An Hfq-dependent post-transcriptional mechanism fine tunes RecB expression in *Escherichia coli*

Irina Kalita[1,2,3,*], Ira Alexandra Iosub[1,4,5], Lorna McLaren[1,2], Louise Goossens[1,2], Sander Granneman[1,4], and Meriem El Karoui[1,2,*]

[1]*Centre for Engineering Biology, University of Edinburgh, UK*
[2]*Institute of Cell Biology, University of Edinburgh, UK*
[3]*Max Planck Institute for Terrestrial Microbiology & Center for Synthetic Microbiology (SYNMIKRO), Marburg, Germany*
[4]*Institute of Quantitative Biology, Biochemistry and Biotechnology, University of Edinburgh, UK*
[5]*The Francis Crick Institute, London, UK*
[*]For correspondence: irykalita@gmail.com, meriem.elkaroui@ed.ac.uk

Abstract

All living organisms have developed strategies to respond to chromosomal damage and preserve genome integrity. One such response is the repair of DNA double-strand breaks (DSBs), one of the most toxic forms of DNA lesions. In *Escherichia coli*, DSBs are repaired via RecBCD-dependent homologous recombination. RecBCD is essential for accurate chromosome maintenance, but its over-expression can lead to reduced DNA repair ability. This apparent paradox suggests that RecBCD copy numbers may need to be tightly controlled within an optimal range. Using single-molecule fluorescence microscopy, we have established that RecB is present in very low abundance at mRNA and protein levels. RecB transcription shows high fluctuations, yet cell-to-cell protein variability remains remarkably low. Here, we show that the post-transcriptional regulator Hfq binds to *recB* mRNA and down-regulates RecB protein translation *in vivo*. Furthermore, specific disruption of the Hfq-binding site leads to more efficient translation of *recB* mRNAs. In addition, we observe a less effective reduction of RecB protein fluctuations in the absence of Hfq. This fine-tuning Hfq-mediated mechanism might have the underlying physiological function of maintaining RecB protein levels within an optimal range.

Key words: RecBCD enzyme, DNA repair, Hfq protein, post-transcriptional regulation, noise suppression.

Introduction

All living organisms rely on multiple molecular mechanisms to repair their chromosomes in order to preserve genome integrity. DNA double-strand breaks (DSBs), simultaneous lesions on both strands of the chromosome, are one of the most detrimental types of DNA damage because they can lead to loss of genetic information. If not repaired or repaired incorrectly, DSBs can trigger cell death, deleterious mutations and genomic rearrangements [1]. In addition to various exogenous sources such as ionizing radiation, mechanical stresses and DNA-damaging agents, DSBs can occur during the normal cell cycle as a results of endogenous metabolic reactions and replication events [2]. For instance, replication forks often encounter obstacles leading to fork reversal, accumulation of gaps that are repaired by the RecFOR pathway [3] or breakage which has been shown to result in spontaneous DSBs in 18% of wild-type *Escherichia coli* cells in each generation [4], underscoring the crucial need to repair these breaks to ensure faithful DNA replication.

In *E. coli*, DSBs are repaired by homologous recombination using an intact copy of the chromosome as a template. Initially, the multi-subunit nuclease/helicase enzyme RecBCD detects a double-strand DNA (dsDNA) end and degrades both strands. Upon recognition of a short DNA sequence, known as a *chi* (χ) site (5′-GCTGGTGG-3′), the RecBCD complex stops cleaving the 3′-ended strand and initiates loading of the RecA protein onto the 3′-single-strand (ssDNA) overhang [5, 6]. This leads to the formation of a RecA-ssDNA nucleoprotein filament [7], which catalyses homology search and strand exchange [8].

Recent *in vitro* and *in vivo* experiments have highlighted the extraordinary processive DNA degradation activity of RecBCD upon recognition of a dsDNA end [9, 10, 11]. Indeed, RecBCD has been shown to process the chromosome for up to ∼100 kb at ∼1.6 kb/s in live bacteria [6]. Such a potent DNA degradation activity is controlled by *chi* recognition [12]. However, because this recognition is probabilistic [13], RecBCD can degrade large chromosome fragments before DNA repair is initiated. As a

result, overproduction of RecBCD has been shown to sensitize wild-type cells to ultraviolet and gamma irradiations [14]. This strongly suggests that an excess of RecBCD imposes a threat to cells upon DNA damage. In contrast, RecBCD expression is essential upon DSB induction [15, 16], hence too low levels might impair DNA repair. Thus, the observation that RecBCD can positively and negatively impact cell fitness upon DNA damage suggests that its activity needs to be tightly controlled.

Whilst the biochemical and *in vivo* activities of RecBCD have been extensively studied ([12, 17, 18, 19, 20], and [8, 21] for review), less is known about the regulation of its expression. RecBCD has been reported to be expressed at very low levels [22, 23] and is present in less than ten molecules per cell [24]. In *E. coli*, the RecBCD subunits are encoded in the same locus by two operons. One operon controls the expression of RecC whilst the other is polycistronic and encodes PtrA, RecB and RecD [25, 26] (Fig. 1A). PtrA is a periplasmic protease with no known function in DNA repair, and the positioning of its gene likely results from a horizontal gene transfer event that has interrupted the usual order *recC-recB-recD* [27]. As sometimes found in bacterial operons [28, 29], the ribosome binding site (RBS) of *recB* is located within the coding sequence of *ptrA*, suggesting a potential translational coupling mechanism between these genes. Previous attempts to explore RecBCD expression upon DNA-damage conditions did not reveal any transcriptional regulation [22, 30] and the *recBCD* genes were shown not to belong to the SOS-response regulon [31]. Thus, RecBCD transcription is considered to be constitutive.

Given that RecBCD is essential for cell survival upon DSB formation and toxic when overproduced, it is likely that its expression needs to be tightly regulated to avoid fluctuations leading to cellular death or deleterious genomic rearrangements. However, its very low abundance, often leading to high stochastic fluctuations, could result in large cell-to-cell variability [32, 33, 34]. Although these fluctuations might not play a significant role during normal cell growth, they may become crucial and determine cell fate upon stress conditions [35, 36, 37]. Thus, the low abundance of RecBCD enzyme molecules raises the following questions: (i) to what extent do RecBCD numbers fluctuate, (ii) are these fluctuations controlled, (iii) if this is the case, at which level (transcription, translation, mRNA or protein degradation) is the control operating.

The lack of evidence of RecBCD transcriptional regulation does not preclude that other mechanisms may be involved in regulating its expression. For example, RNA-binding proteins (RBPs) have been shown to fine tune gene expression both co- and post-transcriptionally during stress responses and adaptation to environmental changes [38, 39]. One of the most studied RBPs in *E. coli*, a Sm-like hexameric ring-shaped protein called Hfq, has been shown to regulate RNA stability and degradation, transcription elongation and translation initiation through various mechanisms (for review [40, 41, 42, 43]). In the most prevalent case, Hfq facilitates base-paring between small non-coding RNAs (sRNAs) and target mRNAs which often results in mRNA translation and/or stability modulation [44]. There is also increasing evidence that Hfq can control mRNA translation directly [45, 46]. This mechanism is typically based on altering the accessibility of the RBS of a target mRNA by direct binding near this region [47, 48, 49].

Here, we show that the expression of RecB is regulated post-transcriptionally in an Hfq-dependent manner in *E. coli*. By quantifying RecB mRNAs and proteins at single-molecule resolution, we found that constitutive transcription leads to low levels of *recB* mRNAs and a noisy distribution. In contrast, fluctuations in RecB protein levels are significantly lower than expected, given the very low number of proteins present in cells. We show that Hfq negatively regulates RecB translation and demonstrate the specificity of this post-transcriptional control *in vivo*. Furthermore, we provide evidence of the role of Hfq in reducing RecB protein fluctuations and speculate that this could contribute into a fine-tuning mechanism to control RecBCD molecules within an optimal range.

Results

recB mRNAs are present at very low levels and are short-lived

To quantitatively measure RecB expression, we first precisely quantified *recB* mRNAs using single-molecule fluorescent *in situ* hybridization (smFISH) [50]. We confirmed that our hybridization conditions are highly specific as *recB* mRNA quantification in a Δ*recB* strain resulted in a negligible error (∼0.007 molecules per cell, Fig. 1B). In wild-type cells, we detected a very low average level of *recB* transcripts: ∼0.62 molecules per cell. Notably, ∼70% of cells did not have any *recB* mRNAs (Fig. 1C). To confirm that this result was not because of low sensitivity of the smFISH protocol, we over-expressed *recB* mRNAs from an arabinose-inducible low-copy plasmid and detected the expected gradual amplification of the fluorescence signal with increased arabinose concentration (Fig. S2).

We next measured the degradation rate of *recB* transcripts in a time-series experiment. Initiation of

transcription was inhibited with rifampicin and *recB* mRNA expression levels were quantified by sm-FISH at subsequent time points. By fitting the mean mRNA value evolution over time to an exponential function, we estimated the *recB* degradation rate as $\gamma_m = 0.62\ min^{-1}$ ($CI_{95\%}$ [0.48, 0.75]) (Fig. 1D). This corresponds to a *recB* mRNA lifetime of 1.6 *min* and is consistent with the genome-wide studies in *E. coli* where transcript lifetimes were shown to be on average ∼2.5 *min* across all mRNA species [51, 52].

recB transcription is constitutive and weak

As noted previously, quantitative analysis of transcription at the single-cell level needs to take into account the variation of gene copy number during the cell cycle [53, 54, 55]. Therefore, we analysed RNA molecule abundance using cell size as a proxy for the cell cycle. Specifically, we binned all bacteria in groups based on cell area and averaged mRNA numbers in each of those size-sorted groups (Fig. 1E). A two-fold increase in mRNA abundance was observed within a narrow cell-size interval (∼3.0-3.5 μm^2), which is consistent with an expected doubling of the transcriptional output as a result of the replication of the *recB* gene. We further showed that *recB* transcription from sister chromosomes happens independently, in full agreement with the hypothesis of an unregulated gene (Fig. S3). Taken together, this evidence indicates that *recB* mRNA levels follow a gene-dosage trend, strongly suggesting that *recB* is constitutively expressed [53].

