Heat Shock Transcription Factor $\sigma^{32}$ Co-opts the Signal Recognition Particle to Regulate Protein Homeostasis in E. coli

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Abstract

All cells must adapt to rapidly changing conditions. The heat shock response (HSR) is an intracellular signaling pathway that maintains proteostasis (protein folding homeostasis), a process critical for survival in all organisms exposed to heat stress or other conditions that alter the folding of the proteome. Yet despite decades of study, the circuitry described for responding to altered protein status in the best-studied bacterium, E. coli, does not faithfully recapitulate the range of cellular responses in response to this stress. Here, we report the discovery of the missing link. Surprisingly, we found that $\sigma^{32}$, the central transcription factor driving the HSR, must be localized to the membrane rather than dispersed in the cytoplasm as previously assumed. Genetic analyses indicate that $\sigma^{32}$ localization results from a protein targeting reaction facilitated by the signal recognition particle (SRP) and its receptor (SR), which together comprise a conserved protein targeting machinery that mediates the cotranslational targeting of inner membrane proteins to the membrane. SRP interacts with $\sigma^{32}$ directly and transports it to the inner membrane. Our results show that $\sigma^{32}$ must be membrane-associated to be properly regulated in response to the protein folding status in the cell, explaining how the HSR integrates information from both the cytoplasm and bacterial cell membrane.

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Abbreviations: HSPs, heat shock proteins; HSR, heat shock response; IM, inner membrane; pBPA, p-benzoylphenylalanine; PhoA, alkaline phosphatase; S.A., specific activity; SR, signal receptor; SRP, signal recognition particle.

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Introduction

The heat shock response (HSR) maintains protein homeostasis (proteostasis) in all organisms. The HSR responds to protein unfolding, aggregation, and damage by the rapid and transient production of heat shock proteins (HSPs) and by triggering other cellular protective pathways that help mitigate the stress. Although the specific HSR is tailored to each organism, chaperones that mediate protein folding and proteases that degrade misfolded proteins are almost always included in the core repertoire of major chaperone systems, DnaK/J/GrpE and GroEL/S. There is extensive support for the model that free chaperones directly inactivate $\sigma^{32}$ and that these chaperones are titrated by unfolded proteins that accumulate and bind chaperones during a HSR. Depletion of either chaperone system or overexpression of
Author Summary

All cells have to adjust to frequent changes in their environmental conditions. The heat shock response is a signaling pathway critical for survival of all organisms exposed to elevated temperatures. Under such conditions, the heat shock response maintains enzymes and other proteins in a properly folded state. The mechanisms for sensing temperature and the subsequent induction of the appropriate transcriptional response have been extensively studied. Prior to this work, however, the circuitry described in the best studied bacterium E. coli could not fully explain the range of cellular responses that are observed following heat shock. We report the discovery of this missing link. Surprisingly, we find that σ^{32}, a transcription factor that induces gene expression during heat shock, needs to be localized to the membrane, rather than being active as a soluble cytoplasmic protein as previously thought. We show that, equally surprisingly, σ^{32} is targeted to the membrane by the signal recognition particle (SRP) and its receptor (SR). SRP and SR constitute a conserved protein targeting machine that normally only operates on membrane and periplasmic proteins that contain identifiable signal sequences. Intriguingly, σ^{32} does not have any canonical signal sequence for export or membrane-integration. Our results indicate that membrane-associated σ^{32}, not soluble cytoplasmic σ^{32}, is the preferred target of regulatory control in response to heat shock. Our new model thus explains how protein folding status from both the cytoplasm and bacterial cell membrane can be integrated to control the heat shock response.

chaperone substrates leads to an increase in σ^{32} activity, and conversely, overexpression of either chaperone system decreases σ^{32} activity [13,14]. Inhibition is likely direct, as DnaK/J and GroEL/S bind σ^{32} in vitro and inhibit its activity in a purified in vitro transcription system [13,15–17]. σ^{32} stability is controlled by the inner membrane (IM) protease FtsH: deletion of the protease stabilizes σ^{32} [18–20], and FtsH degrades σ^{32} in vitro, albeit slowly [18,20]. DnaK/J and GroEL/S also regulate stability, as their depletion leads to σ^{32} stabilization in vivo [13,14,21], although this finding has not yet been recapitulated in vitro [22].

Despite the regulatory complexity of the current model, it inadequately addresses two issues that are central to our understanding of the circuitry controlling the HSR, motivating us to search for additional players in the response: (1) Exhaustive genetic screens for mutations in σ^{32} that result in misregulation have identified a small cluster of four closely spaced amino acid residues (Leu47, Ala50, Lys51, and Ile54), of which three are surface exposed, as well as a somewhat distant fifth residue that abuts this patch in the folded σ^{32} structure. When these residues are mutated, cells have both increased level and activity of σ^{32}, indicating that this region is involved in a central process required for operation of the negative feedback loops that control both the activity and degradation of σ^{32} (Figure 1A) [23–25]. However, the phenotypes of these mutants are not recapitulated in vitro, where both FtsH degradation and chaperone-mediated inactivation of mutant and WT σ^{32} are experimentally indistinguishable [25,26]. Thus, we do not understand how this “homeostatic control region” of σ^{32} functions. (2) σ^{32} is thought to monitor the folding status of IM proteins as well as cytoplasmic proteins, but the mechanism for this additional surveillance is unknown. Their close connection is indicated because (1) the IM protease, FtsH, not only degrades σ^{32}, but also maintains quality control in the IM by degrading unassembled IM proteins; (2) induction of the HSR is a very early response to perturbations in the co-translational membrane-trafficking system that brings ribosomes translating IM proteins to the membrane [27–29]; and (3) IM proteins are significantly overrepresented both in the σ^{32} regulon [30] and in an unbiased overexpression screen for HSR inducers [30].

In this report, we identify the co-translational protein targeting machinery, comprised of the Signal Recognition Particle (SRP; Ffh protein in complex with 4.5S RNA; Figure 2A) and the SRP Receptor (SR; FtsY), as a regulator of σ^{32}. We show that SRP preferentially binds to WT σ^{32} compared to a mutant σ^{32} with a defective homeostatic control region. We further show that a fraction of σ^{32} is associated with the cell membrane and that both the SRP-dependent machinery and the homeostatic control region of σ^{32} are important for this localization. Lastly, the regulatory defects in HSR circuitry caused by mutation of either the σ^{32} homeostatic control region or the co-translational targeting machinery are circumvented by artificially tethering σ^{32} to the IM. We propose that SRP-dependent membrane localization is a critical step in the control circuitry that governs the activity and stability of σ^{32}. Membrane localization is widely used to control σ factors, but this is the first case where the IM-localized state is used for dynamic regulation rather than as a repository for an inactive protein.

