis assays (Fig. S3B, C) and was purified from the solubilised whole cell  
16 lysate with immobilized anti-M2 antibody and separated on SDS-PAGE. In the Hfq-  
17 3xFLAG elution fraction an abundant band migrating just below the 75 kDa marker  
18 could be detected that was absent in the control (Fig. S6). The corresponding protein  
19 could be unambiguously identified as the 75.1 kDa protein PilB1 (encoded by  
20 *slr0063*) by mass spectrometry. Intriguingly, PilB1 is the pilus assembly ATPase  
21 necessary for pilus extension in *Synechocystis* and thus,  $\Delta pilB1$  cells are non-motile  
22 (Yoshihara *et al.*, 2001). Therefore, if Hfq is also important for PilB1 function, this

1 could account for the absence of pili on the cell surface and the non-motile  
2 phenotype of the  $\Delta hfq$  mutant.

3 PilB1 is a member of the secretion ATPase superfamily which typically shows a two  
4 domain architecture with a membrane-facing N-terminal domain and a cytoplasm-  
5 facing C-terminal domain with a variable C2 subdomain at the C-terminus (Yamagata  
6 and Tainer, 2007). We observed that nearly all cyanobacterial PilB homologs  
7 possess a conserved C-terminal region of ~ 40 amino acids with four invariable C  
8 residues in a CXXC-X<sub>10</sub>-CPYC motif which is likely to coordinate a metal ion and  
9 resembles a zinc finger domain (Fig. 2A; Fig. S7). Moreover, the only strains of the  
10 genus *Prochlorococcus* harbouring an Hfq homolog (MIT 9313, MIT 9303; Fig. S1)  
11 are likewise the only two strains of the genus encoding a PilB protein with this  
12 putative zinc-finger domain. To address the significance of this domain and  
13 determine the Hfq-PilB1 interaction more accurately, we assessed the interaction of  
14 Hfq with different PilB1 domains in a yeast two-hybrid system (Fig. 2B). Employing  
15 Hfq as prey we screened for interaction with bait vectors containing either the N-  
16 terminal (PilB1-N; 1-366) or the C-terminal (PilB1-C; 367-672) PilB1 domain, the  
17 putative zinc-finger domain (PilB1-C2; 633-672) or a mutated variant with the  
18 conserved C substituted for A residues (PilB1-C2mut). Selective growth on adenine  
19 minus plates shows that Hfq interacts with both itself and PilB1-C, whereas no  
20 interaction was detected with PilB1-N. In particular, Hfq showed specific interaction  
21 with PilB1-C2 alone. Substitution of the conserved cysteines which allegedly  
22 abolishes the assumed metal coordinating abilities of this domain also abrogates  
23 protein interaction. Subsequently, we investigated the interaction of the non-  
24 functional Hfq variants with single amino acid substitutions (Fig. 1) with the C-  
10

1 terminal domain of PilB1, and additionally tested their ability to interact with a wild-  
2 type Hfq bait protein. Fig. 2C confirms direct binding of R39H, Q15H, D42N, P9T,  
3 W40R and I18F variants to the wild-type Hfq protein implying that these mutations do  
4 not interfere with Hfq hexamerization. By contrast, the V58E mutation abrogates  
5 subunit interaction, which is in accordance with the role of the Sm2 motive in subunit  
6 contact. Regarding the Hfq-PilB1 interaction we found that direct binding of Hfq to  
7 PilB1-C as well as PilB1-C2 is lost in all mutant variants apart from R39H and Q15H.  
8 These results confirm the Hfq-PilB1 interaction detected by co-immunoprecipitation  
9 and suggest that Hfq binds to the C-terminal domain of PilB1 via conserved  
10 functionally important residues on the proximal outer ring.

#### 11 *Hfq is localized to the cell periphery*

12 To determine if Hfq is indeed binding to the pilus base in *Synechocystis* we first  
13 tested its localization employing fluorescence microscopy. To begin with, we  
14 overexpressed N- and C-terminal Hfq-eYFP fusion proteins (eYFP-Hfq and Hfq-  
15 eYFP, respectively) using a conjugative vector and checked if the constructs could  
16 complement the  $\Delta hfq$  phenotype. Northern blot analysis (Fig. 3A) showed that only  
17 the strain expressing the Hfq-eYFP fusion protein accumulated SyR14 and the  
18 putative processing product comparable to the wild type. In agreement with this  
19 result, the strain also showed phototactic motility (Fig. 3B), while the eYFP-Hfq fusion  
20 was not able to re-establish motility in  $\Delta hfq$ . We then used cells grown on phototaxis  
21 plates for fluorescence microscopy. High intensity eYFP-fluorescence could be  
22 observed in both mutant strains (Fig. 3C), clearly indicating that eYFP-Hfq fusion is  
23 expressed but non-functional. Most importantly, only the functional Hfq-eYFP showed

1 distinct localization (Fig. 3C, left panel). In this strain we could observe small  
2 fluorescent spots along the entire cell periphery of the coccoid cells. In addition, we  
3 often detected one large bright spot in each cell, which also appeared to be located  
4 at the cell periphery, while very little fluorescence was detected in the cytoplasm. In  
5 contrast, we observed no membrane localization in the strain expressing non-  
6 functional eYFP-Hfq, where fluorescence was uniformly distributed in the cytoplasm.  
7 Hence, we conclude that Hfq is specifically localized to the cell membrane in  
8 *Synechocystis*. Localization to the cell membrane is disrupted by an eYFP tag at the  
9 N-terminus, and this loss of Hfq localization correlates with loss of phototaxis and  
10 incorrect processing of SyR14. To further explore the connection between  
11 localisation and function we analyzed the localisation of some of the non functional  
12 Hfq variants by constructing C-terminal eYFP fusion proteins. We chose the Hfq  
13 variants P9T, I18F and D42N (as one of the mutants from the highly conserved WQD  
14 motif) because they retained the ability to interact with wild-type Hfq monomers but  
15 showed no interaction with PilB1 in the yeast two-hybrid analysis (Fig. 2C). As shown  
16 in the fluorescence images (Fig. 4) localization in all strains expressing mutant Hfq  
17 variants was altered. Mutants I18F and D42N show very bright dispersed  
18 fluorescence in the cytoplasm, whereas in mutant P9T only one bright spot is visible.  
19 Importantly, the accumulation of small fluorescent spots along the cell periphery, as  
20 seen for wild-type Hfq-eYFP is clearly missing in P9T and I18F.

21

1

2 *Hfq-PilB1 interaction is crucial for the localization of Hfq, phototactic motility and*  
3 *transcript accumulation.*

4 Thus far, we have shown that Hfq localizes to the cell periphery and co-purifies with  
5 PilB1 which is supposed to interact with the pilus base at the cytoplasmic membrane.

6 To investigate whether the Hfq-PilB1 interaction is a prerequisite for the correct  
7 localization of Hfq, we analysed the localization of Hfq-eYFP in a *pilB1* knockout  
8 strain. Fluorescence microscopy revealed that the localization of multiple small  
9 fluorescence spots along the cell periphery is completely lost in the  $\Delta pilB1$   
10 background (Fig. 5A). Instead, Hfq-eYFP is concentrated in very intense spots,  
11 usually no more than one per cell and usually (but not always) at the cell periphery.

12 The change in Hfq-eYFP distribution is quantifiable and highly significant. In the  
13  $\Delta pilB1$  background, the mean number of Hfq-eYFP spots per cell observed in our  
14 confocal optical sections decreases from about 3.3 to 0.9, while the mean spot  
15 intensity increases by a factor of >4. In both cases, t-tests gave *p*-values <10<sup>-10</sup>.  
16 Together with the results from the co-immunoprecipitation experiment, this  
17 observation supports our hypothesis that Hfq is localized to the pilus base at the  
18 cytoplasmic membrane via an interaction with PilB1.