Next, to infer *recB* transcription rate, we used a simple stochastic model of transcription where mRNAs are produced in bursts of an average size b at a constant rate k_m and degraded exponentially at a rate γ_m (Supplementary Information). The expected probability distribution for mRNA numbers at steady-state is given by a negative binomial [56, 57, 58]. We used maximum likelihood estimation to infer the parameters of the negative binomial distribution from the experimental data (Fig. 1F, Methods). For the inference, we restricted our analysis to newborn cells (Fig. 1E) as this model does not take into account cell growth. We then inferred the burst size for the *recB* gene, $b \sim 0.95$, and the rate of transcription, $k_m = 0.21\ min^{-1}$ (Table S7, Methods). The latter is remarkably low and close to the lower-bound reported range of transcription rates in *E. coli*, 0.17-1.7 min^{-1} [59, 60, 61]. Therefore, the low abundance of *recB* mRNAs can be explained by infrequent transcription combined with fast mRNA decay.

Variations in mRNA molecule numbers, as a result of their low abundance and short lifetimes, can significantly contribute to gene expression noise [32, 62, 63]. To evaluate fluctuations of *recB* transcription, we calculated a commonly-used measure of noise, the squared coefficient of variation (CV_m^2), defined as the variance divided by the squared mean $\sigma_m^2/\langle m\rangle^2$. The average CV_m^2 in newborn cells was found to be $CV_m^2 = 5.9$, almost twice larger than the lower bound given by a Poisson process ($CV_{Poisson}^2 = 1/\langle m\rangle = 3.1$). This suggests that *recB* mRNA production is noisy and further supports the absence of a regulation mechanism at the level of mRNA synthesis.

RecB proteins are present in low numbers and are long-lived

To determine whether the mRNA fluctuations we observed are transmitted to the protein level, we quantified RecB protein abundance with single-molecule accuracy in fixed individual cells using the Halo self-labelling tag (Fig. 2A&B). The HaloTag has been translationally fused to RecB in a loop after Ser47 [24] where it is unlikely to interfere with the formation of RecBCD complex [64], the initiation of translation and conventional C-terminal-associated mechanisms of protein degradation [65]. Consistently with minimal impact on RecB production and function, bacterial growth was not affected by replacing the native RecB with RecB-HaloTag, the fusion was fully functional upon DNA damage and no proteolytic processing of the construct was detected [24]. To ensure reliable quantification in bacteria with HaloTag labelling, the technique was previously verified with an independent imaging method and resulted in > 80% labelling efficiency [24, 66]. In order to minimize the number of newly produced unlabelled RecB proteins, labelling and quick washing steps were followed by immediate chemical fixation of cells. By applying this approach, we obtained RecB protein distributions for cells of all sizes and confirmed its low abundance: 3.9 ± 0.6 molecules per cell (Fig. 2C).

To test if RecB is actively degraded by proteases or mainly diluted as a result of cell growth and division, we estimated the RecB protein removal rate. This is usually obtained from either pulse-chase experiments using radio-labelled amino acids or detection of protein concentration decay after treatment with a translation inhibitor [67, 68, 69]. While the first approach is not amenable to single-cell assays, the latter may affect the measurements on long time scales because of the physiological impacts of stopping all protein production. Therefore, we used an alternative approach [70, 71] where we pulse-labelled RecB molecules with the HaloTag ligand, then removed the excess of the dye by extensive washing and measured the abundance of the labelled molecules over time. Assuming protein removal can be described by a first-order reaction, we fitted the mean protein counts over time to an exponential

function on a subset of time points (Fig. 2D, shaded area). The decay rate was extracted from the fit and resulted in $\gamma_p = 0.015\ min^{-1}$ ($CI_{95\%}$ [0.011, 0.019]). Comparison to the population growth rate in these conditions (0.017 min^{-1}) suggests that RecB protein is stable and effectively removed only as a result of dilution and molecule partitioning between daughter cells. This result is consistent with a recent high-throughput study on protein turnover rates in *E. coli*, where the lifetime of RecB proteins was shown to be set by the doubling time [72].

RecB protein cell-to-cell fluctuations are lower than expected

We then extended the transcription model described above by considering protein synthesis and removal. This is known as the *two-stage* or *mRNA-protein* model of gene expression [58, 73, 74]. In this model, a protein is produced from an mRNA at a constant translation rate k_p while protein dilution is implicitly modelled by a first-order chemical reaction with rate γ_p (Supplementary Information). As discussed earlier, we focused on newborn cells and estimated the rate of translation as 0.15 min^{-1} ($CI_{95\%}$ [0.12, 0.18], Table S7, Methods). This value fell below the lower bound of previously reported range of the translation rates in *E. coli* (0.25-1 min^{-1}) [59, 60]. Therefore, we concluded that *recB* mRNAs are translated at low levels compared to other proteins in the cell.

Next, we examined whether the two-stage model can reproduce the cell-to-cell variability in the protein levels observed in our experiments. To this end, we simulated single trajectories of protein abundance over time using the Stochastic Simulation Algorithm (SSA) [75] for the two-stage model with the estimated parameters (Table S7). Strikingly, the simulated distribution had larger fluctuations than the experimentally measured one (Fig. 2E). While both protein means were equal because of the first-moment-based parameter estimation we used (Methods), we found the standard deviations to be higher in simulation (2.2) than in experiments (1.5) (Fig. 2E). In other words, a two-stage protein production model predicts a higher level of fluctuations than what is observed experimentally.

This phenomenon can also be analysed using the squared coefficient of variation of the protein distribution CV_p^2, which is defined as $\sigma_p^2/\langle p\rangle^2$. For the two-stage gene expression model with bursty transcription, it has been established that the following expression gives the coefficient of variation (Supplementary Information) [58, 73, 74, 76]:

$$CV_p^2 = \frac{1}{\langle p\rangle} + \frac{1+b}{\langle m\rangle}\frac{\gamma_p}{\gamma_m+\gamma_p} \tag{1}$$

where $\langle p\rangle$ and $\langle m\rangle$ are the mean protein and mRNA counts (per cell), b is the average (mRNA) burst size, γ_m and γ_p are the mRNA degradation and protein removal rates, respectively. We found that the experimental coefficient of variation (CV^2_{Data} = 0.19±0.06) is approximately half of the analytically predicted one (CV^2_{Theory} = 0.45)(Fig. 2F). On the contrary and as expected, both the theoretically predicted CV^2_{Theory} and the one computed from the simulated data CV^2_{SSA} are in good agreement. This deviation from the theoretical prediction implies a potential regulatory mechanism of RecB expression that actively suppresses protein production variation to maintain protein levels within a certain range.

RecB translational efficiency is increased upon DNA damage

Next, we addressed whether there was any further evidence of this potential regulation once bacterial cells are exposed to stress. We hypothesized that RecB mRNA and/or protein levels may exhibit a concentration change upon DNA damage to stimulate DNA repair. To test this, we treated bacterial cells with a sub-lethal concentration of ciprofloxacin (4 ng/ml), an antibiotic that leads to DNA DSB formation [77]. After two hours of exposure to ciprofloxacin, we quantified proteins by Halo-labelling and mRNAs by smFISH (Fig. 3A). We used mathematical modelling to verify that two hours of antibiotic exposure was sufficient to detect changes in mRNA and protein levels and for RecB mRNA and protein levels to reach a new steady state in the presence of DNA damage (Supplementary Information). Because *E. coli* cells filament upon activation of the DNA damage response (Fig. 3B&C), we measured single-cell distributions of mRNA and protein concentrations (calculated as molecule numbers normalized by cell area) rather than absolute numbers. Cell area is a reasonable proxy for cell volume as rod-shaped *E. coli* cells maintain their diameter while elongating in length upon ciprofloxacin treatment. Under DNA damage conditions, RecB protein concentration remained similar to the intact control (Fig. 3E). In fact, the average cellular RecB concentration was surprisingly conserved: 2.28 $mol/\mu m^2$ ($CI_{95\%}$: [2.21, 2.32]) in perturbed condition and 2.12 $mol/\mu m^2$ ($CI_{95\%}$: [2.01, 2.32]) in intact cells. Additionally, we analysed RecB concentration in size-sorted groups of cells (Fig. S5b). Regardless of moderate fluctuations in

protein concentration over a cell cycle, both samples significantly overlap along the whole range of cell sizes.

In contrast, *recB* mRNA concentration was found to be decreased under exposure to ciprofloxacin (Fig. 3D). Specifically, mRNA concentrations under DNA damage conditions are ∼2.4-fold lower than in the unperturbed control (Fig. 3D: insert): 0.12 $mol/\mu m^2$ ($CI_{95\%}$: [0.06, 0.17]) upon DNA damage versus 0.28 $mol/\mu m^2$ ($CI_{95\%}$: [0.23, 0.32]) in the control. We further confirmed the decrease in all cell-size sorted groups (Fig. S5a). As previously reported, the *recB* gene is not part of the SOS regulon [31], hence its transcription is not necessarily expected to be upregulated upon DNA damage. However, a lower level of *recB* mRNAs is an unforeseen observation and may be a result of lower concentration of *recB* gene copies and/or shortage of resources (such as RNA polymerases) upon SOS response activation.