Results

A Transposon Insertion Mutant at the ftsY Promoter Region Is Defective in Feedback Control

To identify additional players involved in activity control of σ^{32}, we carried out a genetic screen for transposon mutants with increased σ^{32} activity under conditions that inactivate σ^{32} in wild-type cells (see Methods). To impose a condition that mimics the negative feedback control of σ^{32}, the DnaK/J chaperones were overexpressed from an inducible promoter at their chromosomal locus. Under these conditions, a σ^{32}-regulated lacZ reporter (P_{sgrc-lacZ}) is expressed so poorly that cells do not make sufficient β-galactosidase to turn colonies blue on X-gal indicator plates. We screened for blue colonies, indicative of a defect in σ^{32} inactivation. A conceptually similar screen previously identified mutations in the DnaK/J chaperones—key negative regulators of the σ^{32} response [31]. In addition to re-identifying these components, we found an insertion in the promoter region of ftsY (pftsY::Tn5), located 39 bp upstream of the ftsY open reading frame. The pftsY::Tn5 strain had a 3- to 4-fold reduction in the level of FtsY, the SR, and a ~7-fold increase in the activity and amount of σ^{32} relative to WT (Table 1). Defects were complemented by a plasmid carrying ftsY. Unlike WT, in the pftsY::Tn5 strain σ^{32} activity did not respond to increased chaperone expression. Upon chaperone overexpression in WT cells, the specific activity (S.A.) of σ^{32} fell to 0.3, relative to that in cells growing without chaperone overexpression. In contrast, upon chaperone overexpression in pftsY::Tn5 cells, the S.A. of σ^{32} did not change, suggesting a defect in chaperone-mediated activity control in that strain (Table 1). This finding raised the possibility that the high activity of σ^{32} in pftsY::Tn5 resulted from disruption of activity control of σ^{32}, rather than reflecting a cellular response to accumulation of unassembled membrane proteins.

σ^{32} Directly Interacts with SRP

We tested whether σ^{32} binds to either FtsY (SR) or to Ffh, the protein component of SRP. Ffh is a two-domain protein, comprised of an M-domain that binds the signal sequence and 4.5S RNA, and an NG-domain that binds to SR, the ribosome,
and GTP (Figure 2A). We first used co-immunoprecipitation analysis. Interacting proteins were immunoprecipitated with antibodies against either FtsY or Ffh and, following resolution on SDS-PAGE, antibodies against s32 or s70 were used to probe for the presence of these proteins. s32 was detected in the immunoprecipitations (Figure 2B, lanes 7 and 8), and this signal was dependent on the presence of s32 in the strain (Figure 2B, lanes 1–4). By contrast, s70, although much more abundant than s32 in the cell, did not interact with either SRP or SR (Figure 2B, Lanes 3, 4 and 7, 8), indicating that interaction with SRP is not a general property of s70. It was not surprising that s32 was co-immunoprecipitated with both SRP and SR, as the latter two components interact in vivo. To determine the direct binding partner of s32, purified Ffh and FtsY were resolved on SDS-PAGE, transferred to nitrocellulose, and incubated with purified s32. Antibodies against s32 detected s32 present at the molecular weight corresponding to Ffh but not SR (Figure 2C). In a reciprocal experiment, purified s32 was resolved on SDS-PAGE, transferred to nitrocellulose, and incubated with purified Ffh or SR. Ffh, but not SR, bound s32 (unpublished data). Similar studies did not reveal an interaction between s70 and either Ffh or SR (unpublished data). We determined which Ffh domain binds s32 by partially-proteolyzing Ffh to produce an 18 kDa M-domain and a 38 kDa NG-domain, resolving the mixture by SDS-PAGE, transferring to nitrocellulose, and probing with s32. s32 was detected at the position of full-length Ffh and the M-domain, but not at the position of the NG-domain (Figure 2D), indicating that the M-domain contains the determinants mediating the s32-interaction.

We used in vivo crosslinking to validate the direct interaction of SRP (Ffh+4.5S RNA) and s32. We created a s32 derivative with an N-terminal 6×HIS-tag and a photoreactive amino acid analog (pBPA) at amino acid position 52 (6×HIS-s32T52pBPA; see Methods), which is active as WT s32 in expression of the s32 reporter PhtpG-lacZ (activity is 150% that of WT; within the range of the variability of the assay; unpublished data). Following UV irradiation of whole cells, anti-Ffh immunoblotting of the whole cell lysate detected one predominant crosslinked product, which was dependent on UV-irradiation (Figure 3A, lanes 1 and 2) and pBPA at position 52 (Figure 3A, lanes 2 and 4). This UV- and pBPA-dependent product was also detected with anti-s32 immunoblotting (Figure 3A, lane 6). To determine whether the crosslinked product represented 6×HIS-s32T52pBPA-Ffh, we determined whether this product was identified both by co-immunoprecipitation with anti-Ffh antisera (Figure 3B) and by affinity purification of 6×HIS-s32T52pBPA on a TALON resin (Figure 3C). Upon immunoprecipitation with anti-Ffh antiserum, we detected a single higher molecular mass band, which reacted with both anti-Ffh (Figure 3B, lane 2) and -s32 (Figure 3B, lane 6).

**Figure 1. Homeostatic control circuits of s32.** (A) Current and (B) revised model for activity and degradation control of s32. The revised model incorporates SRP-mediated trafficking of s32 to the membrane. Interactions validated in vitro are shown as solid lines; those inferred from in vivo data are shown as dashed lines. Newly identified interactions are shown in red.

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Figure 2. σ^{32} binds to Ffh. (A) Schematic representation of E. coli SRP (Ffh+4.5S RNA), indicating experimentally confirmed functions associated with each domain. (B) σ^{32} co-immunoprecipitates with Ffh and FtsY in vivo, but σ^{70} does not. Immunoprecipitations of Ffh or FtsY were carried out on lysates of Δσ^{32} and ΔftsH cells grown to exponential phase. Immunocomplexes were isolated, analyzed by SDS-PAGE, and immunoblotted with anti-σ^{32} and anti-σ^{70} antibodies. Proteins from approximately 15-fold more cells were loaded onto the gel for the immunoprecipitated samples as compared with the lysate samples. (C) Protein–protein interaction analysis indicates that σ^{32} binds to Ffh, but not FtsY. Purified FtsY and Ffh were run on a 10% SDS-PAGE gel, transferred to nitrocellulose, re-natured, and incubated with purified WTσ^{32}. The Coomassie-stained gel (left) and the nitrocellulose blot probed with polyclonal anti-σ^{32} antibodies (right) are shown. (D) σ^{32} binds to the M-domain of Ffh. Ffh,
partially digested by endoproteinase V8, was resolved on a 10% SDS-PAGE gel, transferred to nitrocellulose, and incubated with σ\(^{32}\). The Coomassie-stained gel (left) and the nitrocellulose membrane, containing transferred Ffh fragments, probed against σ\(^{32}\) (right) are shown.
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Upon affinity purification on a TALON resin, anti-Ffh identified the same predominant UV- and βPA-dependent Ffh-containing crosslinked product (compare Figure 3B and 3C, lane 2). Importantly, no free Ffh was recovered following TALON purification, indicating that the recovery of the Ffh conjugate was mediated by the covalently linked 6× His-σ\(^{32}\), rather than interaction with either the TALON resin or another protein. These results strongly suggest that σ\(^{32}\) directly interacts with Ffh in vivo. Although only a faint band was seen at the same position using anti-σ\(^{32}\) immunoblotting, this was likely a result of high background in this area of the gel, possibly because of extensive interaction between chaperones and σ\(^{32}\) (Figure 3C, lanes 5–6).