19 To explore if the localization of Hfq and/or its interaction with PilB1 is important  
20 for its function, we subsequently analyzed SyR14 accumulation in  $\Delta pilB1/\Delta hfq$  x Hfq-  
21 eYFP as well as in  $\Delta pilB1$  and  $\Delta pilC$  strains. We included the  $\Delta pilC$  mutant in the  
22 analysis because in the current model for T4P assembly it is believed that PilB exerts  
23 its role in pilus extension by interacting with the integral membrane protein PilC  
24 (Crowther *et al.*, 2004). Interestingly, none of the strains with inactivated *pilB1* or *pilC*

genes accumulated the SyR14 processing product in Northern blot experiments (Fig. 5B). Also, in accordance to the fundamental role PilB1 and PilC play in the assembly of T4P, these mutants showed no motility in a phototaxis assay (Fig. 5C). These findings support the notion that the direct interaction between PilB1 and Hfq and/or the localization to the pilus base is critical for Hfq function.

As a further test for direct interaction between Hfq and PilB1, we simultaneously visualised PilB1-sfGFP and Hfq-eYFP expressed in the  $\Delta pilB1$  background (Fig. 6). Phototaxis experiments verified that the C-terminally sfGFP tagged PilB1 protein is functional as it complements the  $\Delta pilB1$  mutant (Fig. S8A). Using appropriate settings on the confocal microscope we were able to visualise sfGFP and eYFP simultaneously, with no significant cross-detection or interference from the fluorescence of the photosynthetic pigments (Fig. S8B). As would be expected, PilB1-sfGFP fluorescence is strongly concentrated around the periphery of the cell (Fig. 6). The Hfq-eYFP fluorescence around the cell periphery shows partial co-localisation with PilB. Some concentrations of PilB-sfGFP fluorescence correspond precisely (at optical resolution) to concentrations of Hfq-eYFP fluorescence, but it is also possible to find concentrations of Hfq-eYFP fluorescence without a corresponding spot of PilB-sfGFP fluorescence, and *vice versa* (Fig. 6). Thus our co-localisation results are consistent with a direct PilB1-Hfq interaction, but they indicate that there is not a simple 1:1 association of the two proteins.

1

2 **Discussion**3 *The proximal outer ring but not the classical RNA-binding pockets are essential for*4 *Hfq function in Synechocystis*

5 The RNA binding pocket of Hfq is well conserved in most bacteria, however

6 sequence conservation of the residues surrounding the central cavity is relatively low

7 in cyanobacteria. Here, we show that mutations of residues (T43, D44) contributing to

8 the structurally conserved proximal RNA binding pocket do not compromise function

9 of the cyanobacterial Hfq protein. Furthermore, none of the other residues (R59, L60,

10 A61) implicated in RNA-binding around the central cavity appear to be critical for

11 function, since mutations in these residues were not found in our random

12 mutagenesis screen. This would be in accordance with the absence of the archetypal

13 Sm2 signature sequence [Y/F]KHAL of Hfq in cyanobacteria (Bøggild *et al.*, 2009).

14 Other bacterial Hfq homologs have highly similar or identical residues in this region

15 (Mikulecky *et al.*, 2004) and mutations of residues around the central cavity in *E. coli*16 Hfq showed severe defects in RNA binding and riboregulation *in vivo* and *in vitro*17 (Mikulecky *et al.*, 2004; Zhang *et al.*, 2013). By contrast, our results show that the

18 proximal RNA-binding pocket around the central cavity that binds preferentially U-rich

19 RNA 3'-ends in Enterobacteria (Sauer and Weichenrieder, 2011) seems not to be

20 involved in Hfq function in *Synechocystis*. Regarding the distal face of the Hfq

21 hexamer, we did not identify any residue contributing to this surface in our screen for

22 non-functional Hfq variants, nor did the G31N mutation abrogate Hfq function.

23 Together with the observed lack of strong conservation of distal face residues (Fig. 1)



1 this implies that this surface also does not form an essential interaction site for RNA.  
2 These results are substantiated by a study showing a very low affinity of two  
3 cyanobacterial Hfq proteins for known Hfq-dependent sRNAs from *E. coli* and the  
4 inability of the cyanobacterial Hfq protein to complement an *E. coli hfq* mutant strain  
5 (Bøggild *et al.*, 2009). Indeed, as we were also unable to detect Hfq-dependent  
6 sRNAs in purified Hfq samples, one has reason to generally question the role of  
7 *Synechocystis* Hfq as a RNA-chaperone (Fig. S3B). However, we observed a strong  
8 correlation between impaired SyR14 processing and motility and therefore we believe  
9 that Hfq exerts some of its function at the level of posttranscriptional gene regulation  
10 by a so far unknown mechanism.

11

#### 12 *The proximal outer ring is critical for Hfq-PilB1 interaction*

13 Given that the most conserved residues among cyanobacterial Hfq proteins are  
14 found in the proximal outer ring (Fig. 1) and all our deleterious amino acid  
15 substitutions were found in this region, we presume that this surface comprises the  
16 essential functional site of Hfq. Mutational studies in *E. coli* led to conflicting results  
17 regarding the requirement of proximal face residues outside the central cavity for  
18 RNA-binding and riboregulation. But for some of these residues a role in stable  
19 hexamer formation was suggested (Mikulecky *et al.*, 2004; Zhang *et al.*, 2013). We  
20 observed that subunit interaction is abrogated in the V58E mutant (Fig. 2C) and we  
21 cannot exclude the possibility that hexamer stability or conformation is altered in  
22 some of the other *Synechocystis* Hfq mutants. However, mutation of residues  
23 contributing to the proximal outer ring did not disturb monomer interaction in yeast

1 two-hybrid analysis (Fig. 2C) and therefore we conclude that loss of function in these  
2 Hfq variants cannot generally be attributed to compromised hexamer formation.  
3 Instead, we could demonstrate that Hfq interacts with PilB1 and that this interaction  
4 was lost in the Hfq variants P9T, I18F, W40R and D42N. Only two mutations (R39H  
5 and Q15H) disturb Hfq function but not binding to PilB1. Three Hfq mutants (P9T,  
6 I18F and D42N) were further analyzed by fluorescence microscopy. In these mutants  
7 Hfq was found to be incorrectly localized (Fig. 4). The mean number of spots per cell  
8 in the P9T mutant resembles the fluorescence distribution in the  $\Delta pilB1$  mutant,  
9 implying that Hfq-PilB1 interaction is lost in this mutant. The diffuse eYFP  
10 fluorescence in the cytoplasm of the I18F and D42N mutants in addition to the  
11 occurrence of bright spots in some cells indicates that Hfq-PilB1 interaction is also  
12 lost. Taking into account that the N-terminal (proximal facing) eYFP-Hfq fusion  
13 protein also was non-functional and did not localize to the cell membrane, we  
14 hypothesize that the proximal outer ring of the Hfq hexamer comprises the interface  
15 for PilB1 interaction and that this interaction is critical for Hfq function. Interestingly,  
16 we observed complete or partial loss of aggregation of Hfq in the form of bright  
17 fluorescent spots in several non-functional Hfq variants. This could be due to  
18 disturbed polymerisation into higher order structures which were demonstrated for *E.*  
19 *coli* Hfq and other Sm-like proteins (Arлуison *et al.*, 2006).

#### 20 *Localization of Hfq to the pilus base*

21 The most novel aspect arising from this study is the interaction of Hfq with the  
22 secretion ATPase PilB1 and the consequent localization of Hfq to the cell periphery.  
23 Furthermore, this interaction is critical both for the accumulation of Hfq-dependent

1 transcripts and for phototactic motility. Direct interaction has been shown for  
2 homologs of PilB and the membrane-integral pilus platform protein PilC in *E. coli*  
3 (Crowther *et al.*, 2004) and localization of *Pseudomonas aeruginosa* PilB to the  
4 cytoplasmic membrane is abolished in a  $\Delta pilC$  background (Chiang *et al.*, 2005).  
5 Assuming a similar interaction of PilB1 and PilC in *Synechocystis*, Hfq would be  
6 localized to the cytoplasmic membrane by interaction with PilB1 bound to PilC. We  
7 found that Hfq-dependent processing of SyR14 is abolished in the *Synechocystis*  
8  $\Delta pilC$  strain as well as in the  $\Delta pilB1$  strain. This indicates that interaction with PilB1 is  
9 not sufficient for Hfq function: the whole complex must also be correctly localized at  
10 the pilus base through interaction with PilC. While we cannot exclude the involvement  
11 of other residues, our results implicate that the Hfq-PilB1 interaction is mediated  
12 primarily by the small (40 amino acids) C-terminal zinc-finger-like domain of PilB1.  
13 Importantly, the high degree of sequence conservation of this domain in conjunction  
14 with the noted co-occurrence with Hfq implies an evolutionarily conserved  
15 mechanism of Hfq-PilB interaction in cyanobacteria.