We then computed the average number of proteins produced per mRNA in both conditions. The ratio between the average protein and mRNA concentrations in damaged cells is 19.7 ($CI_{95\%}$: [17.5, 24.2]) whereas it is 7.9 proteins/mRNA ($CI_{95\%}$: [7.5, 8.3]) in intact cells (Fig. 3F). Thus, a ~ 2.5 increase in translational efficiency was detected under DSB damage conditions (p-value < 0.001, two sample t-test). Taken together, our results show decoupling between mRNA and protein production upon DNA damage, suggesting the existence of a post-transcriptional regulatory mechanism that controls RecB protein level.

Hfq controls the translation of *recB* mRNA

To identify the post-transcriptional mechanism controlling *recB* expression, we focused on Hfq because (i) it has been shown to regulate a vast number of mRNAs [78, 79, 80] and (ii) its activity has been directly linked to the DNA damage and SOS responses [46, 81, 82].

To assess whether Hfq is involved in post-transcriptional regulation of RecBCD, we analysed the available transcriptome-wide dataset, wherein Hfq binding sites were identified in *E. coli* using CLASH (cross-linking and analysis of cDNA) [83]. In a CLASH experiment, Hfq-RNA complexes are cross-linked *in vivo* and purified under highly stringent conditions, ensuring specific recovery of Hfq RNA targets [83]. Analysis of the Hfq binding profile across *recBCD* transcripts showed the interaction of Hfq with the *recBCD* mRNAs (Fig. 4A). Hfq binding is mainly localized at two sites: one in close proximity to the RBS of the *ptrA* gene, and the second further downstream in the coding sequence of *recB* gene. Interestingly, multiple trinucleotide Hfq-binding motifs, A-R(A/G)-N(any nucleotide) [78, 84] were found ∼8 nucleotides upstream of the RBS of the *ptrA* sequence (Fig. S6), suggesting that Hfq may control translation initiation and/or decay of the operon mRNA.

To test this hypothesis, we quantified RecB protein and mRNA levels in an *E. coli* strain lacking Hfq. Δ*hfq* mutant has been reported to have an increased average cell size and various growth defects [85, 86, 87]. Indeed, we confirmed the increased cell size of our Δ*hfq* mutant compared to wild-type cells (Fig. S7a&b). In our conditions, no difference between the growth rates of WT and the Δ*hfq* mutant was observed in the exponential phase of growth (Fig. S7e).

Using Halo-labelling technique, we performed RecB protein quantification in Δ*hfq*. Remarkably, we observed an increase of RecB protein concentration in the Δ*hfq* cells (Fig. 4B&C). The Δ*hfq* mutant showed more fluorescent foci relative to the wild-type strain (Fig. 4B). Even after normalization by cell area to take into account the larger cell size in Δ*hfq* cells, RecB concentration distribution was shifted towards higher values (Fig. 4C). Quantification across five replicated experiments showed a statistically significant 30% increase in RecB protein concentration in the Δ*hfq* strain (Fig. 4C: insert). The concentration was 2.67 $mol/\mu m^2$ ($CI_{95\%}$: [2.54, 2.80]) in Δ*hfq* compared to 2.17 $mol/\mu m^2$ ($CI_{95\%}$: [2.01, 2.32]) in the control.

We further quantified *recB* mRNA molecules in the Δ*hfq* strain using smFISH and analysed mRNA concentration distributions (Fig. 4D&E). Although the average *recB* mRNA concentration is slightly decreased in the Δ*hfq*: 0.21 $mol/\mu m^2$ ($CI_{95\%}$: [0.08, 0.30]) *versus* 0.28 $mol/\mu m^2$ ($CI_{95\%}$: [0.23, 0.32]) in the wild-type cells, this difference is insignificant (p-value = 0.11, two sample t-test). These results were confirmed by RT-qPCR measurements, which did not show any significant changes for *recB* mRNA steady-state levels and lifetime (Fig. S7c,d). Thus, we conclude that Hfq binding to the *recBCD* mRNA does not substantially alter the stability of the transcript.

The observed increase of RecB protein concentration without a significant change in *recB* mRNA steady-state levels is reflected in a rise in RecB translational efficiency (Fig. 4F). The average level of proteins produced per mRNA in the Δ*hfq* mutant is 14.0 ($CI_{95\%}$: [13.0, 18.1]), which is two-fold higher than in the wild-type cells (p-value < 0.05, two-sample t-test). Altogether, this demonstrates that Hfq alters RecB translation *in vivo*.

To test if Hfq regulation contributes to the noise suppression detected in the wild-type cells (Fig. 2F), we looked at the level of fluctuations of RecB copy numbers in Δ*hfq*. To this end, we computed the difference between the analytically predicted noise CV^2_{Theory} and the noise observed experimentally CV^2_{Exp}. Since in the Δ*hfq* mutant *recB* transcription is not affected, the theoretical noise was calculated using Eq. (1) assuming the same mRNA contribution (the second term of the equation). As seen in Fig. 4G, the wild-type strain shows effective noise suppression as the theoretically predicted noise is larger than the one detected experimentally. Interestingly, this suppression is less effective in the Δ*hfq* mutant. In other words, in the absence of Hfq, the mismatch between the model and the experimental data is decreased in comparison to the native conditions of the wild-type cells (p-value = 0.0088, two sample t-test). This provides evidence that, in addition to the average RecB abundance, Hfq affects RecB protein fluctuations.

RecB protein number distribution is recovered by Hfq complementation

To further confirm that Hfq regulates RecB expression, we complemented the Δ*hfq* mutant with a multi-copy plasmid that expresses Hfq, pQE-Hfq [88]. We confirmed the functionality of the Hfq protein expressed from the pQE-Hfq plasmid in our experimental conditions (Fig. S8d). After complementation, the RecB protein concentration distribution was shifted towards lower values (Fig. 5B) whilst no change of RecB protein level was detected in the Δ*hfq* cells carrying the backbone plasmid, pQE80L (Fig. S8a). Although in our experimental conditions, Hfq is expressed at lower levels than in wild-type cells (transcription is $\sim$ a third of the wild-type level, Fig. S8b), RecB protein concentration was nearly fully recovered to the wild-type conditions: 2.57 $mol/\mu m^2$ ($CI_{95\%}$: [2.50, 2.65]). We avoided full induction of Hfq from the plasmid as it can interfere with its self-regulation and lead to cell filamentation. We did not detect significant changes in the *recB* mRNA levels when expressing Hfq from the plasmid (Fig. S8c), confirming that Hfq complementation impacts *recB* mRNA translation but not its stability.

Sequestering Hfq leads to an increase in RecB production

To further ascertain the role of Hfq in controlling RecB expression, we characterized RecB expression while sequestering Hfq proteins away from its *recB* mRNA targets. Hfq is known to bind $\sim$100 sRNAs [78, 80], some of which can reach high expression levels and/or have a higher affinity for Hfq and, thus, compete for Hfq binding [89, 90]. We reasoned that because *recB* mRNA is present in very low abundance, it could be outcompeted for Hfq binding by over-expression of a small RNA that efficiently binds Hfq, such as ChiX [90, 91]. We constructed a multi-copy plasmid pZA21-ChiX to over-express ChiX and obtained a $\sim$30-fold increase in ChiX expression relative to its native expression in wild-type cells (Fig. S9). Given its already high abundance in wild-type cells, ChiX overproduction has been demonstrated to effectively sequester a large number of Hfq molecules [47, 49, 92].

Wild-type cells carrying the pZA21-ChiX plasmid (or the backbone plasmid) were grown to mid-exponential phase, and RecB mRNA and protein levels were quantified with RT-qPCR and Halo-labelling, respectively. No significant changes were detected at the *recB* transcript levels when ChiX was overproduced (Fig. S9). In contrast, a significant increase in RecB protein production was detected upon ChiX over-expression (Fig. 5C). Indeed, RecB concentration in the cells where ChiX was over-expressed almost fully overlapped with the distributions in the Δ*hfq* mutant. This suggests that when fewer Hfq molecules are available for RecB regulation because ChiX is overproduced, RecB is translated at a higher rate. A similar experiment with a plasmid that carried a different sRNA, CyaR which is not expected to titrate Hfq, did not change RecB protein concentration. This indicates that ChiX over-expression specifically impairs RecB post-transcriptional regulation.

To exclude the possibility of a direct ChiX involvement in RecB regulation, we characterized RecB expression in Δ*chiX*. No significant change relative to wild-type cells was detected in RecB mRNA or protein levels (Fig. S10). Thus, we concluded that ChiX does not affect RecB translation. Instead, titration of Hfq proteins from a shared pool by ChiX sRNAs leads to the increased availability of *ptrA-recB* RBS for ribosomes and, thus, results in more efficient RecB translation.

Deletion of an Hfq-binding site results in increased RecB translational efficiency

To further investigate the mechanism of RecB translation, we tested the specificity of Hfq interaction with the mRNA of *recB* without affecting other functions of Hfq. Based on the localization of the Hfq-binding uncovered in the CLASH data (Fig. 4A), we deleted a 36 nucleotide region (5'-TTAACGTGTTGAATCTGGACAGAAAATTAAGTTGAT-3') located in 5'UTR of the operon, in front of *ptrA* gene. This region showed the highest enrichment of Hfq binding (Fig. S6). It is closely located to the RBS site and 76 nucleotides downstream of the promoter sequence of *ptrA-recB*. To get a complete

picture of RecB expression in the mutant strain carrying this deletion (which we further refer to as the *recB*-5′UTR* mutant), we performed single-cell quantification of both mRNA and protein levels of RecB and quantified its translational efficiency.