I54Nσ\(^{32}\) Is Defective in Interacting with SRP

The function of the homeostatic control region of σ\(^{32}\) is not known [23]. I54Nσ\(^{32}\) is a mutation located in this region is severely compromised in both activity and degradation control, but the mechanism responsible for this phenotype had not yet been determined [23]. We therefore compared the binding of WTσ\(^{32}\) and I54Nσ\(^{32}\) to SRP using gel filtration. We incubated WTσ\(^{32}\) or I54Nσ\(^{32}\) either alone or in combination with SRP and subjected the mixture to gel filtration, this was likely a result of high background in this area of the gel, possibly because of extensive interaction between chaperones and σ\(^{32}\) (Figure 3C, lanes 5–6).

Table 1. The altered σ\(^{32}\) phenotypes of the TnS insertion mutant (pftsY::Tn5) are significantly complemented by an ftsY\(^{+}\) plasmid.

| Strain          | FtsY Level (µm) | σ\(^{32}\) Level (µm) | σ\(^{32}\) Activity (%) | None | +DnaK/J/GrpE |
|-----------------|-----------------|------------------------|-------------------------|------|--------------|
| Wild-type       | 1.0±0.2         | 1.0±0.1                | 1.0±1.0                 | 1.0  | 0.3          |
| pftsY::Tn5      | 0.3±0.1         | 6.8±0.6                | 7.7±1.0                 | 1.1  | 1.1          |
| pftsY::Tn5+ftsY\(^{+}\) | 0.9±0.1      | 2.5±0.4                | 2.1±0.5                 | 0.8  | 0.3          |

In this and all other experiments, protein levels were determined by SDS-PAGE followed by quantitative immunoblotting. σ\(^{32}\) activity was determined from a chromosomal β-galactosidase reporter (calculated as a differential rate of synthesis); values presented are from at least 3 experiments. Relative S.A. of σ\(^{32}\) is defined as [I\(^{+}\)σ\(^{32}\) activity/σ\(^{32}\) level] normalized to σ\(^{32}\) S.A. of WT cells grown at 30°C.

*The ftsY\(^{+}\) plasmid inhibits growth of the cells by ~30%.
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Both SecA and SecY Are Important for Membrane Association of σ\(^{32}\)

SecA is an ATP-fueled motor protein that recognizes signal peptides, drives the translocation of secreted proteins through the Sec translocon [33–37], and collaborates with the SRP/SR for integration of a subset of IM proteins into the membrane [35,38]. We previously found that σ\(^{32}\) activity is increased in a SecA\(^{+}\) strain [39]. This observation motivated us to explore the relationship of
SecA to IM trafficking of $\sigma^{32}$. Indeed, using a SecA(ts) mutant with general defects in protein export (SecAL43P) [40,41], we observed that cells displayed a significant defect in membrane localization of $\sigma^{32}$ (Figure 5), as well as increased $\sigma^{32}$ activity ([39] and unpublished data). In addition, purified SecA, resolved on SDS-PAGE and transferred to nitrocellulose, showed binding affinity for $\sigma^{32}$, suggesting that these two proteins interact (Figure S3). We conclude that SecA participates in trafficking of $\sigma^{32}$ to the IM.

SecY forms the core of the SecYEG IM translocon. This multidomain protein has a large cytoplasmic domain (C5) that functionally interacts with SR [42], SecA, and the ribosome [43–50] (Figure 6A). We tested whether 10 previously described secY mutations located in various domains of SecY (Figure 6A) [51] perturb chaperone-mediated control of $\sigma^{32}$ activity and trafficking of $\sigma^{32}$ to the IM (Figure 6B). All mutants had enhanced $\sigma^{32}$ activity. This result was not surprising as secY mutants are expected to accumulate secretory protein precursors that titrate chaperones [52]. Importantly, four mutants (secT24, secT351, secT40, secT292) were also defective in chaperone-mediated control of $\sigma^{32}$ activity (Figure 6B), as indicated by a lack of down-regulation of $\sigma^{32}$ activity in response to overexpression of one or both of the chaperone systems. We examined the secT351 mutant, which had both high $\sigma^{32}$ activity and a significant defect in chaperone-mediated inactivation, and found it to be defective in IM trafficking of $\sigma^{32}$ (Figure 5). secT40 and secT351 affect domain C5 (Figure 6A), implicated in the interaction of SecY with SR, raising the possibility that this interaction is important for both homeostatic control and IM targeting of $\sigma^{32}$.

**An Independent Methodology Indicates Association of $\sigma^{32}$ with the IM**

Alkaline phosphatase is active only in the periplasm, where it forms the disulfide bonds necessary for its activity. Therefore, translational fusions to alkaline phosphatase (PhoA) lacking its own export signal are commonly used as an indicator of membrane targeting by the appended N-terminal sequence [53]. If the appended N-terminal sequence has either an export or insertion signal, the fusion protein will exhibit alkaline phosphatase activity in vivo because it is partly transported to the periplasmic side of the membrane through the SecYEG translocon. Although $\sigma^{32}$ has neither a membrane insertion nor an export sequence, it

![Figure 3. *In vivo* cross-linking between $\sigma^{32}$ and Ffh.](https://www.plosbiology.org/article/10.1371/journal.pbio.1001735.g003)
may contain a sequence that targets it to the cytosolic face of the IM. There is some evidence that the secretory apparatus can recognize the mature domains of exported proteins at low efficiency [54]. If so, proximity of PhoA to the translocon resulting from the IM targeting signal might enable transit of some fraction of PhoA to localize to the periplasmic side of the membrane, where it is active. By random insertion of the transposon probe Tn\_phoA into rpoH, encoding \( \sigma^{32} \) (see Materials and Methods), we found that a \( \text{phoA} \) fusion to the first 52 amino acids of \( \sigma^{32} \) (N52-\( \sigma^{32}\)-PhoA) showed \( \sim 10 \)-fold greater PhoA activity than signal-less PhoA itself, indicating that the N-terminus of \( \sigma^{32} \) facilitates PhoA export (Table 2). Moreover, PhoA activity enhancement is dependent both on the SRP/SR-dependent trafficking system and on SecY, as both \( pftsY::Tn_5 \) and \( \text{secY}^{351} \) decreased the PhoA activity \( \sim 2 \)-fold, whereas leaderless PhoA exhibited little response to these perturbations (Table 2). Thus, this assay is consistent with the idea that the N-terminus of \( \sigma^{32} \) carries an IM-trafficking sequence and that the targeting process is dependent on SRP and SecY.

Membrane-Tethering of Otherwise Deregulated \( \sigma^{32} \)
Restores Homeostatic Control

The I54N \( \sigma^{32} \) mutant and mutants in the IM-targeting machinery \( pftsY::Tn_5, \text{secA}(ts), \text{secY}^{351} \) were both defective in proper regulation of \( \sigma^{32} \) and in \( \sigma^{32} \) association with the IM. This convergence motivated us to test whether artificially tethering \( \sigma^{32} \) to the IM could restore homeostatic control. To this end, we exploited the bacteriophage Pf3 coat protein. With the addition of three leucine residues in its membrane-spanning region, 3L-Pf3 translocates spontaneously in an orientation-specific manner to the IM, where it inserts in an N-out/C-in orientation [55]. We modified \( rpoH \) (encoding \( \sigma^{32} \)) at its chromosomal locus to encode a \( \sigma^{32} \) variant with the 3L-Pf3 membrane-insertion signal attached to its N-terminus (schematized in Figure S4A). Strains carrying Figure 4. SRP (Ffh\_4.5S RNA) preferentially interacts with WT\( \sigma^{32} \).