16 Interaction of Hfq with the T4P pilus base is consistent with the absence of pili  
17 on the cell surface in the  $\Delta hfq$  strain (Dienst *et al.* 2008). Considering that we have no  
18 evidence for specific RNA-Hfq interaction in *Synechocystis*, a direct role of Hfq in  
19 pilus assembly would be the simplest explanation for the observed phenotype.  
20 However, it is clear that Hfq must have additional functions beyond pilus assembly  
21 since  $\Delta hfq$  mutant cells display reduced transcript accumulation of a set of mRNAs  
22 (Dienst *et al.* 2008) and sRNAs (Fig. S4A). Among them are the transcripts of minor  
23 pilins (PilA9-11) and cell surface components (Slr1667 and Slr1668) that are under  
24 the control of the cAMP-dependent transcription factor SyCRP1, known to be  
18

involved in phototactic motility (Yoshimura *et al.*, 2002; Yoshimura *et al.*, 2010). Given our strong evidence for direct interaction between Hfq and PilB1 and the importance of correct Hfq localization we suggest that Hfq could facilitate a differential subcellular accumulation and translation of transcripts encoding gene products that are involved in phototactic motility or require PilB1 for translocation through the T4P.

A vast number of Hfq-dependent sRNAs are involved in the regulation of outer membrane proteins (Vogel and Papenfort, 2006) and there are reports suggesting that Hfq-dependent riboregulation of secreted and membrane proteins takes place in close proximity to the cytoplasmic membrane. For example, in *E. coli* Hfq is localized to the cell membrane (Diestra *et al.*, 2009) and the Hfq/SgrS-dependent regulation of the *ptsG* mRNA is dependent on the localization of the transcript to the cytoplasmic membrane coupled with the insertion of the nascent peptide (Kawamoto *et al.*, 2005). A recent study in *Salmonella* demonstrated that short AU-rich sequences in the 5'-UTR of *gtgA*, *gogB* and *sseL* are sufficient for the translocation of reporter proteins via a type 3 secretion system in a wild-type strain but not in a  $\Delta hfq$  mutant (Niemann *et al.*, 2013). Here, the abrogation of protein secretion in  $\Delta hfq$  was not a consequence of a defective type 3 secretion apparatus or decreased protein expression and *gtgA* mRNA was shown to interact with Hfq. Additionally, *E. coli* Hfq has been copurified with proteins involved in protein translocation, like FliS and SecA (Arifuzzaman *et al.*, 2006). Thus, we speculate that Hfq might play an important function in posttranscriptional regulation as part of a membrane-associated protein complex involved in protein translocation in different bacteria.

## 1 Experimental procedures

### 2 *Bacterial strains and growth conditions*

3 Liquid cultures of *Synechocystis* wild-type (originally obtained from S. Shestakov,  
4 Moscow State University, Russia) and mutant strains were grown in BG11 medium  
5 (Rippka *et al.*, 1979) supplemented with 10 mM TES buffer (pH 8.0) at 30°C under  
6 white light of 50  $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$  and constant shaking. For induction of the  
7 *PpetJ* promoter  $\text{CuSO}_4$  was omitted from the medium.

### 8 *Mutagenesis and plasmid construction*

9 The construction of the  $\Delta hfq$  mutant was described in Dienst *et al.*, 2008 (see also  
10 Fig. S3A). Primers used for mutant constructs are listed in the Supporting  
11 Information. For site directed mutagenesis we used an 1111 bp fragment containing  
12 the *hfq* gene together with its promoter and terminator sequences (Dienst *et al.*,  
13 2008) ligated into the pDrive vector (Qiagen) using PstI and Sall restriction sites. The  
14 resulting plasmid (pDrive-*hfq*) was used as a template for two separate PCR  
15 reactions each involving a primer harbouring the mutated sequence (Q15R: a44g,  
16 a44g\_as; G31N: g91a\_g92a\_g93c, g91a\_g92a\_g93c\_as; T43D: a127g\_c128a,  
17 a127g\_c128a\_as, D44K: g130a\_t132a, g130a\_t132a\_as) and a vector primer  
18 (M13fw or M13rev). These two DNA fragments were used in a third PCR reaction  
19 using both vector primers. The resulting PCR fragment was subcloned into pJET  
20 vector (Fermentas). The pJET vector was then cleaved by PstI and Sall and the  
21 fragment containing the mutagenised *hfq* sequence together with its promoter and  
22 terminator sequences was ligated into the respective restriction sites of the  
23 conjugative vector pVZ321 (pVZ321-*hfq*#AA, Fig. S3B). The pVZ321-*hfq* vector was  
20

1 transferred to *Synechocystis* 6803  $\Delta hfq$  mutant cells via conjugation (Zinchenko *et*  
2 *al.*, 1999) and exconjugants were selected on 40  $\mu\text{g ml}^{-1}$  Km. For construction of  
3 randomly mutagenised *hfq* sequences we again used the pDrive-*hfq* vector as a  
4 template. An error prone PCR reaction (5.0 mM  $\text{MgCl}_2$ , 0.2 mM dATP and dGTP, 1  
5 mM dCTP and dTTP, 0.3 mM  $\text{MnCl}_2$ , 5U Dream Taq (Fermentas) was performed  
6 using the primer pair (hfq-EcoRI-fw and hfq-BamHI-rev) and the PCR products  
7 bearing the coding sequence of *hfq* flanked by EcoRI and BamHI restriction sites  
8 were ligated into the pJET vector. Plasmid DNA was isolated from all obtained  
9 recombinant *E. coli* colonies in one sample and subjected to digestion with EcoRI  
10 and BamHI. The DNA fragments were then ligated into EcoRI and BamHI restriction  
11 sites of the pUR expression vector (Wiegard *et al.*, 2013). This led to a mobilisable  
12 plasmid harbouring randomly mutated *hfq* sequences flanked at the 5' end by the  
13 copper sensitive promoter *PpetJ* and a 3xFLAG sequence and at the 3' end by the  
14 *oop* terminator from phage Lambda (pUR-*hfq*#AA, Fig. S3C).

15         Insertion of Hfq-3xFLAG in a neutral locus of the  $\Delta hfq$  strain was carried out  
16 using a similar strategy as for the Ycf34-3xFLAG strain (Wallner *et al.*, 2012). Briefly,  
17 the *hfq* ORF and the 200nt downstream sequence was amplified using primers hfq-  
18 NdeI-fw and hfq-BglII-rev and the PCR-fragment was subcloned into the pJET vector.  
19 It was excised with NdeI and BglII and ligated into a modified pSK9 vector (Kuchmina  
20 *et al.*, 2012) (the Km resistance cassette from pUC4K vector was excised with EcoRI  
21 and ligated into the pSK9 EcoRI site, disrupting the chloramphenicol resistance  
22 cassette) resulting in pSK9-Hfq. Subsequently oligonucleotides containing the 3x-  
23 FLAG epitope (FLAG-fw and FLAG-rev) were hybridized and ligated into the pSK9-

1 Hfq vector, linearised with NdeI (pSK-3xFLAG-*hfq*, Fig. S3D). This construct was  
2 introduced into  $\Delta hfq$  mutant cells by electroporation (Thiel and Poo, 1989).