Firstly, we quantified *recB* mRNAs molecules in the *recB*-5′UTR* strain using smFISH (Fig. 5D). We detected a significant decrease in the concentration of mRNAs in the strain with the modified sequence: 0.14 $mol/\mu m^2$ ($CI_{95\%}$: [0.07, 0.17]) compared to 0.28 $mol/\mu m^2$ ($CI_{95\%}$: [0.23, 0.32]) in the wild type. This is not an unexpected outcome as the 5′-untranslated region of a transcript can control its degradation and stability [93, 94]. Despite the mRNA steady-state level decreasing two-fold in the modified *recB*-5′UTR* strain, only a slight decrease in RecB protein concentration was detected in comparison with the wild type (Fig. 5E): 1.96 $mol/\mu m^2$ ($CI_{95\%}$: [1.56, 2.36]) in *recB*-5′UTR* and 2.17 $mol/\mu m^2$ ($CI_{95\%}$: [2.01, 2.32]) in the wild type.

We calculated the translational efficiency of *recB* transcripts based on our single-cell mRNA and protein quantification. The resulting translational efficiency in the *recB*-5′UTR* strain was found to be significantly higher than in the wild-type cells: 16.3 ($CI_{95\%}$: [14.3, 19.7]) in *recB*-5′UTR*, whereas it is 7.9 ($CI_{95\%}$: [7.5, 8.3]) proteins/mRNA in the wild type (Fig. 5F). Remarkably, the RecB translational efficiency in the *recB*-5′UTR* strain is comparable with the values observed in the Δ*hfq* mutant (a two-sample t-test confirmed that the difference is not significant). Thus, when the main Hfq binding site was deleted, the lower abundance of the mRNA was compensated by more efficient translation. This demonstrates that the translation of *recB* mRNA is limited by a specific interaction with Hfq.

Discussion

Despite its essential role in DSB repair, over-expression of RecBCD decreases cell viability upon DNA damage induction [14]. Such a dual impact on cell fitness suggests the existence of an underlying regulation that controls RecBCD expression within an optimal range. Using state-of-the-art single-molecule mRNA and protein quantification, we (i) characterize RecB expression at transcriptional and translational levels in normal and DNA-damaging conditions and (ii) describe a novel post-transcriptional mechanism of RecB expression mediated by the global regulator Hfq *in vivo*. Utilizing stochastic modelling, we provide evidence that Hfq contributes to suppressing fluctuations of RecB molecules. To our knowledge, this is the first experimental evidence of an RNA-binding protein involved in noise suppression in bacterial cells under native conditions.

While a few recent studies have shown evidence for direct gene regulation by Hfq in a sRNA-independent manner [46, 47, 48, 49, 95], we attempted to investigate whether a small RNA could be involved in the Hfq-mediated regulation of RecB expression. We tested Hfq mutants containing point mutations in the proximal and distal sides of the protein, which were shown to disrupt either binding with sRNAs or with ARN motifs of mRNA targets, respectively [79, 96]. Hfq mutated in either proximal (K56A) or distal (Y25D) faces were expressed from a plasmid in Δ*hfq* background. In both cases, Hfq expression was confirmed with qPCR and did not affect *recB* mRNA levels (Fig. S11b). When the proximal Hfq binding side (K56A) was disrupted, RecB protein concentration was nearly similar to that obtained in Δ*hfq* (Fig. S11a, top panel). This observation suggests that the repression of RecB translation requires the proximal side of Hfq, and that a small RNA is likely to be involved as small RNAs (Class I and Class II) were shown to predominantly interact with the proximal face of Hfq [97]. When we expressed Hfq mutated in the distal face (Y25D) which is deficient in binding to mRNAs, less efficient repression of RecB translation was detected (Fig. S11a, bottom panel). This suggests that RecB mRNA interacts with Hfq at this position. We did not observe full de-repression to the Δ*hfq* level, which might be explained by residual capacity of Hfq to bind its *recB* mRNA target in the point mutant (Y25D) (either via the distal face with less affinity or via the lateral rim Hfq interface). Taken together, these results suggest that Hfq binds to *recB* mRNA and that a small RNA might contribute to the regulation. Therefore, we next attempted to looked for potential candidates of sRNAs, using the available Hfq CLASH and RIL-seq datasets [83, 98]. CyaR and ChiX sRNAs were identified as potentially interacting with the *ptrA-recB-recD* mRNA. However, we did not find any significant changes in RecB expression when CyaR was overexpressed (Fig. S9) and we showed that ChiX was involved indirectly by sequestering Hfq (Fig. 5 & Fig. S10). Altogether, we could neither rule out the possibility of another small RNA candidate nor identify one involved, but the low abundance of the *recB* transcripts is likely to make it particularly difficult to detect RNA-RNA interactions in bulk experiments, making it necessary to develop a specific approach with single-molecule resolution.

Hfq-mediated control of RecB expression may be just a hint of a larger bacterial post-transcriptional

stress-response programme to DNA damage. Indeed, post-transcriptional regulation has already been shown to impact DNA repair and genome maintenance pathways [81, 82, 99, 100]. For instance, the DNA mismatch repair protein, MutS, was shown to be regulated by a sRNA-independent Hfq binding mechanism and its translation repression was linked to increased mutagenesis in stationary phase [46]. In addition, a modest decrease ($\sim$30%) in Hfq protein abundance has been seen in a proteomic study in *E. coli* upon DSB induction with ciprofloxacin [101]. While Hfq is a highly abundant protein, it has many mRNA and sRNA targets, some of which are also present in large amounts [102]. As recently shown, the competition among the targets over Hfq proteins results in unequal (across various targets) outcomes, where the targets with higher Hfq binding affinity have an advantage over the ones with less efficient binding [89]. In line with these findings, it is conceivable that even modest changes in Hfq availability could result in significant changes in gene expression, and this could explain the increased translational efficiency of RecB under DNA damage conditions. Therefore, Hfq-mediated regulation might play a larger role in DNA repair pathways than previously considered. As many metabolic pathways are regulated through Hfq [103, 104, 105], the control of RecB (and other DNA repair proteins) expression may even provide a way of coordinating DNA repair pathways with other cellular response processes upon stress.

Mechanisms similar to the one described in our study might underlay the expression networks of other DNA repair proteins, many of which are known to be present in small quantities. In this regard, it is worth emphasizing the valuable methodological insights which can be taken from our study. By utilizing single-molecule mRNA and protein quantification techniques, we show the importance of detailed investigation of both mRNA and protein levels when establishing novel regulation mechanisms of gene expression. Indeed, measuring the protein level only would not be sufficient to draw the correct interpretation, for example, in the case of *recB*-5'UTR* mutants (Fig. 5). Moreover, single-molecule measurements combined with stochastic modelling allow access to cell-to-cell variability, which can be a powerful and, possibly, the only approach to investigate the regulation mechanisms of gene expression for low abundant molecules.

In agreement with a previous report [14], we confirm that RecBCD overproduction led to less efficient repair when DSBs were induced using ciprofloxacin (Fig. S12). We note that another study did not detect any toxicity of RecBCD overproduction upon DNA damage [106]. However, this is likely because the level of UV irradiation was too low to detect a significant reduction in cell viability. We only observed the effect at a relatively high level of ciprofloxacin exposure (>10 ng/ml with the minimum inhibitory concentration at 16 ng/ml in our conditions). It is worth noting that the observed decrease in cell viability upon DNA damage was detected for relatively drastic perturbations such as *recB* deletion and RecBCD overexpression. Verifying these observations in the context of more subtle changes in RecB levels would be important for further investigation of the biological role of the uncovered regulation mechanism. However, the extremely low numbers of RecB proteins make altering its abundance in a refined, controlled, and homogeneous across cells manner extremely challenging and would require the development of novel synthetic biology tools.

In addition to the regulation of the average number of proteins per cell, fluctuation control might be crucial for a quasi-essential enzyme with very low abundance, such as RecBCD. Our analysis of RecB fluctuations suggests that Hfq is playing a role in protein copy number noise reduction (Fig. 4G). Post-transcriptional regulation has been proposed theoretically as an effective strategy for reducing protein noise [63, 107, 108, 109, 110]. Conceptually, this is achieved via buffering stochastic fluctuations of mRNA transcription and enabling more rapid response. Based on these principles, suppression of protein fluctuations via post-transcriptional control has been demonstrated in synthetically engineered systems in bacteria, yeast and mammalian cells [111, 112, 113]. However, do cells employ these strategies under native conditions? Suppressed fluctuations of protein expression were experimentally detected for hundreds of microRNA-regulated genes in mammalian cells [114]. Remarkably, those genes were expressed at a very low level. This is precisely the case for the regulation of *recB* translation by Hfq, where a very abundant post-transcriptional regulator can effectively buffer the fluctuations of the low-abundant RecB enzyme. Taken together, these previous observations and our results could hint at a universal functional 'niche' for post-transcriptional regulators across a wide range of organisms.

Methods

Strains and plasmids

All strains and plasmids, used in the study, are listed in Table S1 and Table S2, respectively. The *E. coli* MG1655 and its derivatives were used for all conducted experiments except the one with arabinose-inducible expression where we used *E. coli* BW27783 background strain. The strains with RecB-HaloTag fusion were used in Halo-labelling experiments while the corresponding parental strains were used in smFISH experiments. MEK1329 and MEK1938 were built using the plasmid-mediated gene replacement method [115] with pTOF24 derivative plasmids, pTOFΔ*recB* [116] and pTOF*recB*-5′UTR, respectively. The deletion of *recB* and a 36 bp region (5′-TTAACGTGTTGAATCTGGACAGAAAATTAAGTTGAT-3′) were verified with Sanger sequencing. MEK1902 and MEK1457 were constructed with lambda red cloning technique using hfq_H1_P1 and hfq_H2_P2 primers (Table S3). MEK1888 and MEK1449 were constructed with lambda red using ChiX_H1_P1 and ChiX_H2_P2 primers (Table S3). *hfq* and *chiX* deletions were verified by PCR.

pIK02 was constructed to induce *recB* expression. The backbone was amplified with the primers oIK03/oIK04 from pBAD33 vector while the *recB* gene was amplified from *E. coli* chromosome using oIK10/oIK11 oligos (Table S3). The PCR products were ligated using Gibson. Plasmid construction was verified by sequencing. pZA21-ChiX plasmid was constructed to allow overproduction of a small RNA ChiX. The plasmid, derived from the pZA21MCS (Expressys), was generated according to the protocol described here [83] using chiX_ZA21 and pZA21MCS_5P primers (Table S3). To construct pTOF*recB*-5′UTR, the pTOF24 plasmid was digested with XhoI and SalI and ligated using Gibson assembly with the gBlock *recB*-5′UTR (Table S4). The deletion of a 36 bp region was confirmed by sequencing.