(A) \( A_{280}\) elution profiles of WT\( \sigma^{32} \), I54N\( \sigma^{32} \), Ffh, and SRP alone or in complex. WT\( \sigma^{32} \) or I54N\( \sigma^{32} \) was incubated with a 10-fold molar excess of purified SRP on ice for 10 min, and complexes were analyzed by gel filtration on a Superdex 200 PC3.2/30 column. Protein elution was monitored by \( A_{280}\). Gel filtration of purified WT\( \sigma^{32} \), I54N\( \sigma^{32} \), and SRP alone was carried out to determine the migration of each individual protein on the column. (B) Eluted fractions were separated on SDS-PAGE and probed with polyclonal antibodies against Ffh and \( \sigma^{32} \); Western blots of \( \sigma^{32} \) are shown. Experiments were performed at least four times, and a representative experiment is shown. doi:10.1371/journal.pbio.1001735.g004

Figure 5. \( \sigma^{32} \) is partially membrane associated.
The extent of association of \( \sigma^{32} \) and the \( \beta' \) subunit of RNA polymerase with the membrane fraction was determined by quantitative immunoblotting of the soluble and nonsoluble fractions. Membrane association of \( \sigma^{32} \) and \( \beta' \) was assessed in several relevant strain backgrounds. In addition to endogenous \( \sigma^{32} \), all strains contained a plasmid-encoded variant of \( \sigma^{32} \) lacking its 21 C-terminal amino acids (\( \sigma^{32}\Delta21aa \)). Ectopically expressed \( \sigma^{32}\Delta21aa \) or I54N\( \sigma^{32}\Delta21aa \) were present at levels comparable to native \( \sigma^{32} \) and were distinguished from endogenous \( \sigma^{32} \) on a 12% SDS-PAGE gel. All fractionation experiments were performed \( \sim 8 \) times, and % fractionation was calculated from experiments where probed cytoplasmic (RuvB) and membrane (RseA) proteins separated properly. doi:10.1371/journal.pbio.1001735.g005
3L-Pf3-σ32 (IM-WTσ32) or 3L-Pf3-I54Nσ32 (IM-I54Nσ32) as their sole source of σ32 were viable, even though 99% of IM-WTσ32 was inserted in the membrane as judged by fractionation studies (Figure S4B). Thus, σ32 functions when it is tethered to the IM.

We determined whether IM-WTσ32 was subject to homeostatic control circuitry exhibited by WTσ32. σ32 is maintained at a low level by FtsH degradation, and its activity is decreased by chaperone-mediated inactivation. Both phenotypes are evident by comparing the amount and activity of σ32 in a WT versus a ΔftsH strain. In a ΔftsH strain, the level of WTσ32 increases ~30-fold because the major protease degrading σ32 is removed (Table 3; Figure S5 [compare lanes 3 and 4]). Additionally, the level of IM-WTσ32 was significantly lower in ΔftsH than in a ΔftsH strain, indicating that IM-WTσ32 was efficiently degraded by FtsH (Table 3 and Figure S5 [compare lanes 2 and 4]). The presence of a contaminating band prevented absolute quantification of IM-WTσ32 levels via Western blot analysis (Figure S5). However, if the relative S.A. of IM-WTσ32 and WTσ32 are equivalent in the ΔftsH strain as we found in the ΔftsH strain, then the 2-fold decrease in activity of IM-WTσ32 relative to WTσ32 implies a slight increase in the rate of degradation of IM-WTσ32 relative to WTσ32. Note that the 3L-Pf3 membrane-insertion tag itself is not a signal for FtsH degradation, as the stability of the FliA σ factor, which is closely related to σ32, was unchanged when expressed as

![Figure 6](https://www.plosbiology.org/article/f6.png)

**Figure 6.** The SecY translocon plays a role in chaperone-mediated activity control of σ32. (A) Schematic of SecY topology in the IM by highlighting in yellow the locations/allele names of the mutated residues used in this study [51]. The region that interacts with FtsY (Domain C5) is boxed in green. (B) Mutations in secY show higher σ32 activity and affect chaperone-mediated activity control of σ32. The activity of σ32 was measured in WT and secY mutant cells growing at 30°C in LB medium (column 1) or in the same cells following induction of DnaK/J (column 2) or GroEL/S (column 3). Activity is calculated as the differential rate of β-galactosidase synthesis from a chromosomal P_{sgc-lacZ} reporter in each cell type relative to that of WT cells.

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3L-P3-FliA (Figure S6). In summary, both the chaperone-mediated activity control circuit and the FtsH-mediated degradation control circuit are active on IM-tethered σ^{32}.

Next, we asked whether the forced and stable tethering of σ^{32} to the IM bypassed the regulatory defects of I54N^{32} and the reduced-level SR mutant pftsY::Tn5. I54N^{32} is degraded poorly by FtsH as its level was 11-fold higher than that of WT σ^{32} (Table 3; Figure S5 [compare lanes 1 and 6] and [25]). I54N^{32} also had compromised chaperone-mediated activity control as the high chaperone levels in this strain did not reduce the S.A. of a N52-PhoA protein through the SRP-Sec pathway. Indeed, IM-WT^{32} was degraded and subject to chaperone-mediated activity control. This feed-forward mechanism allows the σ^{32} homeostatic control to sense the state of cytosolic and IM proteostasis before unfolded proteins accumulate to a significant extent. Interestingly, fth (encoding the protein subunit of the SRP) is a σ^{32} regulon member as its expression increases at least 3-fold following induction of σ^{32} either by heat shock or by deletion of dnaK [30 and unpublished data]. This could provide an additional connection between σ^{32} and protein flux to the IM.

Finally, and more speculatively, given the demonstrated involvement of SecA in IM targeting of σ^{32} and its direct interaction with σ^{32}, the σ^{32} homeostatic control circuit may also monitor protein flux through SecA to the periplasm and outer membrane.

The idea that the high activity of σ^{32} in the I54N^{32} homostatic control mutant and in SRP/SR mutants (eg. pftsY::Tn5) results from σ^{32} mislocalization to the cytosol and consequent homeostatic dysregulation, rather than from chaperone titration by a buildup of unfolded proteins, is supported by our data. First, forced IM-tethering overcomes the inviability of the I54N^{32} mutation in the ΔftsH strain background (Table 3), as well as the growth defects of I54N^{32} and pftsY::Tn5 (Figure S7), suggesting that high expression of σ^{32} is aberrant and deleterious to cells, rather than required to remodel misfolded proteins. This is reminiscent of previous findings that reduced-function σ^{32} mutants suppress physiological defects of a ΔdtnA strain [59] and that overexpression of HSPs was deleterious to growth [13,60]. Second, secT mutants dysregulated in chaperone-mediated activity control were not distinguished by their extent of σ^{32} induction. This is contrary to the prediction of the chaperone titration model, which posits that secT mutants with the highest σ^{32} induction would have the highest level of unfolded proteins. These mutants would then be refractory to activity control because the additional chaperones resulting from chaperone overexpression would actually be needed to remodel the misfolded protein burden. We conclude that homeostatic dysregulation of σ^{32} results from σ^{32} mislocalization, rather than from the buildup of unfolded proteins.