3 The *hfq* gene was amplified using primers hfqYFPNde and hfqYFPXho and  
4 the gene encoding eYFP was amplified from pEYFP-His<sub>6</sub>-Sp<sup>R</sup> plasmid described in  
5 (Birungi *et al.*, 2010) using primers eYFPXho and eYFPBgIII generating NdeI, XhoI  
6 and BgIII restriction sites at the 5' and 3' ends, respectively. The fragments were  
7 subcloned the psk9 based expression vector pSDC01 (Savakis *et al.*, 2012) to  
8 generate a C-terminal YFP tagged Hfq fusion. A PvuII fragment bearing the  
9 expression cassette together with eYFP tagged *hfq* was then ligated into a modified  
10 pVZ321 vector (Zinchenko *et al.* 1999) where the chloramphenicol resistance  
11 cassette was exchanged by the *aadA* gene conferring streptomycin resistance (pVZ-  
12 *hfq*-eYFP, Fig. S3E). To generate the eYFP tagged Hfq variants carrying single amino  
13 acid substitutions the same strategy was used but the mutated *hfq* sequence from  
14 the clones generated by error prone PCR was used. For N-terminal fusion of eYFP to  
15 *hfq* the eYFP gene was amplified using the primer pair NdeI-eYFP-fw and eYFP-  
16 EcoRI-rev; the *hfq* sequence was amplified with primers hfq-Eco-fw and hfq-BamHI-  
17 rev generating EcoRI and BgIII restrictions sites at the ends and both fragments were  
18 subsequently ligated into the pVZ321 based conjugative expression vector pUR  
19 allowing expression from the *PpetJ* promoter (pVZ-eYFP-*hfq*, Fig. S3F).  $\Delta pilB1$  and  
20  $\Delta pilC$  mutants were generated by transformation of the wild-type strain with genomic  
21 DNA of *slr0063::zeo* or *pilC::aadA* strains obtained from Roman Sobotka (Department  
22 of Phototrophic Microorganisms, Academy of Sciences, Trebon).

23 To generate a C-terminal GFP tagged PilB1 fusion protein we amplified the  
24 sequence encoding "superfolder" sfGFP fused to a 12 amino acid linker from plasmid  
22

1 pXG10-SF (Corcoran *et al.*, 2012) using primers XhoI-L-sfGFP-fw and BglII-L-sfGFP-  
2 rev and the *pilB1* gene using primers NdeI-pilB-fw and XhoI-pilB-rev. These  
3 fragments were cloned into the respective NdeI/XhoI and XhoI/BglII restriction sites  
4 of vector pSDC01 which was subsequently used to transform *Synechocystis*.

#### 5 *Motility assays*

6 Phototactic movement was analysed on 0.5% (w/v) BG11 agar plates with 0.2%  
7 glucose added. Cell suspensions were spotted on the plates and incubated for 2-3  
8 days under diffuse white light ( $50 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ ) before being placed into  
9 non-transparent boxes with an one-sided opening ( $>5 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ ) for 10  
10 days.

#### 11 *RNA isolation and northern blot hybridization*

12 Approximately 30 ml of *Synechocystis* liquid cultures were collected at an  $\text{OD}_{750\text{nm}}$  of  
13 0.6-0.8 by quenching on ice and immediate centrifugation at 4 °C. RNA was isolated  
14 using the PGTX method (Pinto *et al.*, 2009) with an additional  
15 phenol/chloroform/isoamyl alcohol (25:24:1 v/v) extraction preceding the RNA  
16 precipitation. Total RNA was diluted in 30  $\mu\text{l}$   $\text{H}_2\text{O}$  and 5-10  $\mu\text{g}$  were separated by  
17 electrophoresis on 10% denaturing urea polyacrylamide gels and blotted onto Roti-  
18 Nylon plus (Roth) membranes. Hybridization probes were generated either by *in vitro*  
19 transcription of PCR fragments from the T7 promoter in the presence of  $[\alpha\text{-}^{32}\text{P}]\text{UTP}$   
20 using the T7 polymerase Maxiscript kit (Ambion) or by labeling DNA oligonucleotides  
21 with  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$  using T4 polynucleotide kinase (Fermentas). Signals were detected  
22 and analysed on a Personal Molecular Imager FX (Bio-Rad). All DNA  
23 oligonucleotides are listed in the table at the end of this section.



## 1 *Yeast two-hybrid analysis*

2 The yeast two-hybrid analysis was carried out as described previously (Watanabe *et*  
3 *al.*, 2007). The DNA fragments of *hfq* and *pilB1* were amplified from genomic DNA of  
4 a *Synechocystis* wild-type strain or plasmid DNA in case of the Hfq variants carrying  
5 single amino acid substitutions and cloned into pGBTK, a GAL4 DNA-binding domain  
6 fusion vector, or pGAD424, a GAL4 activation domain fusion vector, respectively.  
7 Bait-prey pairs for specificity were streaked onto SC-LW and -LWA plates and  
8 incubated for 6 days. Primers for cloning are listed in the Supporting Information.

## 9 *Fluorescence microscopy*

10 Fluorescence micrographs were recorded at room temperature (20°C) using a Leica  
11 TCS SP5 laser-scanning confocal microscope. For visualisation of YFP in single-  
12 labelled cells, excitation was at 496 nm and emission was recorded simultaneously at  
13 520-540 nm (for eYFP) and 670-720 nm (for chlorophyll, showing the location of the  
14 thylakoid membranes). For co-localisation of GFP and YFP in dual-labelled cells,  
15 sequential images were recorded with excitation at 488 nm, emission 503-515 nm  
16 (for GFP) and excitation at 514 nm, emission at 520-540 nm (for YFP). Controls with  
17 single-labelled cells show that there is no significant cross-detection of GFP and YFP  
18 with these wavelength settings (Supplementary Fig. S8B). For all images, a 63x oil-  
19 immersion objective lens (numerical aperture 1.4) was used. The confocal pinhole  
20 was set to give an optical section in the z-direction of about 1 µm. Cells were taken  
21 from agar plates, resuspended in growth medium and then droplets of the cell  
22 suspension were adsorbed onto fresh BG11 agar plates. When the liquid had dried,  
23 small blocks of agar with the cells on the surface were pressed onto glass cover-slips

and mounted in a custom-made sample holder. Fluorescence intensities were quantified with ImageJ software (National Institutes of Health). Statistical tests were with SigmaPlot 10.0 software (Jandel Scientific).

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## References

- Argaman, L., Elgrably-Weiss, M., Hershko, T., Vogel, J., and Altuvia, S. (2012) RelA protein stimulates the activity of RyhB small RNA by acting on RNA-binding protein Hfq. *Proc Natl Acad Sci* **109**: 4621–4626.
- Arifuzzaman, M., Maeda, M., Itoh, A., Nishikata, K., Takita, C., Saito, R., *et al.* (2006) Large-scale identification of protein-protein interaction of *Escherichia coli* K-12. *Genome Res* **16**: 686–691.
- Arluison, V., Mura, C., Guzmán, M.R., Liquier, J., Pellegrini, O., Gingery, M., *et al.* (2006) Three-dimensional structures of fibrillar Sm proteins: Hfq and other Sm-like proteins. *J Mol Biol* **356**: 86–96.