Growth media and conditions

In microscopy and population experiments, cell cultures were grown in M9-based medium supplemented with 0.2% glucose, 2 mM $MgSO_4$, 0.1 mM $CaCl_2$ and either 10% Luria Broth (Figures 1&2) or MEM amino acids (from Gibco) (Figures 3–5). In the *recB* induction smFISH experiments, 0.2% glucose was replaced with 0.2% glycerol, and arabinose ranging from 10^{-5}% to 1% was added to the medium. For mRNA lifetime measurements with smFISH, rifampicin (Sigma-Aldrich) was added to cell cultures to a final concentration of 500 $\mu g/ml$. In the DSB induction experiments, a sub-lethal concentration of ciprofloxacin 4 ng/ml was used. Ampicillin (50 $\mu g/ml$), kanamycin (50 $\mu g/ml$) or chloramphenicol (30 $\mu g/ml$) were added to cultures where appropriate.

Cell cultures were grown in medium overnight (14–16 hours) at 37°C. The overnight cultures were diluted (1:300) and grown at 37°C until the mid-exponential phase (optical density OD_{600} = 0.2–0.3). Unless otherwise stated, cells were further treated according to the smFISH or Halo-labelling procedures described below. In the *recB* induction smFISH experiments, arabinose was added after one hour of overday growth, the cultures were grown for one more hour, and then samples were collected. For the smFISH experiments with rifampicin, the antibiotic was added once the cultures had reached OD_{600} = 0.2. Cells were harvested at 1 min intervals and fixed in 16% formaldehyde. In the DSB induction experiments, ciprofloxacin was added at $OD_{600} \sim 0.1$ and cell cultures continued growing for one hour. For mRNA quantification with smFISH, the cultures were grown for one more hour before being harvested and fixed in the final concentration of 3.2% formaldehyde. For protein quantification, the Halo-labelling protocol was followed as described below with ciprofloxacin being kept in the medium during labelling and washing steps.

Single-molecule RNA FISH (smFISH)

Single-molecule fluorescent *in situ* hybridization (smFISH) experiments were carried out according to the established protocol [50]. Bacterial cells were grown as described above, fixed in formaldehyde solution, permeabilised in ethanol and hybridized with a *recB* specific set of TAMRA-labelled RNA FISH probes (LGC Biosearch Technologies). Probe sequences are listed in Table S6. Unbound RNA FISH probes were removed with multiple washes, and then samples were visualized as described below.

Halo-labelling

For single-molecule RecB protein quantification, we followed the Halo-labelling protocol described previously [24]. *E. coli* strains with RecB-HaloTag fusion, grown as described above, were labelled with Janelia Fluor 549 dye (purchased from Promega) for one hour, washed with aspiration pump (4-5 times), fixed in 2.5% formaldehyde and mounted onto agar pads before imaging. In each experiment, a parental strain which does not have the HaloTag fusion ('no HaloTag') was subjected to the protocol in parallel

with the primary samples as a control.

Microscopy set-up and conditions

Image acquisition was performed using an inverted fluorescence microscope (Nikon Ti-E) equipped with an EMCCD Camera (iXion Ultra 897, Andor), a Spectra X Line engine (Lumencor), dichronic mirror T590LPXR, 100X oil-immersion Plan Apo objective (NA 1.45, Nikon) and 1.5X magnification lens (Nikon). A TRITC (ET545/30nm, Nikon) filter was used for imaging Halo-labelling experiments while smFISH data were acquired with an mCherry (ET572/35nm, Nikon) filter.

Once a protocol (smFISH or Halo-labelling) had been carried out, fixed cells were resuspended in 1X PBS and mounted onto a 2% agarose pad. The snapshots were acquired in bright-field and fluorescence channels. In all experiments, an electron-multiplying (EM) gain of 4 and an exposure time of 30 ms were used for bright-field imaging. In the fluorescence channel, smFISH data were acquired with a 2 s exposure time and a gain of 4, while RecB proteins were visualized with the same exposure time but EM gain 200. For each XY position on an agarose pad, a Z-stack of 6 images centred around the focal plane (total range of 1 μm with a step of 0.2 μm) was obtained in both channels. A set of multiple XY positions were acquired for each slide to visualize $\sim$1000 cells per sample.

Cell segmentation

Microscopy bright-field images were used to identify positions of bacterial cells with an automated MATLAB-based pipeline [117]. Briefly, the segmentation algorithm is based on detection of cell edges by passing an image through a low-pass filter. In comparison to the original version, we applied this analysis to defocused bright-field images (instead of fluorescence signal from a constitutive reporter [117]) and tuned segmentation parameters for our conditions. The segmentation outputs were manually corrected by discarding misidentified cells in the accompanied graphical interface. The corrected segmentation results were saved as MATLAB matrices (cell masks) containing mapping information between pixels and cell ID numbers. The cell masks were used as an input for the spot-finding software described below.

Spot detection

The Spätzcell package was utilized to detect foci in fluorescent smFISH images [50]. In principle, the analysis consists of the following steps: (i) identification of local maxima above a chosen intensity threshold, (ii) matching the maxima across frames in a Z-stack, (iii) performing 2D-Gaussian fitting of the detected maxima. Based on the peak height and spot intensity, computed from the fitting output, the specific signal was separated from false positive spots (Fig. S1a). To identify the number of co-localized mRNAs, the integrated spot intensity profile was analyzed as previously described [50]. Assuming that (i) probe hybridization is a probabilistic process, (ii) binding each RNA FISH probe happens independently, and (iii) in the majority of cases, due to low-abundance, there is one mRNA per spot, it is expected that the integrated intensities of FISH probes bound to one mRNA are Gaussian distributed. In the case of two co-localized mRNAs, there are two independent binding processes and, therefore, a wider Gaussian distribution with twice higher mean and twice larger variance is expected. In fact, the integrated spot intensity profile had a main mode corresponding to a single mRNA per focus, and a second one representing a population of spots with two co-localized mRNAs (Fig. S1b). Based on this model, the integrated spot intensity histograms were fitted to the sum of two Gaussian distributions (see equation Eq. (2), where a, b, c, and d are the fitting parameters), corresponding to one and two mRNA molecules per focus. An intensity equivalent corresponding to the integrated intensity of FISH probes in average bound to one mRNA was computed as a result of multiple-Gaussian fitting procedure (Fig. S1b), and all identified spots were normalized by the one-mRNA equivalent.

$$f(x) = ae^{-\frac{(x-b)^2}{c^2}} + de^{-\frac{(x-2b)^2}{2c^2}} \tag{2}$$

To detect fluorescent single-molecule foci in the Halo-labelling experiments, the first step of Spätzcell analysis was modified (similar to the modification for fluorescent protein detection implemented here [53]). For the Halo-labelling single-molecule data, we tested two modifications: (i) removing a Gaussian filter (that was used to smooth raw signal in the original version) or (ii) calculating the Laplacian of a Gaussian-filtered image. Both versions gave similar and consistent quantification results. Peak height intensity profiles allowed the separation of specific signal from false positive spots (Fig. S4). As a result of low-abundance of the protein of interest and single-molecule labelling, the analysis of integrated intensity was skipped for Halo-labelling images. All other steps of the Spätzcell software remained

unchanged.

Total (mRNA or protein) molecule numbers per cell were obtained by matching spot positions to cell segmentation masks and quantifying the number of (mRNA or protein) spots within each cell. Molecule concentration was calculated for each cell as total number of (mRNA or protein) molecules (per cell) divided by the area of the cell ($1/\mu m^2$).

Bacterial RNA extraction and RT-qPCR

Bacterial cells were grown as described earlier, harvested in the equivalent of $volume \times OD_{600}$ = 2.5 ml and flash-frozen in liquid nitrogen. In the Halo-labelling experiments, samples for RNA extraction were grown at 37°C and collected before the fixation step. Total RNA extraction was performed with the guanidium thiocyanate phenol protocol [118]. Primers for real-time qPCR experiments are listed in Table S5. RT-qPCR quantification was carried out on 20 ng of RNA with the Luna One-Step RT-qPCR Kit (NEB). All qPCRs were performed in technical triplicates. The qPCR reactions were carried out using LightCycler 96 (Roche). The *rrfD* gene (5S rRNA) was used as a reference gene to compute a fold-change relative to a control sample with the $2^{-\Delta\Delta Ct}$ method. Outliers with the standard deviation larger than $std(Ct) > 0.3$ across technical replicates were removed from the analysis.

Population growth and viability assays

Bacterial cells, seeded by inoculating from fresh colonies, were grown in an appropriate medium at 37°C overnight. The cultures were diluted (1:1000) and grown in a shaking incubator at 37°C. Optical density measurements were taken overday at OD_{600} with the interval of 15 min. The exponential phase of growth, OD_{600} = [0.08, 0.4], was used for the fitting procedure. The fitting was performed with a linear regression model in MATLAB.