Discussion

Our work has led to a revised model of the HSR circuitry (Figure 1B). σ^{32} first transits to the IM via an SRP/SR-dependent process and is then subjected to the chaperone-mediated activity control and FtsH-mediated degradation control that have been previously described. This revised model enables the homeostatic control circuit to integrate information on both cytosolic and IM status. Importantly, the efficiency of co-translational protein targeting depends on the cumulative effect of multiple SRP checkpoints including differences in cargo binding affinities, kinetics of SRP-SR complex assembly, and GTP hydrolysis [57]. Multiple checkpoints and the fact that SRP is sub-stoichiometric relative to translating ribosomes (~1:100; SRP molecules to translating ribosomes [58]) may allow SRP to modulate the extent of IM-localization of σ^{32} during times of stress and/or increased protein flux. Thus, σ^{32} down-regulation through its localization to the membrane could be alleviated when the IM is disturbed or SRP is overloaded in assisting membrane protein biogenesis.

Table 2. N-terminal segment of σ^{32} directs activation of PhoA protein through the SRP-Sec pathway.

| Strain         | N52-σ^{32} PhoA Activity | Protein Level | Signal-Less σ^{32} PhoA Activity | Protein Level |
|----------------|--------------------------|---------------|----------------------------------|---------------|
| Wild-type      | 1.00±0.11*               | 1.0           | 0.11±0.03                        | 0.15          |
| pftsY::Tn5     | 0.43±0.05                | 0.8           | 0.14±0.03                        | 0.2           |
| sec735T        | 0.46±0.07                | 1.5           | 0.32±0.05                        | 0.2           |

Wild-type strain (MG1655) and its derivatives carrying the mutation as indicated were transformed by the plasmid containing each PhoA construct. The resulting transformants were grown to log phase in LB medium at 30 °C. PhoA activity and protein levels were determined by standard procedures (see Materials and Methods).

*The activity of N52-σ^{32}-phoA was set to 1.00 in the wild-type strain.

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Table 3. IM-insertion of σ^{32} significantly restores homeostatic control to mutant cells.

| σ^{32} Variant | σ^{32} Level | ΔftsH | WT σ^{32} Activity | Relative S.A. of σ^{32} |
|----------------|--------------|-------|---------------------|-------------------------|
| IM-σ^{32}      | 26.7±4.5     | 0.7±0.1| 2.0±0.1             | N.D. 0.1               |
| I54N^{32}      | 11.4±2.1     | 6.7±0.5| N.D. 0.6            | N.D.                  |
| IM-I54N^{32}   | 29.5±6.0     | 1.3±0.6| 4.3±0.8            | N.D. 0.1              |
| pftsY::Tn5     | 6.8±0.6      | 7.7±1.0| 1.1                 | N.D.                  |
| pftsY::Tn5:IM-σ^{32} | 0.7±0.1 | N.D.  |

Protein levels were determined by SDS-PAGE followed by immunoblotting, and averages from 3–4 expts are presented. Relative S.A., relative Specific Activity, is calculated as described in Table 1. N.D., not determined, denotes that values could not be determined because of a and b.
aLevels of the σ^{32} variants could not be measured accurately.
bAn IM45N^{32}-ΔftsH strain is inviable.

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σ^{32} interacts with the Signal Recognition Particle

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The molecular nature of IM-localized σ^32 remains unclear. Prediction programs [61,62] do not detect either a signal peptide-like or transmembrane sequence in σ^32. We favor the idea that following transit to the IM, σ^32 is maintained at the membrane via interactions with other proteins and/or lipid head groups during its short half-life in the cell (30-60min). Indeed, we have already demonstrated interactions between σ^32 and several membrane-associated or IM proteins, including SRP, SecA, and FtsH itself. Moreover, the chaperone systems regulating σ^32 (DnaK/J/GroE and GroEL/S) show partial distribution to the membrane [63-69], whereas other potential membrane-associated protein partners have not yet been tested for σ^32 interaction (e.g., SecY and additional members of the Sec machinery). Each of these proteins could result in partial membrane localization of σ^32, as was shown for FtsH where depletion of the protein decreased localization relative to cells with the protease-dead mutation FtsH E415A. Importantly, if σ^32 is membrane associated via transient protein–protein and/or protein–lipid interactions, some σ^32 may dissociate from the membrane during cell lysis, as was demonstrated for FtsY, another peripheral membrane protein [69,70]. Therefore, although we report that ~50% of σ^32 is membrane-associated, the fraction of σ^32 that is actually IM-localized may be significantly higher.

IM-associated σ^32 may provide regulatory flexibility not possible for IM-tethered σ^32. For example, during times of high stress, σ^32 may be able to dissociate from the membrane to escape homeostatic control. These excursions could be transient if SRP were able to transport σ^32 posttranslationally, a possibility suggested by the fact that full-length, fully folded σ^32 binds to SRP (Figures 2 and 3 and Figure S1). Additionally, IM-tethered σ^32 is more rapidly degraded than IM-associated σ^32, suggesting that tethering makes σ^32 a better FtsH substrate. This could diminish the ability of the cell to regulate the rate at which FtsH degrades σ^32, which is of physiological significance during temperature upshift [8]. The transient reduction in σ^32 degradation following increased temperature contributes significantly to the rapid build-up of σ^32 during heat shock [8].

Membrane localization is widely used to control σ factors [71,72]. The inactive B. subtilis SigK pro-protein is membrane inserted; cleavage of its N-terminal pro-sequence releases SigK [73,74]. Cleavage is coordinated with passage of a checkpoint in spore development to provide just-in-time SigK activity [75]. Additionally, many σ factors are held in an inactive state at the membrane by cognate membrane-spanning anti-σ factors and released as transcriptionally active proteins when stress signals lead to degradation of their anti-σ [71,76]. IM-localization of σ^32 serves a conceptually distinct role as σ^32 is equally active in the cytoplasm or at the IM. Instead, the localization process itself is the key regulatory step in two ways: localization is both regulated by protein folding status and is prerequisite for proper function of the homeostatic control circuit.

The SRP-SR co-translational targeting system has an important role in maintaining proteostasis. SRP-SR minimizes aggregation of the approximately 20%–30% of proteins destined for the IM, by making their translation coincident with membrane lipid interactions, some of which are also SRP-like or transmembrane sequence in σ^32. The prevailing paradigm suggests that the M-domain interacts only with nascent polypeptides with particularly hydrophobic signal sequences. It is possible that σ^32 is detected co-translationally, as the Region 2.1 N-terminal α-helical structure, which resembles a hydrophobic signal sequence, may be recognized by the SRP. Alternatively, we note that the SRP chloroplast homolog (cpSRP54) has a dedicated posttranslational targeting mechanism for several fully translated membrane proteins [77], and E. coli SRP, alone or in combination with additional accessory factors (e.g., other σ^32 interactors, such as chaperones or SecA), may target mature σ^32 to the membrane in vivo. It remains to be determined whether an interaction between full-length σ^32 and SRP, or a novel co-translational targeting interaction by the SRP-SR system, mediates transit of σ^32 to the membrane.