- 1 Ashkenazy, H., Erez, E., Martz, E., Pupko, T., and Ben-Tal, N. (2010) ConSurf 2010:  
2 calculating evolutionary conservation in sequence and structure of proteins and  
3 nucleic acids. *Nucleic Acids Res* **38**: W529–W533.
- 4 Azam, T.A., Hiraga, S., and Ishihama, A. (2000) Two types of localization of the  
5 DNA-binding proteins within the *Escherichia coli* nucleoid. *Genes Cells* **5**: 613–626.
- 6 Bhaya, D., Watanabe, N., Ogawa, T., and Grossman, A.R. (1999) The role of an  
7 alternative sigma factor in motility and pilus formation in the cyanobacterium  
8 *Synechocystis* sp. strain PCC6803. *Proc Natl Acad Sci USA* **96**: 3188–3193.
- 9 Birungi, M., Folea, M., Battchikova, N., Xu, M., Mi, H., Ogawa, T., *et al.* (2010)  
10 Possibilities of subunit localization with fluorescent protein tags and electron  
11 microscopy exemplified by a cyanobacterial NDH-1 study. *Biochim Biophys Acta*  
12 **1797**: 1681–1686.
- 13 Bøggild, A., Overgaard, M., Valentin-Hansen, P., and Brodersen, D.E. (2009)  
14 Cyanobacteria contain a structural homologue of the Hfq protein with altered RNA-  
15 binding properties. *FEBS J* **276**: 3904–3915.
- 16 Butland, G., Peregrín-Alvarez, J.M., Li, J., Yang, W., Yang, X., Canadien, V., *et al.*  
17 (2005) Interaction network containing conserved and essential protein complexes in  
18 *Escherichia coli*. *Nature* **433**: 531–537.
- 19 Chiang, P., Habash, M., and Burrows, L.L. (2005) Disparate subcellular localization  
20 patterns of *Pseudomonas aeruginosa* Type IV pilus ATPases involved in twitching  
21 motility. *J Bacteriol* **187**: 829–839.
- 22 Corcoran, C.P., Podkaminski, D., Papenfort, K., Urban, J.H., Hinton, J.C.D., and  
23 Vogel, J. (2012) Superfolder GFP reporters validate diverse new mRNA targets of the  
24 classic porin regulator, MicF RNA. *Mol Microbiol* **84**: 428–45.
- 25 Crowther, L.J., Anantha, R.P., and Donnenberg, M.S. (2004) The inner membrane  
26 subassembly of the enteropathogenic *Escherichia coli* bundle-forming pilus machine.  
27 *Mol Microbiol* **52**: 67–79.
- 28 Dienst, D., Dühring, U., Mollenkopf, H.J., Vogel, J., Golecki, J., Hess, W.R., and  
29 Wilde, A. (2008) The cyanobacterial homologue of the RNA chaperone Hfq is  
30 essential for motility of *Synechocystis* sp. PCC 6803. *Microbiology* **154**: 3134–3143.
- 31 Diestra, E., Cayrol, B., Arluison, V., and Risco, C. (2009) Cellular electron  
32 microscopy imaging reveals the localization of the Hfq protein close to the bacterial  
33 membrane. *PLoS One* **4**: e8301.
- 34 Georg, J., Voss, B., Scholz, I., Mitschke, J., Wilde, A., and Hess, W.R. (2009)  
35 Evidence for a major role of antisense RNAs in cyanobacterial gene regulation. *Mol*  
36 *Syst Biol* **5**: 305.

- 1 Kajitani, M., Kato, A., Wada, A., Inokuchi, Y., and Ishihama, A. (1994) Regulation of  
2 the *Escherichia coli* *hfq* gene encoding the host factor for phage Q beta. *J Bacteriol*  
3 **176**: 531–534.
- 4 Kawamoto, H., Morita, T., Shimizu, A., Inada, T., and Aiba, H. (2005) Implication of  
5 membrane localization of target mRNA in the action of a small RNA: mechanism of  
6 post-transcriptional regulation of glucose transporter in *Escherichia coli*. *Genes Dev*  
7 **19**: 328–338.
- 8 Kuchmina, E., Wallner, T., Kryazhov, S., Zinchenko, V. V., and Wilde, A. (2012) An  
9 expression system for regulated protein production in *Synechocystis* sp. PCC 6803  
10 and its application for construction of a conditional knockout of the ferrochelatase  
11 enzyme. *J Biotechnol* **162**: 75–80.
- 12 Link, T.M., Valentin-Hansen, P., and Brennan, R.G. (2009) Structure of *Escherichia*  
13 *coli* Hfq bound to polyriboadenylate RNA. *Proc Natl Acad Sci USA* **106**: 19292–  
14 19297.
- 15 Mikulecky, P.J., Kaw, M.K., Brescia, C.C., Takach, J.C., Sledjeski, D.D., and Feig,  
16 A.L. (2004) *Escherichia coli* Hfq has distinct interaction surfaces for DsrA, rpoS and  
17 poly(A) RNAs. *Nat Struct Mol Biol* **11**: 1206–1214.
- 18 Mitschke, J., Georg, J., Scholz, I., Sharma, C.M., Dienst, D., Bantscheff, J., *et al.*  
19 (2011) An experimentally anchored map of transcriptional start sites in the model  
20 cyanobacterium *Synechocystis* sp. PCC6803. *Proc Natl Acad Sci USA* **108**: 2124–  
21 2129.
- 22 Mohanty, B.K., Maples, V.F., and Kushner, S.R. (2004) The Sm-like protein Hfq  
23 regulates polyadenylation dependent mRNA decay in *Escherichia coli*. *Mol Microbiol*  
24 **54**: 905–920.
- 25 Morita, T., Maki, K., and Aiba, H. (2005) RNase E-based ribonucleoprotein  
26 complexes: mechanical basis of mRNA destabilization mediated by bacterial  
27 noncoding RNAs. *Genes Dev* **19**: 2176–2186.
- 28 Niemann, G.S., Brown, R.N., Mushamiri, I.T., Nguyen, N.T., Taiwo, R., Stufkens, A.,  
29 *et al.* (2013) RNA type III secretion signals that require Hfq. *J Bacteriol* **195**: 2119–  
30 2125.
- 31 Pinto, F.L., Thapper, A., Sontheim, W., and Lindblad, P. (2009) Analysis of current  
32 and alternative phenol based RNA extraction methodologies for cyanobacteria. *BMC*  
33 *Mol Biol* **10**: 79.
- 34 Puerta-Fernández, E., and Vioque, A. (2011) Hfq is required for optimal nitrate  
35 assimilation in the Cyanobacterium *Anabaena* sp. strain PCC 7120. *J Bacteriol* **193**:  
36 3546–3555.

- 1 Rabhi, M., Espéli, O., Schwartz, A., Cayrol, B., Rahmouni, A.R., Arluison, V., and  
2 Boudvillain, M. (2011) The Sm-like RNA chaperone Hfq mediates transcription  
3 antitermination at Rho-dependent terminators. *EMBO J* **30**: 2805–2816.
- 4 Rippka, R., Deruelles, J., Waterbury, J.B., Herdman, M., and Stanier, R.Y. (1979)  
5 Generic assignments, strain histories and properties of pure cultures of  
6 cyanobacteria. *J Gen Microbiol* **111**: 1–61.
- 7 Sauer, E., Schmidt, S., and Weichenrieder, O. (2012) Small RNA binding to the  
8 lateral surface of Hfq hexamers and structural rearrangements upon mRNA target  
9 recognition. *Proc Natl Acad Sci USA* **109**: 9396–9401.
- 10 Sauer, E., and Weichenrieder, O. (2011) Structural basis for RNA 3'-end recognition  
11 by Hfq. *Proc Natl Acad Sci USA* **108**: 13065–13070.
- 12 Savakis, P., DeCausmaecker, S., Angerer, V., Ruppert, U., Anders, K., Essen, L.-O.,  
13 and Wilde, A. (2012) Light-induced alteration of c-di-GMP level controls motility of  
14 *Synechocystis* sp. PCC 6803. *Mol Microbiol* **85**: 239–251.
- 15 Schumacher, M.A., Pearson, R.F., Møller, T., Valentin-Hansen, P., and Brennan,  
16 R.G. (2002) Structures of the pleiotropic translational regulator Hfq and an Hfq-RNA  
17 complex: a bacterial Sm-like protein. *EMBO J* **21**: 3546–3556.
- 18 Shih, P.M., Wu, D., Latifi, A., Axen, S.D., Fewer, D.P., Talla, E., *et al.* (2013)  
19 Improving the coverage of the cyanobacterial phylum using diversity-driven genome  
20 sequencing. *Proc Natl Acad Sci USA* **110**: 1053–1058.
- 21 Sukhodolets, M. V, and Garges, S. (2003) Interaction of *Escherichia coli* RNA  
22 polymerase with the ribosomal protein S1 and the Sm-like ATPase Hfq. *Biochemistry*  
23 **42**: 8022–8034.
- 24 Thiel, T., and Poo, H. (1989) Transformation of a filamentous cyanobacterium by  
25 electroporation. *J Bacteriol* **171**: 5743–5746.
- 26 Valentin-Hansen, P., Eriksen, M., and Udesen, C. (2004) The bacterial Sm-like  
27 protein Hfq: a key player in RNA transactions. *Mol Microbiol* **51**: 1525–1533.
- 28 Vogel, J., and Luisi, B.F. (2011) Hfq and its constellation of RNA. *Nat Rev Microbiol*  
29 **9**: 578–589.
- 30 Vogel, J., and Papenfort, K. (2006) Small non-coding RNAs and the bacterial outer  
31 membrane. *Curr Opin Microbiol* **9**: 605–611.
- 32 Wallner, T., Hagiwara, Y., Bernát, G., Sobotka, R., Reijerse, E.J., Frankenberg-  
33 Dinkel, N., and Wilde, A. (2012) Inactivation of the conserved open reading frame