In the viability assays, cells were grown overnight in Luria Broth at 37°C in a shaking incubator. The optical density of the overnight cultures was normalized to OD_{600} = 1.0. 10-fold serial dilutions were then performed in 96-well plates for the range of OD_{600} from 1.0 to 10^{-7}. 100 μl of cells of a specific dilution were plated onto LB agar plates containing ciprofloxacin and ampicillin at a concentration of 0–16 ng/ml and 100 $\mu g/ml$, respectively. For each condition, two dilutions were optimally chosen to have 30-300 colonies per plate. After 24 hours of incubation at 37°C, the colonies on each plate were counted and colony forming units (CFU) per ml were calculated using the following formula:

$$\frac{\text{CFU}}{ml} = \frac{\text{Number of colonies per plate x dilution}}{100\ \mu l} \tag{3}$$

The survival factor (SF) was calculated by normalizing against the CFU/ml in the absence of ciprofloxacin.

Data analysis of CRAC data

The CLASH data (NCBI Gene Expression Omnibus (GEO), accession number GSE123050) were analysed in the study [83] with the entire pipeline available here [119, 120]. The SGR file, generated with `CRAC_pipeline_PE.py`, was used to plot the genome browser track in Fig. 4A.

Fitting and parameter estimation

The smFISH data from Fig. 1C was conditioned on cell size (cell area < 3.0 μm^2, *newborns*) and the updated distribution was fitted by a negative binomial distribution. In mathematical terms, a negative binomial $NB(r, p)$ is defined by two parameters: r, the number of successful outcomes, and p, the probability of success. Mean and variance of $X \sim NB(r, p)$ are given as:

$$\langle X \rangle = \frac{pr}{(1-p)} \qquad \sigma_X^2 = \frac{pr}{(1-p)^2} \tag{4}$$

The probability density function of the fitted distribution was found with the maximum likelihood estimation method in MATLAB. The fitted parameters, r and p, are given as $r = 0.335$ and $p = 0.513$.

The average mRNA burst size b and transcription rate k_m were calculated using the fitted parameters, r and p, and the *recB* mRNA degradation rate ($\gamma_m = 0.615\ min^{-1}$, measured in an independent time-course experiment: Fig. 1D) according to the following analytical expressions (Eq.(A4), Eq.(A5) & Eq.(3)):

$$b = \frac{1-p}{p} = 0.95\, molec \tag{5}$$

$$k_m = \gamma_m r = 0.21\, min^{-1} \tag{6}$$

The RecB protein distribution from Fig. 2C was conditioned on cell size (cell area < 3.0 μm^2, *newborns*). Then, the average protein count number in *newborns* (∼3.2 molecules per cell) was matched to the theoretical expression for the protein mean Eq.(A4). The rate of translation k_p was calculated as follows (using protein removal rate, $\gamma_p = 0.015\, min^{-1}$, and mean mRNA number in *newborns*, $\langle m \rangle = 0.323$):

$$k_p = \frac{\gamma_p \langle p \rangle}{\langle m \rangle} = 0.15\, min^{-1} \tag{7}$$

All parameters of the model are listed in Table S7.

Statistical analysis

Unless otherwise stated, histograms and RT-qPCR results represent the average across at least three replicated experiments and error bars reflect the maximum difference between the repeats. The Kullback–Leibler divergence (D_{KL}) between simulated and experimental distributions was calculated with the MATLAB function [121]. Comparison between the average protein or mRNA concentrations among different conditions were performed with a two-sample *t*-test and *P* values were calculated in the MATLAB built-in function `ttest2`. Average translational efficiency was calculated as the ratio between the mean protein concentration c_p and the mean mRNA concentration c_m (Fig. 3F & Fig. 4F). The error bars are standard deviations across replicated experiments.

Stochastic simulations

Single trajectories of mRNA and protein abundance over time were generated for the reaction scheme Eq.(A1) with the Gillespie's algorithm, executed in MATLAB environment [122]. Parameters, used in simulations, are listed in Table S7. 10,000 simulations were performed for a given condition and probabilistic steady-state distributions were recovered for population snapshot data.

Data and code availability

All data that support this study including microscopy data, RT-qPCRs, growth rates, viability assays, and source codes for image analysis are available on Zenodo 10.5281/zenodo.10209982.The modified versions of the image analysis used for spot detection in Halo-labelling experiments and bright-field segmentation pipeline are also available on GitLab https://gitlab.com/ikalita/spotdetection_spatzcells and https://gitlab.com/ikalita/cellsegmentation_mek, respectively. The CRAC/CLASH dataset (NCBI Gene Expression Omnibus ID GSE123050) [83], used in this study, is available from GSE123050. The pipeline for the analysis of the CRAC/CLASH data is available from https://git.ecdf.ed.ac.uk/sgrannem and https://pypi.org/user/g_ronimo/.

Article and author information

Author contributions

Irina Kalita: Conceptualization, Methodology, Software, Investigation (experiments), Formal analysis (computational analysis & modelling), Writing – Original Draft Preparation, Writing – Review & Editing, Visualization;

Ira Alexandra Iosub: Conceptualization, Methodology, Software, Investigation (experiments), Formal analysis (computational analysis), Writing – Review & Editing, Visualization;

Lorna McLaren: Investigation (experiments), Writing – Review & Editing;

Louise Goossens: Investigation (experiments), Writing – Review & Editing;

Sander Granneman: Conceptualization, Methodology, Software, Formal analysis (computational analysis), Writing – Review & Editing, Supervision, Funding Acquisition;

Meriem El Karoui: Conceptualization, Methodology, Formal analysis (modelling), Writing – Original Draft Preparation, Writing – Review & Editing, Supervision, Funding Acquisition.

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Competing interests

The authors have no competing interests.

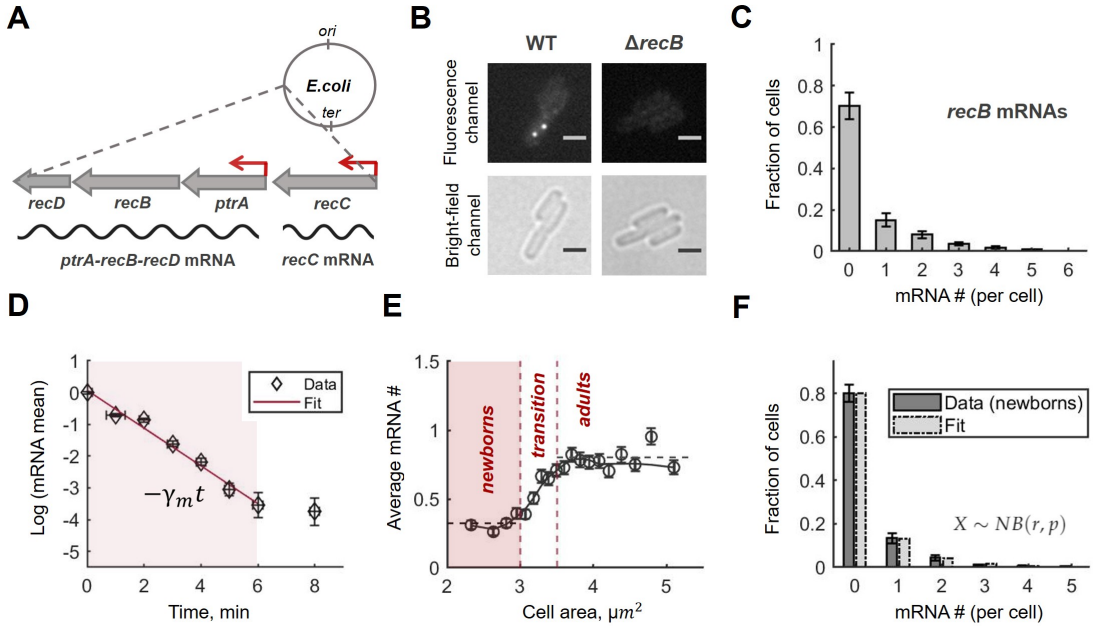

Figure 1: *recB* mRNAs are low abundant, short-lived and constitutively expressed.
A: Schematic description of the *recBCD* locus, its location on the *E. coli* chromosome and the corresponding mRNAs. **B:** Examples of fluorescence and bright-field images of *recB* mRNA FISH experiments in wild-type (WT) and Δ*recB* strains. Scale bars represent 2 μm. **C:** Total *recB* mRNA distribution quantified with smFISH and presented in molecule numbers per cell. The histogram represents the average across three replicated experiments; error bars reflect the maximum difference between the repeats. Total number of cells, included in the distribution, is 15,638. **D:** *recB* mRNA degradation rate measured in a time-course experiment where transcription initiation was inhibited with rifampicin. Mean mRNA counts, calculated from the total mRNA distributions for each time point, were normalized to the mean mRNA number at time $t = 0$ and represented in the natural logarithmic scale. Vertical error bars represent the standard error of the mean (s.e.m.); horizontal errors are given by experimental uncertainty on time measurements. Shaded area shows the time interval used for fitting. The red line is the fitted linear function, $-\gamma_m t$, where γ_m is the *recB* mRNA degradation rate. The final degradation rate was calculated as the average between two replicated time-course experiments (Table S7). **E:** *recB* mRNA molecule numbers per cell from the experiments in Fig. 1C shown as a function of cell area. The black circles represent the data binned by cell size and averaged in each group (mean +/- s.e.m.). The solid line connects the averages across three neighbouring groups. Based on the mean mRNA numbers, all cells were separated into three sub-populations: *newborns*, *cells in transition* and *adults*. **F:** Experimental data from Fig. 1C conditioned on cell size (<3.0 μm^2, *newborns*; total cell number is 2,180) fitted by a negative binomial distribution, $NB(r,p)$. The Kullback–Leibler divergence between experimental and fitted distributions is $D_{KL} = 0.002$.