Materials and Methods

Strains, Plasmids, and Growth Conditions

All strains used were derivatives of the E. coli K-12 strain MG1655, CAG48238 [25,39]. For chaperone overexpression experiments, mutations were transduced with phage P1 into strains carrying chromosomal P_{araC}::gfpEL/S [78] or P_{araC}::lacIq-dnaK/J-lacP [14]. Mutant alleles in secY [51] and secD [39] were transferred to various strain backgrounds through P1 transduction. The SecA43P mutant used here is a SecA(α) allele, with general defects in protein export [40,41]. For propagation and transfer of the R6K plasmid, pKNG101, strains DH5α and SM10 λpro were used, respectively. Plasmids pET21a and pTrc99A were used as expression plasmids. For construction of pRM5 (6×His-6pkoF), the pkoF gene was PCR-amplified from the chromosomal DNA of W3110 and cloned into the EcoRI-Sall sites of pTTQ18 [79]. Then, the T32amber mutation was introduced into pRM5: by site-directed mutagenesis, yielding pRM17 (6×His-6σ^32T52amber), pEVOLO-pBpfF (Addgene) carried evolved Methanocaldococcus janaschii aminocyl-tRNA synthetase/suppressor tRNA for incorporation of a photoreactive amino acid analog, 5-benzoylphenylalanylamine (5BPA), into the amber codon site. All strains were grown in LB medium. When required, antibiotics were added to the medium as follows: 100 μg/ml ampicillin, 30 μg/ml kanamycin, 20 μg/ml chloramphenicol, and 25 μg/mL streptomycin.

Isolation of pfsY::Tn5 Mutant

Strain CAG48275 [25], which is ΔlacX74, contains the prophase JW2 (P_{araC-lacZ}) and a chromosomal dnaK/J locus driven from P_{araC-lacO}, under control of lacP [14] was grown in LB, induced with 1 mM IPTG to overexpress DnaK/J chaperones, treated with Tn5, and plated at 30°C on X-gal indicator plates containing kanamycin to select for strains containing Tn5. Blue colonies were picked and tested for higher σ^32 activity and for feedback resistance to excess DnaK/J [25]. Tn5 insertion sites were determined by DNA sequencing.

β-Galactosidase Assay

Overnight cultures (LB medium) were diluted 250-fold and grown to exponential phase (OD600 = 0.05–0.5). Samples were taken at intervals starting at OD600 = 0.05, and β-galactosidase activity was measured by measuring β-galactosidase activity expressed from the σ^32-dependent hppG promoter, as done previously [25].

Protein Purification

The following proteins were purified essentially as described: 6×H-tagged, Strep-6×H-tagged, and untagged WTσ^32 or 154Ne[52] [80], FtsY, Ffh, 4.5S RNA [81], and SecA [82]. Chaperones were removed from σ^32 with an additional wash containing 10 mM ATP, 10 mM MgCl₂, and 25 μM of both peptides, CALLA3R and MQERILKKDYAM, synthesized by Elin Biopharmaceuticals, Inc (Hayward, CA).

σ^32 Interacts with the Signal Recognition Particle
In Vivo Co-Immunoprecipitations

Cells were grown to OD_{600}=0.3 to 0.35 in LB medium at 30°C, harvested, washed two times with 1× PBS, resuspended in Lysis Buffer (20 mM Heps-KOH, 150 mM NaCl, 10 mM EDTA, 10% glycerol, pH 7.5), and lysed by passing 4× through an Avestin EmulsiFlex-C5 cell homogenizer at 15,000 psi. Cellular debris was spun out and the supernatants were incubated with anti-Ffh or anti-FsY antibodies at 4°C for 14 h by rotation. TrueBlot anti-Rabbit Ig IP Beads (eBioscience) were added and the supernatants rotated for an additional 2 h at 4°C. Immunocomplexes were isolated by centrifugation and washed 5× in Lysis Buffer without EDTA, and eluted in TCA Resuspension Buffer (100 mM Tris (pH 11.0), 3% SDS) containing LDS Sample buffer (Invitrogen). Proteins were separated by 10% SDS-PAGE, analyzed by immunoblotting using anti-σ^{30} and anti-σ^{32} antibodies, and imaged using fluorescent secondary antibodies (as described below).

Identification of Direct Protein–Protein/Domain Interactions

Detection of a direct protein–protein/domain interaction was carried out exactly as previously described [83]. Proteins were separated on 10% SDS-PAGE. Partially polyethylene glycol (PEG) purified Ffh was obtained by incubating 400 μg of purified Ffh with 4 μg of Glu-C endopeptidase (New England Biolabs) at 25°C in 10 mM Na-Hepes (pH 7.5), 150 mM NaCl, 1 mM DTT, 10 mM MgCl_{2}, and 10% glycerol. An aliquot of the reaction was taken out at various times (0, 5, 10, 15, 30, 45, 60, 120, 180, and 330 min) and stopped by addition of 5× volume of 5× SDS-sample loading buffer. The samples were then analyzed by blot overlay with σ^{32} as the probe.

In Vivo Crosslinking, 6×His-tag Affinity Isoolation and Co-Immunoprecipitation

In vivo crosslinking experiments were carried out essentially as described previously [84]. Strains of CAG48238 carrying pEVOL-pBPφ were further transformed with pRM5 or pRM17. Cells were grown at 30°C in L medium containing 0.2% arabinose and 1 mM pBPφ, induced with 1 mM IPTG for 1 h, and UV-irradiated for 0 or 10 min at 4°C. For analysis of whole cell samples, total cellular proteins were precipitated with 5% trichloroacetic acid, solubilized in SDS sample buffer, and analyzed by 7.5% SDS-PAGE and immunoblotting.

Co-immunoprecipitations were carried out as follows: UV-irradiated cells were suspended in 10 mM Tris-HCl (pH 8.1) and disrupted by sonication at 0°C. After removal of total membranes by ultracentrifugation, proteins were precipitated with 5% trichloroacetic acid, washed with acetone, and solubilized in buffer containing 50 mM TrisHCl (pH 8.1), 1% SDS, 1 mM EDTA. The samples were then diluted 32-fold with NP40 buffer (50 mM TrisHCl (pH 8.1), 150 mM NaCl, 1% NP40). After clarification, supernatants were incubated with anti-Ffh antibodies and TrueBlot anti-Rabbit IgG IP Beads (eBioscience) at 4°C for 15 h with rotation. Immunocomplexes were isolated by centrifugation, washed 2× with NP40 buffer and then once with 10 mM TrisHCl (pH 8.1), and dissolved in SDS sample buffer. Proteins were separated by 7.5% SDS-PAGE and analyzed by immunoblotting using anti-Ffh and anti-σ^{32} antibodies, TrueBlot anti-Rabbit IgG (eBioscience), and Can Get Signal immunoreaction enhancer solution (TOYOBO Life Science, Japan).

For 6×His-tag affinity isolation, UV-irradiated cells were suspended in 10 mM Tris-HCl (pH 8.1) containing 6 M urea and disrupted by sonication at 0°C. After clarification by ultracentrifugation, the soluble fraction was loaded onto the TALON resin (TAKARA BIO, Inc., Japan). After washing the resin with wash buffer (50 mM TrisHCl (pH 7.0), 300 mM KCl, 6 M urea, 20 mM imidazole), bound proteins were eluted with wash buffer containing 300 mM imidazole. Proteins were precipitated with 5% trichloroacetic acid, solubilized in SDS sample buffer, and analyzed by 7.5% SDS-PAGE and immunoblotting.