- 1 *ycf34* of *Synechocystis* sp. PCC 6803 interferes with the photosynthetic electron  
2 transport chain. *Biochim Biophys Acta* **1817**: 2016–2026.
- 3 Watanabe, S., Kobayashi, T., Saito, M., Sato, M., Nimura-Matsune, K., Chibazakura,  
4 T., *et al.* (2007) Studies on the role of HtpG in the tetrapyrrole biosynthesis pathway  
5 of the cyanobacterium *Synechococcus elongatus* PCC 7942. *Biochem Biophys Res*  
6 *Commun* **352**: 36–41.
- 7 Wiegard, A., Dörrich, A.K., Deinzer, H.T., Beck, C., Wilde, A., Holtzendorff, J., and  
8 Axmann, I.M. (2013) Biochemical analysis of three putative KaiC clock proteins from  
9 *Synechocystis* sp. PCC 6803 suggests their functional divergence. *Microbiology* **159**:  
10 948–58.
- 11 Windbichler, N., Pelchrzim, F. Von, Mayer, O., Csaszar, E., and Schroeder, R. (2008)  
12 Isolation of small RNA-binding proteins from *E. coli*: evidence for frequent interaction  
13 of RNAs with RNA polymerase. *RNA Biol* **5**: 30–40.
- 14 Yamagata, A., and Tainer, J.A. (2007) Hexameric structures of the archaeal secretion  
15 ATPase GspE and implications for a universal secretion mechanism. *EMBO J* **26**:  
16 878–890.
- 17 Yoshihara, S., Geng, X., Okamoto, S., Yura, K., Murata, T., Go, M., *et al.* (2001)  
18 Mutational analysis of genes involved in pilus structure, motility and transformation  
19 competency in the unicellular motile cyanobacterium *Synechocystis* sp. PCC 6803.  
20 *Plant Cell Physiol* **42**: 63–73.
- 21 Yoshimura, H., Kaneko, Y., Ehira, S., Yoshihara, S., Ikeuchi, M., and Ohmori, M.  
22 (2010) CccS and CccP are involved in construction of cell surface components in the  
23 cyanobacterium *Synechocystis* sp. strain PCC 6803. *Plant Cell Physiol* **51**: 1163–  
24 1172.
- 25 Yoshimura, H., Yoshihara, S., Okamoto, S., Ikeuchi, M., and Ohmori, M. (2002) A  
26 cAMP receptor protein, SYCRP1, is responsible for the cell motility of *Synechocystis*  
27 sp. PCC 6803. *Plant Cell Physiol* **43**: 460–463.
- 28 Zhang, A., Schu, D.J., Tjaden, B.C., Storz, G., and Gottesman, S. (2013) Mutations  
29 in interaction surfaces differentially impact *E. coli* Hfq association with small RNAs  
30 and their mRNA targets. *J Mol Biol* **425**: 3678–97.
- 31 Zinchenko, V.V., Piven, I.V., Melnik, V.A., and Shestakov, S.V. (1999) Vectors for the  
32 complementation analysis of cyanobacterial mutants. *Russ J Genet* **35**: 228–232.

33

1

2 **Figure legends**

3

4 **Fig. 1. Sequence conservation and mutational analysis of single amino acid**5 **residues.** (A) The *Synechocystis* Hfq sequence coloured by sequence conservation6 among cyanobacterial Hfq homologs. The secondary structure of *Synechocystis* Hfq

7 (PDB ID: 3hfo) is displayed on the top and residues involved in RNA-binding in other

8 bacterial Hfq proteins are marked as followed: \* putative proximal RNA binding site of

9 *Synechocystis* Hfq determined by structural superposition with the known *S. aureus*10 RNA-binding site (Schumacher *et al.*, 2002; Bøggild *et al.*, 2009); • distal RNA11 binding site seen in the crystal structure of a Hfq-RNA complex in *E. coli* (Link *et al.*,12 2009); + charged residues contributing to the lateral RNA binding site (Sauer *et al.*,13 2012). Amino acid substitutions that did not abolish Hfq function in *Synechocystis* are

14 shown in grey and deleterious substitutions in black. (B) Sequence conservation

15 superimposed on the molecular surface of *Synechocystis* Hfq shows an unilateral

16 arrangement of conserved residues along an “outer proximal ring”. Mutated residues

17 that contribute to the surface area of the Hfq hexamer are indicated. (C) Site directed

18 mutagenesis of specific residues. All mutants showed positive phototaxis towards an

19 unidirectional light source in a phototaxis assay. (D) Northern blot analysis showing

20 that SyR14 transcript accumulation is comparable to the wild type in all mutant

21 strains. (E-F) Random mutagenesis clones with single amino acid substitutions that

22 abolished *Synechocystis* Hfq function and were not able to restore phototactic



1 motility and SyR14 processing. A sequence alignment of cyanobacterial Hfq proteins  
2 is shown in Fig. S1.

3 **Fig. 2. Hfq interacts with the secretion ATPase PilB1.** (A) Schematic  
4 representation of PilB1 highlighting the domains used in the yeast two-hybrid  
5 experiment and showing the sequence motif of the putative zinc-finger domain found  
6 in cyanobacterial PilB homologs. The conserved cysteines are marked with red  
7 asterisks. (B) Yeast two-hybrid results showing interactions between Hfq and the  
8 different domains of PilB1 as indicated above including a C2 variant with the  
9 conserved C substituted for A residues (PilB1-C2mut). Empty prey and bait vectors  
10 pGAD424 and pGBTK are included as controls. For auxotrophic selection of diploid  
11 cells harbouring prey and bait vectors cells were grown on restrictive growth media  
12 lacking tryptophan (-Trp) and Leucine (-Leu). To test specific interaction Adenine (-  
13 Ade) was also omitted. (C) Protein-protein interaction screen of the non functional  
14 Hfq variants described in Fig. 1 with wild-type Hfq and the C-terminal PilB1 domains  
15 as above.

16  
17 **Fig. 3. Complementation of  $\Delta hfq$  with C- and N-terminal eYFP fusion proteins**  
18 **and its localization.** Cultures were grown in copper depleted medium to induce  
19 protein expression. (A) Altered transcript accumulation of SyR14 as seen in the  $\Delta hfq$   
20 strain is only restored to wild-type levels by Hfq-eYFP. (B) Phototaxis assay showing  
21 that only Hfq-eYFP complements the motility phenotype. (C) Subcellular localization  
22 of the fusion proteins (Hfq-eYFP, C-terminal fusion; eYFP-Hfq, N-terminal fusion).



Fluorescence images from cells grown on agar plates. eYFP fluorescence is shown in green and chlorophyll fluorescence in magenta.

**Fig. 4. Localization of non functional Hfq variants.** Fluorescence images showing the alteration in subcellular localization of Hfq variants with a C-terminal eYFP fusion compared to the wild type Hfq-eYFP fusion protein. Cells were grown on agar plates without copper to induce gene expression. eYFP fluorescence is shown in green and chlorophyll fluorescence in magenta. In the wild type Hfq-eYFP strain we observed bright and also multiple weaker spots (mean spots per cell =  $3.69 \pm 2.44$  (SD,  $n = 78$ )). In contrast, in mutant P9T, predominantly only one bright spot per cell was visible (mean spots per cell =  $1.06 \pm 0.33$  (SD,  $n = 71$ )). Mutants I18F and D42N showed some bright spots as well as intense dispersed eYFP fluorescence in the cytoplasm.