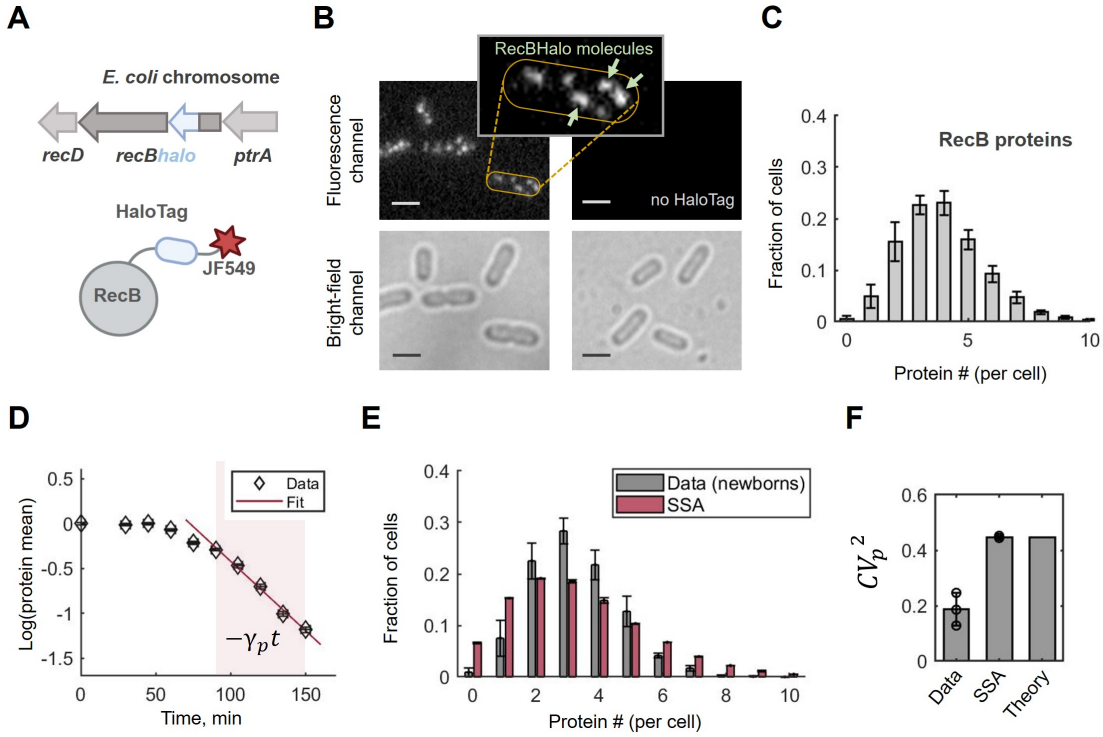

Figure 2: RecB proteins are low abundant, long-lived and show an evidence of noise reduction.
A: Schematic of RecB-HaloTag fusion at the endogenous *E. coli* chromosomal locus of the *recB* gene. RecB-HaloTag is conjugated to the Janelia Fluor 549 dye (JF549). **B:** Examples of fluorescence and bright-field Halo-labelling images for the strain with the RecB-Halo fusion and its parental (no HaloTag) strain. Both samples were labelled with JF549 dye and the images are shown in the same intensity range. Scale bars represent 2 μm. **Zoom-in:** An example of a cell with five RecBHalo molecules (several single RecBHalo molecules are shown with light-green arrows). **C:** Total RecB protein distribution quantified with Halo-labelling and presented in molecules per cell. The histogram represents the average of three replicated experiments; error bars reflect the maximum difference between the repeats. Total number of analysed cells is 10,964. Estimation of false positives in no HaloTag control resulted in ∼0.3 molecules/cell. **D:** RecB removal rate measured in a pulse-chase Halo-labelling experiment where the dye was removed at time $t = 0$. Mean protein counts, calculated from the total protein distributions for each time point, were normalized to the mean protein number at time $t = 0$ and represented in the natural logarithmic scale. Shaded area shows the time interval used for fitting. The red line is the fitted linear function, $-\gamma_p t$, where γ_p is the RecB removal rate. The final removal rate was calculated as the average between two replicated pulse-chase experiments (Table S7). **E:** Comparison between the experimental RecB molecule distribution from Fig. 2C conditioned on cell size (in grey) and the results of Gillespie's simulations (SSA) for a two-stage model of RecB expression (in red). Parameters, used in simulations, are listed in Table S7. The Kullback–Leibler divergence between the distributions is $D_{KL} = 0.15$. **F:** Comparison of the coefficients of variation, $CV_p^2 = \sigma_p^2/\langle p \rangle^2$, for experimental (Data), simulated (SSA) data and analytical prediction (Theory). The experimental and simulated data from Fig. 2E were used; error bars represent standard deviations across three replicates; the black circles show the mean CV_p^2 in each experiment. Theoretical prediction was calculated using Eq. (1).

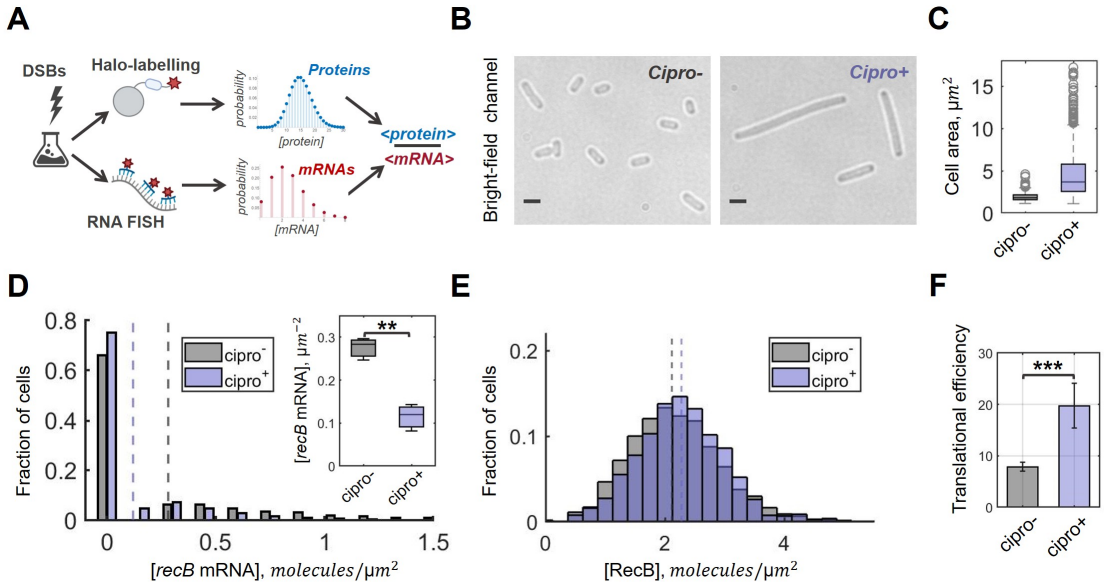

Figure 3: Translational efficiency of RecB is increased under DNA damage conditions.
A: Schematic of the experimental workflow. DSBs were induced with 4 ng/ml of ciprofloxacin for two hours, and protein (mRNA) quantification was performed with Halo-labelling (smFISH). Mean protein and mRNA concentrations were calculated from the distributions, and the average protein-to-mRNA ratio (translational efficiency) was estimated. **B:** Examples of bright-field images for unperturbed ($Cipro^-$) and perturbed ($Cipro^+$) conditions. Scale bars represent 2 μm. **C:** Box plot with cell area distributions for perturbed (blue) and unperturbed (grey) samples. The medians of cell area in each sample are 1.9 μm^2 ($Cipro^-$) and 3.6 μm^2 ($Cipro^+$). **D:** *recB* mRNA concentration distributions quantified with smFISH in intact (grey) and damaged (blue) samples. The histograms represent the average of three replicated experiments. The medians of the *recB* mRNA concentrations are shown by dashed lines: 0.28 $mol/\mu m^2$ ($Cipro^-$) and 0.12 $mol/\mu m^2$ ($Cipro^+$). Total numbers of analysed cells are 11,700 ($Cipro^-$) and 6,510 ($Cipro^+$). **Insert:** Box plot shows significant difference between the average *recB* mRNA concentrations (P value = 0.0023, two-sample t-test). **E:** RecB concentration distributions quantified with Halo-labelling in unperturbed and perturbed conditions. The histograms represent the average of three replicated experiments. The medians are shown by dashed lines: 2.12 $mol/\mu m^2$ ($Cipro^-$) and 2.28 $mol/\mu m^2$ ($Cipro^+$). Total number of analysed cells are 1,534 ($Cipro^-$) and 683 ($Cipro^+$). The difference between the average RecB concentrations was insignificant (P value = 0.36, two-sample t-test). **F:** The average number of proteins produced per one mRNA in intact and damaged conditions. Average translational efficiency for each condition was calculated as the ratio between the mean protein concentration c_p and the mean mRNA concentration c_m. The error bars indicate the standard deviation of the data; statistical significance between the conditions was calculated with two-sample t-test (P value = 0.0001).