Gel Filtration

Purified proteins were run on a Superdex 200 26/30 column, pre-equilibrated with Buffer A (20 mM Tris-HCl pH 8.0, 150 mM NaCl, 10 mM MgCl_{2}, 2 mM DTT). Purified proteins or protein complexes were run with Buffer A at a flow rate of 40 μL/min, and collected fractions were analyzed by SDS-PAGE and immunoblotting for σ^{32}. SRP was formed by incubating purified Ffh with 1.5× molar excess of purified 4.5S RNA on ice for 10 min. To form SRF-σ^{32} complexes, 3 μg of purified WT σ^{32} or E54Ntσ^{32} was mixed with 10× molar excess of SRP; proteins were incubated on ice for 30 min before analysis by gel filtration.

Construction of σ^{32}-PhoA Fusion

A 52-σ^{32}-His tag PhoA fusion was initially isolated by random screening for PhoA^+ clones on PhoA indicator plates—using a strain carrying a TnphoA transposon probe [85] on a low-copy plasmid and P_{lac}-rpoH (encoding σ^{32}) on a multicopy plasmid. The fusion used in this article (N52-σ^{32}-PhoA lacking the transposon but containing the first 52 amino acids of WT σ^{32}) was subsequently constructed by standard recombinant DNA techniques. Direct construction of fusions past amino acid 52 of σ^{32} was very unstable, precluding their analysis.

Cell Fractionation

Cells were grown to OD_{600}=0.3–0.4, harvested, and resuspended in ice-cold Buffer B (10 mM Tris-Acetate (pH 7.4), 10 mM Mg(OAc)_{2}, 60 mM NH_4Cl, 1 mM EDTA, supplemented with 1 mM PMSF) to an OD_{600} of 15. Cells were immediately lysed by passage of the extracts through an Avestin EmulsiFlex-C5 cell homogenizer at 15,000 psi, and subjected to low-speed centrifugation to remove cell debris and un-lysed cells. Membranes were collected by ultracentrifugation in an Optima benchtop centrifuge (Beckman–Spinco) with a TLA 100.3 rotor (60 min; 100,000 rpm). The supernatant was saved as the soluble fraction, while the pellet was washed 3× with Buffer B and then resuspended in Buffer C (50 mM HEPES-KOH pH 7.6, 50 mM KCl, 1 mM EDTA, 1 mM EGTA, 0.5% n-Dodecyl β-D-maltoside, and 5% glycerol). Both the soluble and membrane fractions were precipitated in trichloroacetic acid (13% vol/vol), incubated on ice for 30 min, and then overnight at 4°C. Precipitated proteins were then washed with ice-cold acetone and analyzed by SDS-PAGE and immunoblotted for σ^{32} (Neocline), β′ (Neocline), σ^{70} (Neocline), RscA [86], and RuvB (Abcam) with fluorescent secondary antibodies (LI-COR Biosciences) used for detection. The percentage of σ^{32} in each fraction was determined by direct scanning and analyzing bands with ImageJ software (National Institutes of Health).

RNA Polymerase Pull-Downs

Cells were grown to OD_{600}=0.35–0.45, harvested, and resuspended in ice-cold Buffer D (50 Tris-HCl, pH 8.0, 0.1 mM EDTA, 150 mM NaCl, and 5% glycerol) to an OD_{600} of 20. Lysozyme was added to 0.75 mg/mL and cells were incubated on ice for 30 min, followed by sonication, then subjected to low-speed
centrifugation to remove cell debris and unlysed cells. Lysates were then incubated with pre-equilibrated, pre-blocked (Buffer D containing 5% Bovine Serum Albumin, 0.1 mg/mL dextran) Softag 4 Resin (Neoclone) overnight at 4°C. Bound proteins were washed 3× with Buffer D and eluted with 4× LDS NuPAGE Buffer (Life Technologies). To collect lysates and eluted proteins, 0.05 μM of Strep-6×H-tagged σ32 was added as a loading and blotting control during analysis by SDS-PAGE and Western blotting against Figure S1.

Construction of 3L-Pf3 Fusion Proteins

The 3L-Pf3 genetic sequence was created by carrying out standard polymerase chain reaction using the following overlapping oligos: 5'-atgcaatccgtgattactgatgtgacaggccaactgacagcggtgca-3', 9'-taacattggggtgcattctctcgattgtcttgccgctgtgtgctggg-3', 5'-aaaggagttggcttcatgacgaatccacagacagcggccagaa-3', and 5'-aagtaagcttgaatccacagacagcggccagaa-3', and 5'-aagtaagcttgaatccacagacagcggccagaa-3', and 5'-aagtaagcttgaatccacagacagcggccagaa-3'. The 3L-Pf3 sequence was then integrated 5' and in-frame with the chromosomal tfdP gene by double homologous recombination. Counterselection of sacB on pKNG101 was carried out on 10% sucrose media (5 g/L Yeast Extract, 10 g/L Tryptone, 15 g/L Bacto Agar, 10% sucrose) [25,87]. Clones were sequenced to verify chromosomal integration of the 3L-Pf3 sequence. To construct pTrc99A expressing 3L-Pf3-FliA, fliA and fliC (in that order) were cloned as an operon, with the sequence 5'-ccgtctagaattaaagAGGAGaaaggtacc-3' added between the two genes in the vector; the Shine-Dalgarno site is designated in 5-open reading frame and 500 base pairs upstream of the start codon, and subsequently cloned into the pKNG101 suicide vector. The 3L-Pf3 sequence was then integrated 5' and in-frame with the chromosomal tfdP gene by dual homologous recombination. Counterselection of sacB on pKNG101 was carried out on 10% sucrose media (5 g/L Yeast Extract, 10 g/L Tryptone, 15 g/L Bacto Agar, 10% sucrose) [25,87]. Clones were sequenced to verify chromosomal integration of the 3L-Pf3 sequence in the correct reading frame.

To construct pTrc99A expressing 3L-Pf3-FliA, fliM and fliC (in that order) were cloned as an operon, with the sequence 5'-ccgtctagaattaaagAGGAGaaaggtacc-3' added between the two genes in the vector; the Shine-Dalgarno site is designated in uppercase. Two plasmids were created—one with just fliM and fliC, unmodified, and one where the 3L-Pf3 sequence was cloned 5' to and in-frame with fliC. Clones were sequenced to verify correct sequences and proper reading frame. Expression was from the leaky pTrc promoter, and experiments were only carried out after fresh transformation into the parental CAG48238 strain. Levels of FliA were analyzed by SDS-PAGE and immunoblotting with antibodies against FliA (Abcam).

Immunoblotting

Cells were re-suspended in equal volumes of Buffer C, with the addition of trichloroacetic acid (final 13% vol/vol), kept on ice overnight, and the precipitate collected by centrifugation. Pellets were washed with acetone and resuspended in 1x LDS NuPAGE Buffer (Life Technologies). Serial dilutions of WT and mutant samples were loaded onto a polyacrylamide gel, and proteins transferred to nitrocellulose membranes. The blots were first probed with primary antibodies and then with anti-primary fluorescence-conjugated secondary antibody (Loric). Immunoblots were scanned at the appropriate wavelengths for detection. Fold increase (protein level experiments) was estimated by comparison with a dilution series of samples from the WT strain. Fold decrease after addition of chloramphenicol (protein stability experiments) was determined by direct scanning and analyzing bands with ImageJ software (National Institutes of Health).