**Fig. 5. Significance of PilB1-Hfq interaction for Hfq function and localization.**

(A) Fluorescence images showing different Hfq-eYFP distribution in the  $\Delta pilB1$  background. eYFP fluorescence is shown in green and chlorophyll fluorescence in magenta. The differences in mean number of spots/cell ( $\Delta hfq \times$  Hfq-eYFP =  $3.3 \pm 2.1$  (SD,  $n = 110$  cells));  $\Delta pilB1/\Delta hfq \times$  Hfq-eYFP =  $0.85 \pm 0.69$  (SD,  $n = 86$  cells)) and mean spot intensity ( $\Delta hfq \times$  Hfq-eYFP =  $51 \pm 41$  (a.u.) (SD,  $n = 166$  spots));  $\Delta pilB1/\Delta hfq \times$  Hfq-eYFP =  $240 \pm 180$  (a.u.) (SD,  $n = 35$  spots)) are statistically significant (Student's  $t$ -test,  $p < 10^{-10}$  in both cases). For the  $\Delta pilB1$  background, mean spot intensity is a minimum estimate because many spots were at saturating intensity with the settings used. (B) Altered transcript accumulation of SyR14 is

1 evident in  $\Delta hfq$  as well as in all  $\Delta pilB1$  and  $\Delta pilC$  strains. (C) Phototaxis assay of  
2 analyzed mutants.

3 **Fig. 6. Simultaneous visualisation of PilB1-sfGFP and Hfq-eYFP in**  
4 ***Synechocystis* cells.** A  $\Delta pilB1$  mutant complemented with a sfGFP tagged version  
5 of *pilB1* (Fig. S8A) was transformed with the pVZ-*hfq*-eYFP plasmid and grown under  
6 inducing conditions. See Experimental Procedures for settings and Supplementary  
7 Fig. S8B for controls. sfGFP fluorescence (PilB1) is shown in magenta and eYFP  
8 fluorescence (Hfq) in green. Scale-bars 5 microns.

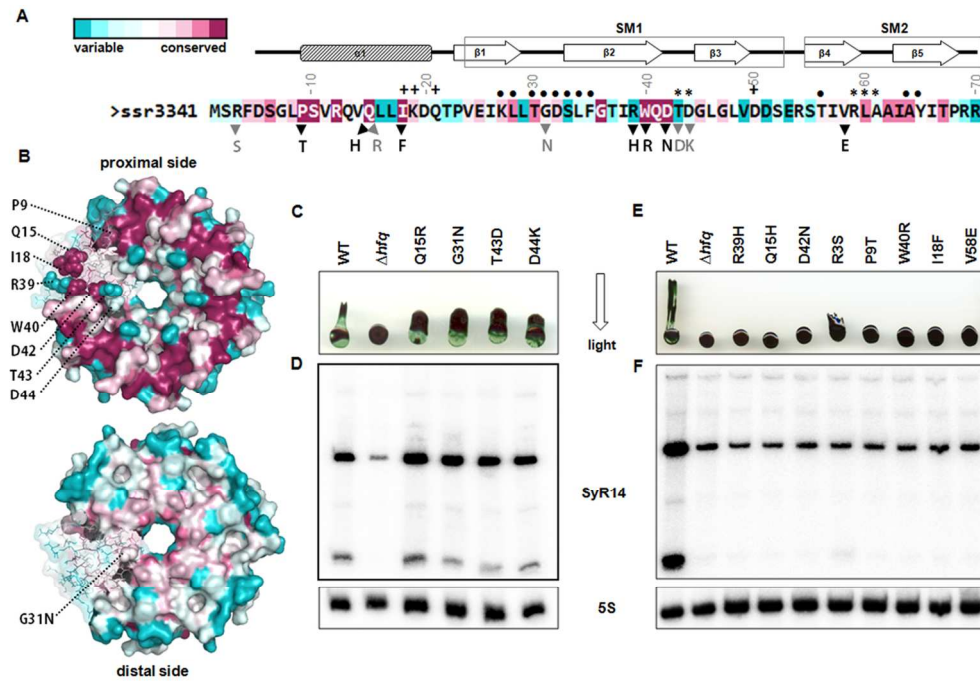


Figure 1  
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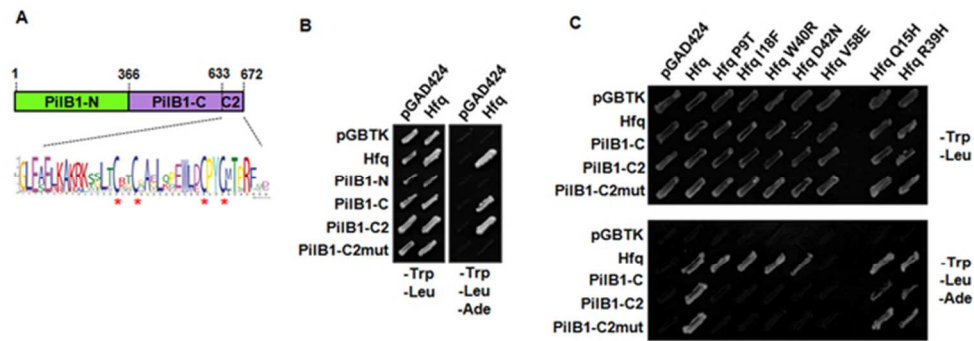
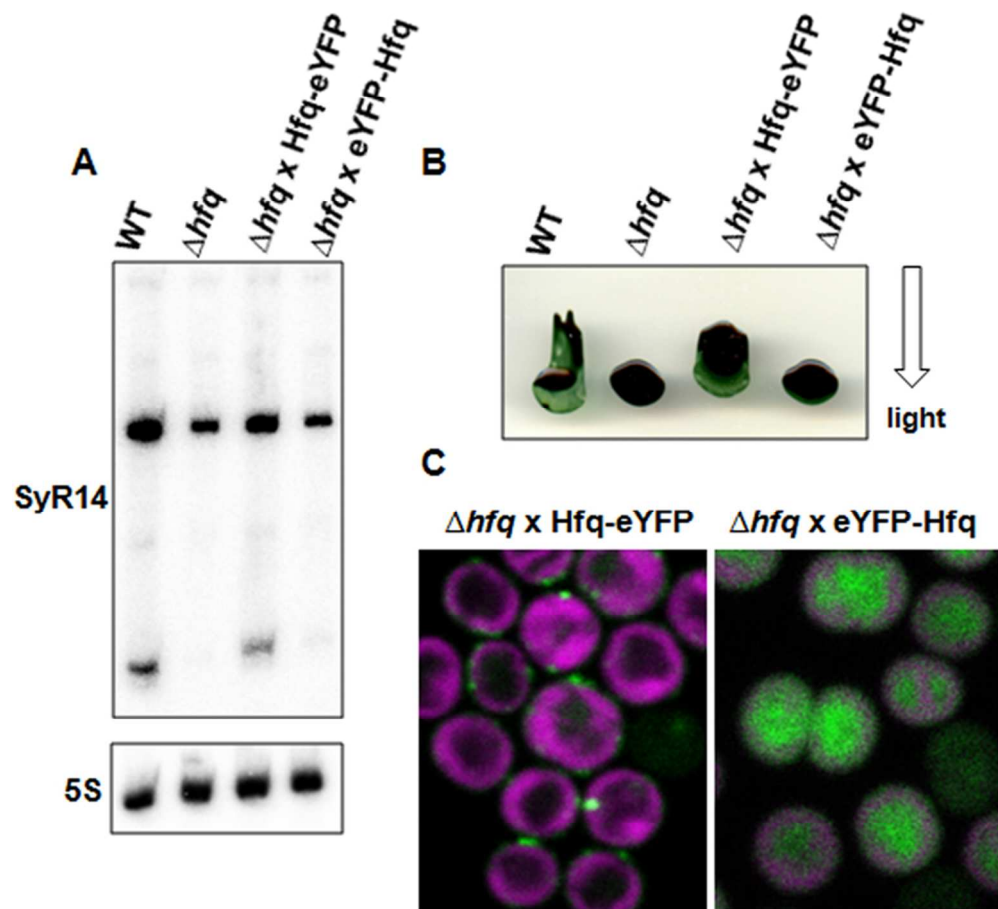
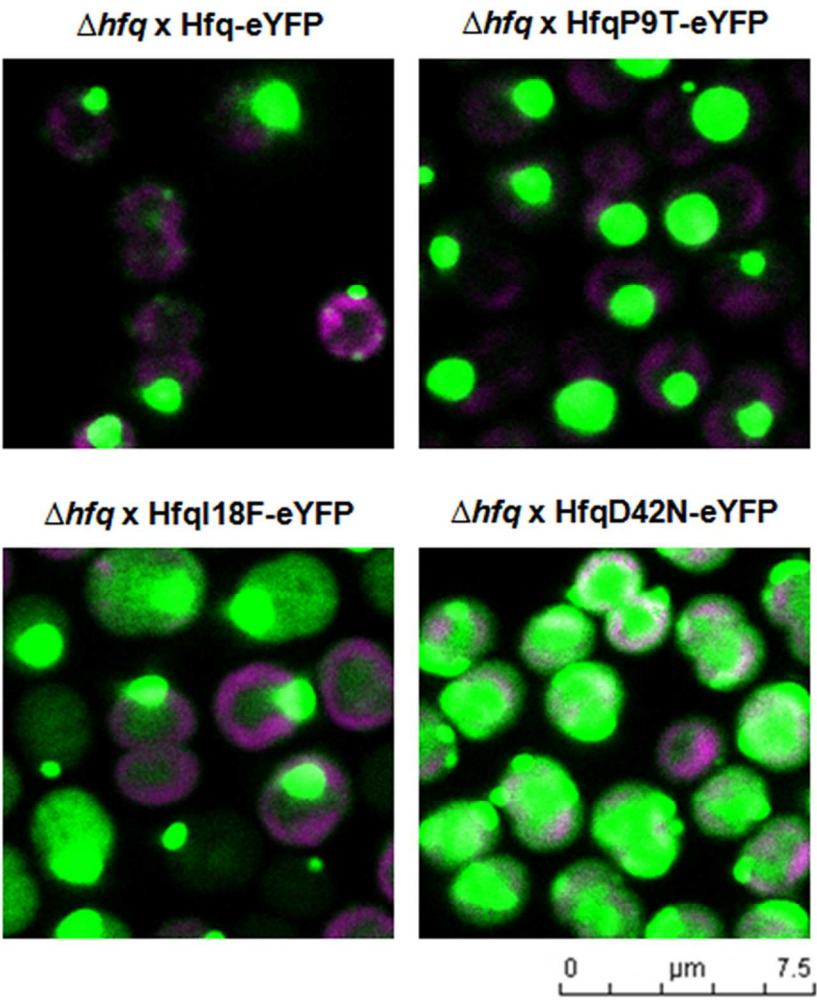