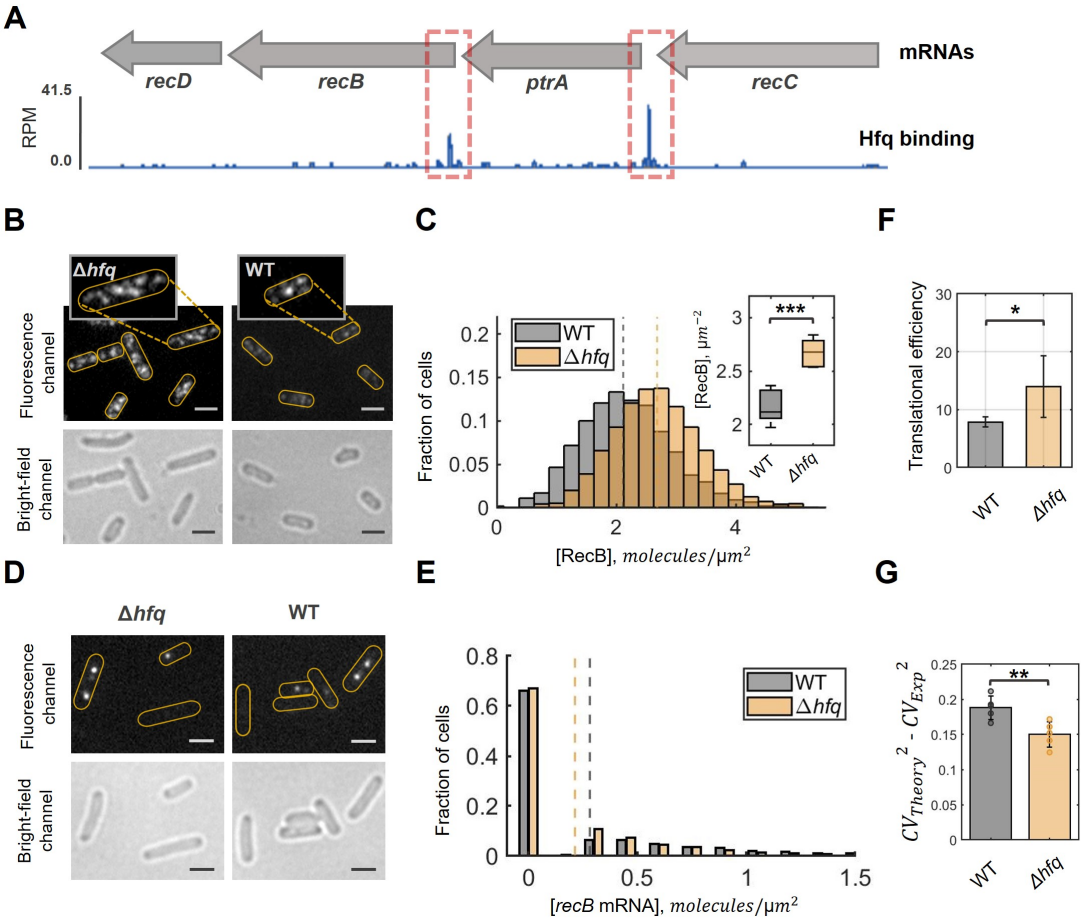

Figure 4: RecB expression is regulated by Hfq protein *in vivo*.
A: Genome browser track showing Hfq binding to *recC-ptrA-recB-recD* mRNAs. The coverage is normalized to reads per million (RPM). The major peaks of interest are highlighted by red dashed boxes. **B:** Examples of fluorescence and bright-field images of RecB quantification experiments in Δ*hfq* and wild-type strains. Yellow outlines indicate rough positions of bacterial cells in the fluorescence channel. Scale bars represent $2\mu m$. Both fluorescence images are shown in the same intensity range while different background modulation was applied in the zoom-in figures (for better spots visualization). **C:** RecB concentration distributions quantified with Halo-labelling in wild-type cells and the Δ*hfq* mutant. The histograms represent the average of five replicated experiments. The medians are shown by dashed lines: 2.12 $mol/\mu m^2$ for WT and 2.68 $mol/\mu m^2$ for Δ*hfq*. Total number of analysed cells are 3,324 (WT) and 2,185 (Δ*hfq*). **Insert:** Box plot shows significant difference (P value = 0.0007) between the average RecB concentrations in wild-type and Δ*hfq* cells verified by two-sample *t*-test. **D:** Examples of fluorescence and bright-field images of *recB* mRNA FISH experiments in Δ*hfq* and wild-type cells. Yellow outlines indicate rough positions of bacterial cells in the fluorescence channel. Scale bars represent 2 μm. **E:** *recB* mRNA concentration distributions quantified with smFISH in the Δ*hfq* mutant and wild-type cells. The histograms represent the average of three replicated experiments. The medians are shown by dashed lines: 0.28 $mol/\mu m^2$ for WT and 0.21 $mol/\mu m^2$ for Δ*hfq*. Total number of analysed cells are 7,270 (WT) and 5,486 (Δ*hfq*). The insignificant difference between the average *recB* mRNA concentrations in both strains was verified by two-sample *t*-test (P value = 0.11). **F:** RecB translational efficiency in wild-type and Δ*hfq* cells. Average translational efficiency (for each strain) was calculated as the ratio between the mean protein concentration c_p and the mean mRNA concentration c_m across replicated experiments. The error bars indicate the standard deviation of the data; statistical significance between the strains was calculated with two-sample t-test (P value = 0.014). **G:** The difference between theoretically predicted (CV^2_{Theory}) and experimentally measured (CV^2_{Exp}) coefficient of variation (squared). The theoretical values were computed according to Eq. (1). Error bars represent s.e.m. calculated from the replicated experiments. Statistical significance between the samples was calculated with two-sample t-test (P value = 0.0088).

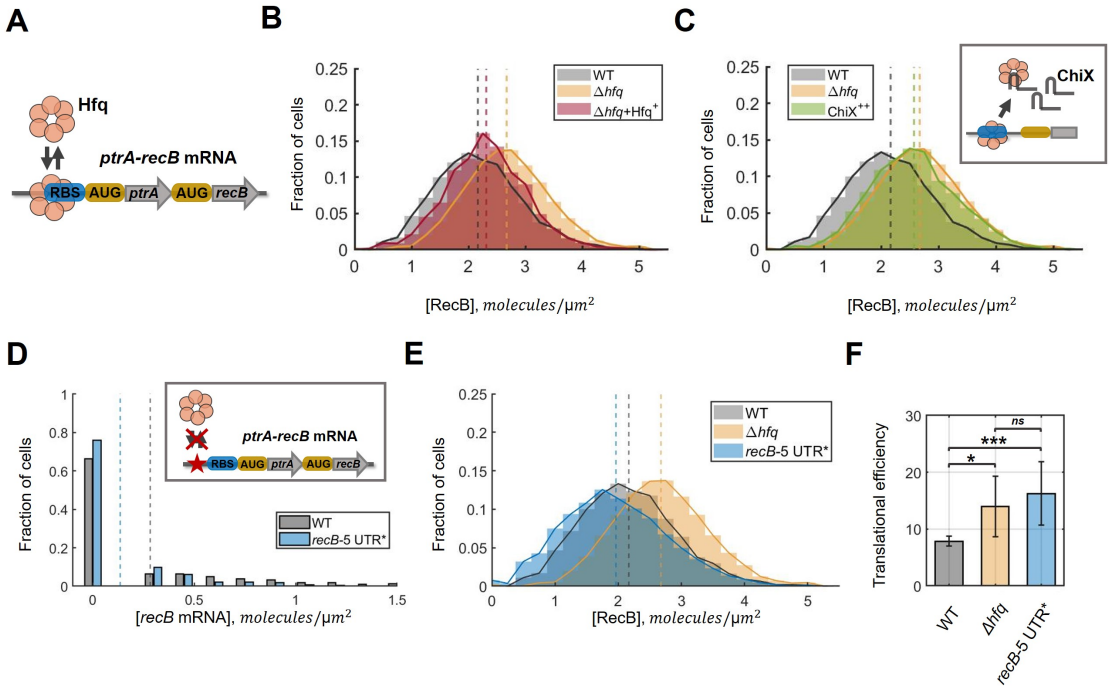

Figure 5: Specific alteration of RecB translation by Hfq *in vivo*.
A: A model of Hfq downregulating RecB translation by blocking the ribosome binding site of the *ptrA-recB* mRNA. **B:** Partial complementation of RecB expression to the wild-type level demonstrated by expression of a functional Hfq protein from a multicopy plasmid (pQE-Hfq) in Δ*hfq* (shown in red). The histogram represents the average of two replicated experiments. RecB concentration histograms in wild type and Δ*hfq* mutant from Fig. 4C are shown for relative comparison. Dashed lines represent the average RecB concentration in each condition. **C:** An increase in RecB protein production caused by sequestering of Hfq proteins with highly abundant small RNA ChiX (shown in green). The histogram represents the average of two replicated experiments. RecB concentration histograms in wild type and Δ*hfq* mutant from Fig. 4C are shown for relative comparison. Dashed lines represent the average RecB concentration in each condition. **D:** *recB* mRNA concentration distribution quantified with smFISH in the strain with the deletion of the main Hfq binding site *recB*-5′UTR* (shown in blue). The histogram represents the average of three replicated experiments. The *recB* mRNA distribution in the wild-type cells is shown for relative comparison. The medians are indicated by dashed lines. An approximate location of the removed sequence is schematically shown in the insert (red star). **E:** RecB protein concentration distribution quantified with RecBHalo-labelling in *recB*-5′UTR* strain (shown in blue). The histogram represents the average of three replicated experiments. RecB concentration histograms in wild type and Δ*hfq* mutant from Fig. 4C are shown for relative comparison. Dashed lines represent the average RecB concentration in each condition. **F:** Translational efficiency of RecB in wild type, Δ*hfq* mutant and the strain with the deletion of the main Hfq binding site, *recB*-5′UTR*. Average translational efficiency was calculated as the ratio between the mean protein concentration c_p and the mean mRNA concentration c_m across replicated experiments. The error bars indicate the standard deviation of the data; statistical significance between the samples was calculated with two-sample t-test. The P value for WT and *recB*-5′UTR* is 0.0007; while the difference between Δ*hfq* and *recB*-5′UTR* is non-significant (P value > 0.05).

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