Supporting Information

Figure S1 σ32Δ21aa, a C-terminal truncation of σ32, is defective in binding to RNA polymerase in vivo. [A] Immunoprecipitation of RNA polymerase-bound native σ32 and σ32Δ21aa. σ32Δ21aa was expressed from pTrc99A in ΔftsH cells, induced to levels comparable to endogenous σ32, grown to mid-exponential at 30°C in LB medium and the amount of σ32 bound to the anti-σ’ resin (Softag; Neoclone) and remaining σ32 in the supernatant was quantified by immunoblotting using a polyclonal antibody against σ32. Comparable amounts of total cellular lysates (TCL; left lane) and corresponding RNA-polymerase immunoprecipitations (RNAP IP; right lane) are shown. Purified σ32, tagged at the N-terminus with a Strep and 6×Histidine (Strep-6×H tag), was used as a loading and blotting control. Results of a representative experiment are shown. [B] Quantification of RNA polymerase-bound native σ32 and σ32Δ21aa expressed in the same strain background (ΔftsH). Averages of four independent experiments are shown.

Figure S2 Membrane fractionation of σ32 is independent of RNA polymerase binding. ftsH E415A cells expressing either WT σ32Δ21aa or Δ34Nσ32Δ21aa were subjected to cellular fractionation (see Materials and Methods), and soluble and membrane fractions were resolved by SDS-PAGE and analyzed by immunoblotting for σ32, σ20, and the β’ subunit of RNA polymerase. Ectopically expressed σ32Δ21aa or Δ34Nσ32Δ21aa were present at levels comparable to native σ32 and were distinguished from endogenous σ32 on a 10% SDS-PAGE gel. All fractionation experiments were performed ≥8 times, and % fractionation was calculated from experiments where probed cytoplasmic (RuvB) and membrane (RseA) proteins separated properly.

Figure S3 σ32 interacts with SecA through protein–protein interaction analysis. Purified SecA was run on a 10% SDS-PAGE gel (along with FtsY and Ffh), transferred to nitrocellulose, re-natured, and incubated with purified WT σ32. The Coomassie-stained gel of the prey proteins (FtsY, Ffh, and SecA; left) and the nitrocellulose membrane containing the transferred prey proteins, probed with polyclonal anti-σ32 antibodies (right), are shown. The Coomassie-stained gel section of FtsY and Ffh and the corresponding σ32-incubated nitrocellulose membrane probed with anti-σ32 antibodies are also shown in Figure 2C.

Figure S4 Fusing the 3L-Pf3 peptide to the N-terminus of WT σ32 coding sequence significantly increases its membrane localization. (A) Schematic representation of membrane-tethered 3L-Pf3-WT σ32 (IM-WT σ32). The amino acids corresponding to the 3L-Pf3 and σ32 are shown as open or enclosed dark circles, respectively. (B) Soluble (lanes 1 and 3) and membrane (lanes 2 and 4) fractions from cellular fractions (described in Materials and Methods) were separated by SDS-PAGE and immunoblotted for the indicated proteins shown on the right.

Figure S5 Levels of σ32 and σ32 variants in varying strain backgrounds. Strains were grown to OD600~0.35, precipitated by addition of TCA to 13% final (vol/vol). Levels of σ32 and σ32 variants were determined by quantitative immunoblotting (see Materials and Methods). The experiment was carried out ≥5 times, with an example blot shown. These are the raw data used to obtain level values for σ32 and its variants shown in Table 3. Averaged quantification of the amount β’ served as a loading control, and levels of FtsH and FtsY are additionally shown. The genetic backgrounds of the mutant strains are shown below the
blobs. The specific protein probed on each blot is shown to the right. Note that IM-σ32 and IM-I54Nσ32 run as a smear, most likely because the membrane localization signal adopts multiple conformations during SDS-PAGE electrophoresis. To minimize this problem, gels were run very slowly (60–80 volts). Amount of IM-σ32 variants was calculated over the entire smear. Additionally, there is a contaminating band in all samples marked with an asterisk (*) that runs approximately at the same molecular weight as IM-σ32. This contaminating band prevents accurate quantification of samples with low amounts of IM-σ32 (lanes 2, 7, and 9).

Figure S6 The 3L-Pf3 peptide does not alter the stability of the fluA σ. (A) Addition of the 3L-Pf3 peptide to the N-terminus of FluA σ does not affect its cellular levels. Total cellular lysates were separated on SDS-PAGE and immunoblotted for FluA. WT fliA 3L-Pf3, or 3L-Pf3-fldA was expressed from uninduced pTrc99A in the MG1655 background, and the fliA variants expressed are shown (at top). MG1655 carrying only pTrc99A (Vector) shows the endogenous levels of FluA. The lower band present in the 3L-Pf3-FluA lysate is endogenous FluA. Experiments were performed at least three times. The representative experiment shown demonstrates that addition of the 3L-Pf3 peptide does not alter the amount of the FluA present in the lysate. As both FluA and 3L-Pf3-FluA are expressed from the same transcriptional and translational start points, we conclude that the 3L-Pf3 tag does not destabilize FluA. Thus, even though targeted to the membrane, 3L-Pf3FluA is not degraded by the membrane localized FtsH protein, which preferentially degrades membrane proteins. (B) Addition of the 3L-Pf3 peptide to the N-terminus of FluA increases its membrane localization. Soluble and membrane fractions from cellular fractionations of MG1655 carrying fliA or 3L-Pf3-fldA expressed on pTrc99A were separated on SDS-PAGE and immunoblotted for FluA. Percentage of membrane-localized FluA is plotted. Averages of four independent experiments are shown. (TIF)

Figure S7 Growth defects in I54Nσ32 and pflaTn:5 are relieved when the endogenous σ32 is membrane- tethered. (A) Early exponential growth comparison of WT, IM-WTσ32, IM-I54Nσ32, and IM-I54Nσ32. (B) Early exponential growth comparison of WT, IM-WTσ32, pflaTn:5 mutant, and the double mutant pflaTn:5, IM-WTσ32. Cellular density (OD600) was plotted over time in (A) and (B). Experiments for both (A) and (B) were carried out three times, and an example growth curve obtained is shown. (C) IM-tethering of σ32 in mutant strains restores growth rates to that of WT. Doubling times were calculated as the inverse of the slope of the cultures growing in early exponential phase in LB at 30°C. Strain mutations are shown on the left. The exact values of the doubling times for each strain are shown on the right and are an average of three experiments. (D) Membrane-tethering of σ32 in the pflaTn:5 mutant restores transition into stationary phase growth to that of WT. The pflaTn:5 mutant transitions into stationary phase growth significantly earlier and at a lower OD600 than both WT and the double mutant pflaTn:5, IM-WTσ32. Cellular density (OD600) was plotted over time. Growth curves are an average of three biological replicates. (TIF)

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Author Contributions

The author(s) have made the following declarations about their contributions: Conceived and designed the experiments: BL SN YA PW TY CAG. Performed the experiments: BL RM TY DAS. Analyzed the data: BL RM SN YA TY. Wrote the paper: BL KI YA PW TY CAG.

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