Figure 2  
59x20mm (300 x 300 DPI)



74x69mm (300 x 300 DPI)



82x84mm (300 x 300 DPI)

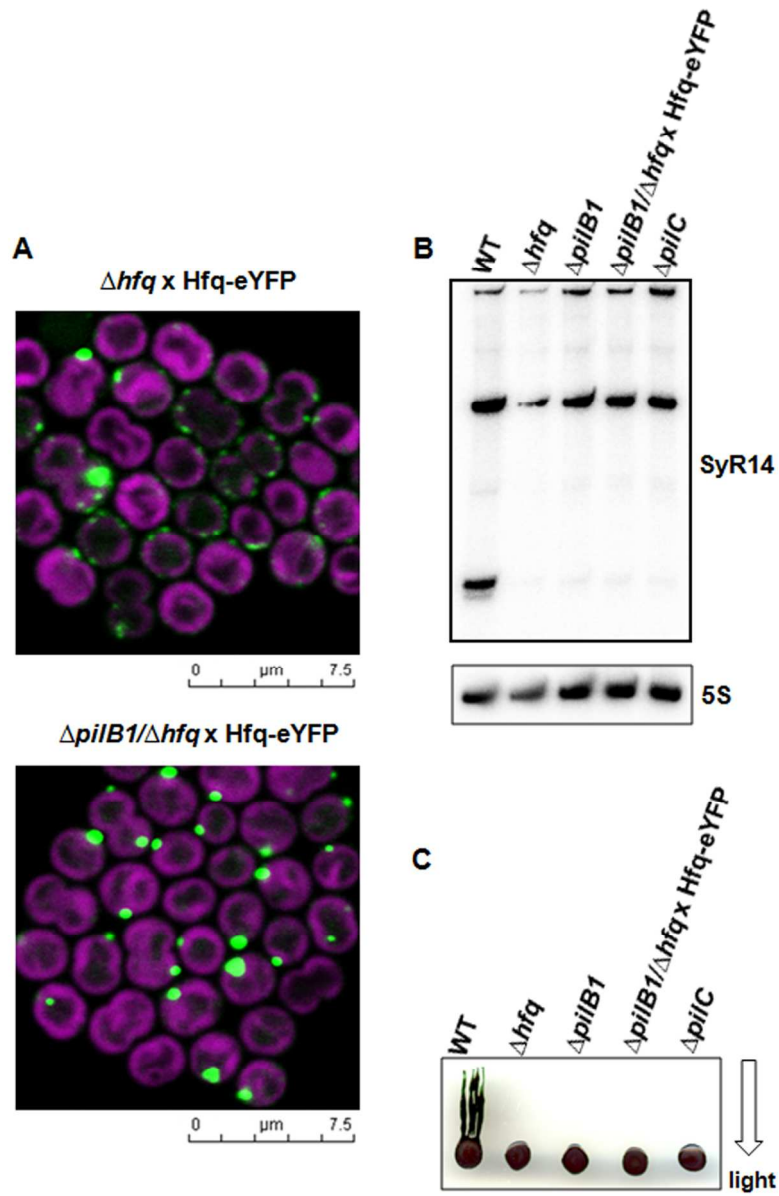


Figure 5  
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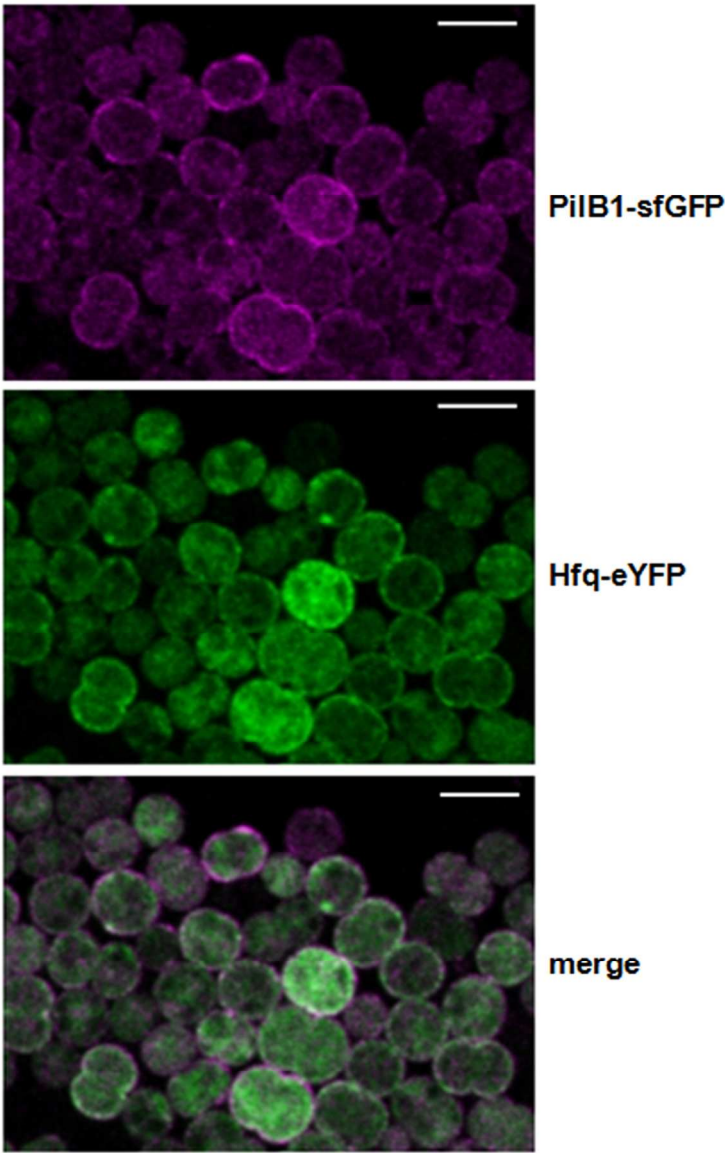


Figure 6  
99x124mm (300 x 300 DPI)